Effect of enzyme pretreatment in the ultrasound assisted extraction of finger millet polyphenols

V. Geetha Balasubramaniam¹ · P. Ayyappan¹ · S. Sathvika¹ · Usha Antony¹

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Abstract Polyphenols from plant sources are increasingly recognized as functional ingredients with multiple health benefits. Finger millet (*Eleusine coracana*), a major millet grown in Asia and Africa is a significant source of polyphenols. The extraction of polyphenols from millets which are under-utilized has received less research attention compared to other plant matrices. Therefore, the present study was carried out to identify an effective method of extracting the polyphenols from finger millet (v. GPU 28) seed coat (FMSC). Two eco friendly methods namely ultrasonication (UA) and enzyme treatment followed by ultrasonication (EUA) were compared with the conventional heat reflux method (HR). The polyphenolic profile and content along with the antioxidant potential of the extract were evaluated. Phenolic yield increased 2.3 fold using EUA with xylanase (XUA) compared to heat reflux extraction (HR). However, yield with UA was equivalent to the conventional method. Total flavonoids increased 1.4 fold in UA and 1.3 fold in XUA, similarly, tannins also showed a significant increase (1.1 fold in UA and 1.2 fold in XUA). FTIR spectra revealed the presence of all phenolic functional groups and ESI-MS showed 80% similarity in the individual polyphenols in the extracts. Catechins, luteolin and cyanidin were identified in the UA and XUA treated samples, whereas, shikimic acid

derivatives- caffeoyl and di caffeoyl were present only in XUA extracts. This is the first report on enhanced extraction of polyphenols from FMSC using a combination of enzyme treatment and ultrasonication, providing a green technology for utilization of polyphenols in nutraceuticals and functional foods.

Keywords Polyphenols · Finger millet seed coat · Ultrasonication · Xylanase pretreatment · Total polyphenol content · Cyandins

Abbreviations

ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
CCD	Central composite design
CE	Catechin equivalents
CUA	Cellulase ultrasonic assisted
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EC	Enzyme cellulase
ESI MS	Electrospray ionization mass spectrum
EX	Enzyme xylanase
EUA	Enzyme treatment and ultrasonication
FMSC	Finger millet seed coat
GAE	Gallic acid equivalent
HPLC	High performance liquid chromatography
HR	Heat reflux
RSM	Response surface methodology
TPC	Total polyphenol content
UA	Ultrasonic assisted
XUA	Xylanase ultrasonic assisted

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✉ Usha Antony
usha.antony@gmail.com; ushaantony@annauniv.edu

¹ Department of Biotechnology, Centre for Food Technology, A. C. Tech. Campus, Anna University, Chennai, Tamil Nadu 600025, India

Introduction

Polyphenols are a large and diverse group of phytochemicals mostly found as secondary metabolites in plants. They contain one or more aromatic rings with one or more hydroxyl groups attached (Chandrasekara and Shahidi 2010). The antioxidant activity of polyphenols has preventive and therapeutic value with respect to degenerative diseases, particularly neurodegenerative diseases, cardiovascular diseases and cancers (Chandrasekara and Shahidi 2010).

Sorghum and millet have a wide variety of phenolic acids. Their bran is threefold richer in these compounds compared to the endosperm. Finger millet is a rich source of dietary fibre and polyphenols (0.2–3.0%) (Antony and Chandra 1999) and the seed coat (FMSC) fraction contains nearly 90% of the total polyphenols (Chethan and Malleshi 2007).

The effective utilization of the phytochemicals or bioactive compounds depends on potential of the extraction process carried out. Heat reflux and soxhlet extraction are the commonly followed conventional methods for polyphenol extraction (Hao et al. 2002). They have low efficiency due to 1) their increased time and temperature requirements and 2) potential environmental hazards on human health due to the toxic solvent residues that may remain in the final product (Ajila et al. 2011). These problems may be overcome with alternate green technology processes with environmentally benign and non-toxic food grade solvents (Ajila et al. 2011).

A number of novel methodologies such as microwave extraction, ultrasound-assisted extraction (UAE), supercritical fluid extraction, pressurized fluid extraction and pulsed electric field have been developed to obtain the polyphenols from different plant samples. These methodologies have short extraction times; consume low energy and organic solvent (Ajila et al. 2011).

The low frequency energy (20 kHz–1 MHz) of ultrasonication results in increased rates of mass transfer due to the generation of localized pressure which breaks down the plant tissue and releases the cellular bioactive components (Ajila et al. 2011). Furthermore, the products generated by ultrasonic treatment would be a good source of ingredients for use as nutraceuticals and in functional foods.

With hydrolytic enzymes, the cellulosic network is cleaved, the solvent distribution is improved, particle size is reduced, mass transfer rate is improved, all of which leads to enhanced recovery of bound phenolics (Ajila et al. 2011).

There are very few studies on polyphenol extraction from cereals and millets. Ease of phenolic extraction in rice bran (Tabaraki and Nateghi 2011), wheat bran (Wang et al.

2008) and sorghum (Pascal Agbangnan et al. 2012) using ultrasonication have been documented. Till date there have been no reports on the non-conventional techniques and enzyme treatment in the extraction of bioactives from finger millet. The study is therefore designed to optimize and enhance the extraction of phenolic contents by enzyme treatments and ultrasound assisted extraction.

Materials and methods

Materials

Chemicals

The standard polyphenols such as gallic acid, catechin hydrate, quercetin and enzymes such as glucoamylase (EC 3.2.1.3) from *Aspergillus niger*, α -amylase (EC 3.2.1.1) from *Bacillus licheniformis*, xylanase and cellulase were purchased from Sigma Aldrich (St. Louis, USA). 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and Folin–Ciocalteu reagent were obtained from Merck (Darmstadt, Germany) and all other chemicals and reagents used for the study were of analytical grade.

Procurement of sample

Finger millet variety GPU 28 was procured from University of Agricultural Sciences, Bangalore, India as most agricultural universities grow and maintain specific varieties of crops.

Methods

Separation of seed coat rich fraction from finger millet (FMSC)

The finger millet seed coat fraction was separated as described by Shobana et al. (2009). The millet was tempered with 7% water and milled at a local mill. The meal was sieved through a 60 micron sieve shaker and the tailings weighed to calculate the seed coat yield. They were stored in air tight containers at room temperature (25 ± 2 °C) until further use.

The FMSC sample was de-fatted and de-starched before extraction of the polyphenols. For de-fatting, the sample was treated with petroleum ether (boiling point 60–80 °C) in a Soxhlet apparatus for 16 h at 70 °C and de-starching was done using enzyme treatment. De-fatted sample (50 g) was mixed in 100 mL of sodium acetate buffer (0.1 M, pH 4.8) and 1 mL of α -amylase (1000 U) and incubated at 95 °C for 1 h. The mixture was cooled to room temperature (25 ± 2 °C) followed by addition of 100 mg of

glucoamylase (7000 U) and further incubated at 55 °C for 48 h. The mixture was then centrifuged at 3000 rpm for 20 min at room temperature (25 ± 2 °C). The supernatant containing released glucose was decanted and the residue was used for extraction of polyphenols.

Extraction of polyphenols

Conventional Heat reflux (HR) Sample (1 g) was refluxed with 30 mL of 1% HCl methanol at 60 °C for 60 min/cycle. The residue was tested for polyphenols using Folin–Ciocalteu Phenol reagent and then re-extracted with fresh solvent until all the polyphenols were extracted. The extracts were pooled, concentrated and used for further analysis. The extraction was repeated twice.

Ultrasound assisted (UA) extraction The polyphenol extraction was performed using an ultrasonication unit (SONICS Vibracell VCX750 Ultrasonic Cell Disrupter, Newtown, USA) equipped with a probe (13 mm titanium alloy). The sonication frequency and power were adjusted to 20 kHz and 250 W, pulse was set at 20 s on and off and amplitude at 30%. The UA process was optimized with three independent variables: A—extraction temperature (35–65 °C); B—extraction time (10–40 min) and C—solvent concentration (0–100%) at three levels (−1, 0 and 1) central composite design (CCD) using 1 g of FMSC with 30 mL of extraction solvent.

The dependent variable (response variable) was total polyphenol content (TPC) expressed as milligrams (mg) gallic acid equivalent (GAE)/g of FMSC. The optimal conditions and experimental values were predicted from an empirical second order polynomial regression model using Design-Expert 10 software.

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4AB + \beta_5AC + \beta_6BC + \beta_7A^2 + \beta_8B^2 + \beta_9C^2 \quad (1)$$

Average response values (Y) were obtained from the experiments performed in triplicates. The optimal conditions predicted with the RSM were validated by additional experiments and compared to values predicted by the model.

Following ultrasonication, the flask was instantly cooled to room temperature, centrifuged (10,000 rpm for 15 min) and the supernatant was concentrated using Heidolph rotary evaporator (Laborota 4001, Germany), lyophilized (Scanvac-110 Cool Safe Freeze Drier, Lynge, Denmark) and stored for further analysis (25 ± 2 °C).

As ultrasonic power is known to have a positive effect on the extraction of bioactives, additional experiments were carried out with enhanced ultrasonic power of 500 W

under the optimized conditions derived from the CCD model.

Enzyme pretreated ultrasonication In a separate set of experiments, enzyme treatment (EX, EC) was performed to evaluate the effect of the enzymes on the yield of polyphenols from the sample matrix. To 1 g of seed coat, 10 mL of sodium citrate buffer (pH 5.0) was added. Enzymes such as xylanase (50,000 U/mL) and cellulase (187,500 U/mL) at different concentrations (U) were added separately, incubated at 50 °C for 2 h and further deactivated (90 °C, 5 min). After decanting, the supernatant was concentrated using rotovap, lyophilized and stored (25 ± 2 °C) until further analysis.

In another set of experiments, the combined effect of enzymatic treatment followed by ultrasonication was studied. Samples were pretreated with enzymes as described above and the residues were dried at 70 °C in a hot air oven and subjected to ultrasonication at 500 W under the optimized conditions (55 °C, 30 min, 50% ethanol). The two supernatants (XUA, CUA) were pooled, concentrated using rotovap, lyophilized and stored (25 ± 2 °C) until further analysis.

The enzyme pretreatment of the sample and enzyme pretreatment followed by ultrasonication was repeated twice.

Analysis of FMSC extracts

Determination of total phenolics content (TPC) The Folin–Ciocalteu method described by Singleton and Rossi (1965) was employed to determine the TPC. The absorbance was measured at 765 nm using a spectrophotometer (Synergy H1 multi-mode microplate reader, Biotek, Germany) against a respective reagent blank. Gallic acid in the concentration range of 0.05–0.25 mg/mL was used to plot the standard curve and the results are expressed as mg GAE/100 g of sample.

Determination of total flavonoids Total flavonoid content was determined as described by Zhishen et al. (1999). Absorbance (510 nm) was measured using multi-mode microplate reader (Synergy H1 Biotek, Germany). The flavonoid concentration in the sample was determined by plotting the calibration curve (0.05–0.25 mg/mL) and expressed as mg catechin equivalents (CE)/100 g of sample.

Determination of total tannins The tannin content in the extract was determined using Vanillin assay (Price et al. 1978). Different concentrations of catechin ranging from 0.05 to 0.25 mg/mL were used to prepare the standard

curve and the total condensed tannins are expressed as mg of CE/100 g sample.

Scavenging activity of DPPH radical The DPPH free radical-scavenging assay was used to establish the antioxidant capacity of the extracted polyphenols (de Ancos et al. 2002). Percentage inhibition of the DPPH was calculated as follows using quercetin as standard (0.5–2.5 mg/mL)

$$\% \text{ Inhibition} = [(Ac - As)/Ac] \times 100,$$

where, As is the absorbance of the sample and Ac is the absorbance of the control.

ATR-FTIR analysis The Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy technique was used for determining the IR spectra of the extracts obtained by different methods. The IR spectrum in the range 4000–400 cm^{-1} at a resolution of 4 cm^{-1} using a Diamond ATR was investigated. Spectra were recorded on an FTIR-4100 spectrometer (Jasco Products, Easton Pennsylvania, USA).

HPLC characterization The qualitative analysis of phenolics were performed by HPLC (Shimadzu, SPD 20A) with a C18-4E (5 μm , Shodex, Japan) column using a binary gradient system (Rødtjer et al. 2006) with modifications. The mobile phase consisted of 2.5% acetic acid (Solvent A) and a mixture of acetic acid, methanol and water (2.5:95:2.5—Solvent B). The gradient was applied at a flow rate of 1.0 mL/min: 0–10 min, 100% A; and 10–110 min, 100% A to 50% A. The detector employed was a diode array detector (DAD) at 280 nm and identification was achieved by comparison with retention times of standard phenolic compounds.

ESI-MS spectra Electrospray Ionization Mass spectrum measured by Q-TOF (Micromass spectrometer, Waters, UK) to obtain the fingerprints of the FMSC phenolics. The conditions used were capillary voltage of 3.5 kV, core voltage 100 V, source temperature 120 °C, desolvation temperature 200 °C, desolvation gas (Nitrogen) 250 $\mu\text{L}/\text{min}$. The m/z values in the spectra obtained for the millet seed coat phenolics were matched with the m/z values obtained for the different standard phenolics.

Statistical analysis

Experiments were performed in triplicates and results obtained are expressed as mean $\pm$ standard deviation of error (figures). Karl Pearson's coefficient of correlation was derived using statistical tool Origin version 7.0; regression analysis with Design Expert software version

10.0 (Stat-Ease, Minneapolis). Differences were considered statistically significant when $p < 0.05$ (ANOVA).

Results and discussion

Optimization of UA variables by CCD

The effect of the independent and dependent process variables of the design matrix are presented in Table 1. The quadratic model for predicting the optimal point for UA was:

$$\begin{aligned} \text{Yield} = & 15133.72 + 286.33A + 560.220B + 56.56C \\ & + 726.54AB + 696.16AC + 442.00BC \\ & - 3247.58A^2 - 3412.20B^2 - 3269.53C^2 \end{aligned} \quad (2)$$

The ANOVA (Table 2) for the fitted quadratic model shows good agreement between R^2 pred (0.9235) and R^2 adj which confirms the significance of the model. The p value for lack of fit exceeding 0.05 suggests that the model fits the experimental data. Further, the coefficient of variance (CV) which was low (8.72%) indicated precision, reliability and reproducibility of the experimental data. Therefore, the model ($p < 0.0001$), can be applied for prediction in the range of the selected experimental

Table 1 Coded CCD and response values in ultrasonication

Run	A (°C)	B (min)	C (% v/v)	Response (mg GAE/100 g)
1	0.000	0.000	0.000	14,589.1
2	1.682	0.000	0.000	6953.1
3	0.000	0.000	0.000	14,465.0
4	0.000	0.000	0.000	15,272.0
5	− 1.000	− 1.000	1.000	3580.0
6	0.000	0.000	0.000	16,058.4
7	0.000	− 1.682	0.000	4428.4
8	0.000	0.000	0.000	15,362.4
9	1.000	− 1.000	1.000	4449.1
10	− 1.000	− 1.000	− 1.000	7077.2
11	0.000	0.000	− 1.682	5152.7
12	1.000	1.000	1.000	7015.1
13	0.000	0.000	1.682	6684.0
14	− 1.000	1.000	− 1.000	4969.1
15	− 1.682	0.000	0.000	5007.8
16	1.000	− 1.000	− 1.000	3621.4
17	1.000	1.000	− 1.000	5959.8
18	0.000	0.000	0.000	15,044.4
19	0.000	1.682	0.000	6601.3
20	− 1.000	1.000	1.000	4780.2

CCD central composite design

Table 2 ANOVA for response surface quadratic model in ultrasonication

Source	Sum of squares	Degree of freedom	Mean square	F value	<i>P</i> value
Model	4.105	9	4.561	85.99	0.0001
A-temperature	1.120	1	1.120	2.11	0.1769
B-Time	4.286	1	4.286	8.08	0.0175
C-solvent conc	43,684.66	1	43,684	0.082	0.7800
AB	4.223	1	4.223	7.96	0.0181
AC	3.877	1	3.877	7.31	0.0222
BC	1.563	1	1.563	2.95	0.1168
A ²	1.520	1	1.520	286.53	< 0.0001
B ²	1.678	1	1.678	316.32	< 0.0001
C ²	1.541	1	1.541	290.42	< 0.0001
Residual	5.305	10	5.305	–	–
Lack of Fit	3.626	5	7.253	2.16	0.2089

EX enzyme xylanase, *EC* enzyme cellulase, *XUA* enzyme xylanase ultrasonicated, *CUA* enzyme cellulase ultrasonicated, *HR* heat reflux, *UA* ultrasonic assisted extraction, *TPC* total polyphenol content

Mean $\pm$ standard deviation of three independent estimations

variables and suitable for efficient extraction of polyphenols from FMSC.

Analysis of response surfaces

The independent variables namely extraction temperature, extraction time and solvent concentration (A, B, C) for UA process had a positive impact on the polyphenols yield.

TPC increased with an increase in temperature of up to 50 °C (Fig. 1b), as a mild increase in local temperature may weaken the cell wall integrity, soften the plant tissue and hydrolyze the bonds of bound phenolic compounds (phenol–protein or phenol–polysaccharide) (Spigno et al. 2007). However, extraction temperature above 60 °C resulted in a decrease in the polyphenol content (Fig. 1b) which might be due to decrease in the number of cavitation bubbles which reduces the impact of cavity collapse on homogenized samples thereby decomposing thermo-labile constituents (Li et al. 2017). The effect of temperature in antioxidants extraction processes has been reported to be variable. Studies by Jerman et al. (2010) on olive fruit polyphenols reported that efficiency increases up to 45 °C after which a reduction in yield results. Zhang et al. (2009) in their studies on almond powder found that extraction yields were enhanced as the temperature increased from 60 to 80 °C. It was observed by Teh and Brich (2014) that inducing heat (70 °C) increases the phenomena of mass transport thereby maximizing the phenolic recovery and antioxidant capacity of canola seed cake extracts. This underscores the fact that the optimum temperature might be product/matrix dependent, since food/plant materials are complex and polyphenols are known to have interactions with fiber components as well as proteins (Ajila et al. 2011).

The ethanol/water mixture remains a unique extraction solvent, because water enables swelling of the plant material, and ethanol facilitates disruption of bonds between the solutes and plant matrices (d'Alessandro et al. 2012). Phenolics extracted using 50% ethanol was higher than that with 90% as seen from Fig. 1a. The polarity of the solvents is critical for the effective extraction of the polyphenols, since dilute solvent (water: ethanol/methanol) resulted in enhanced extraction compared to mono component solvents (d'Alessandro et al. 2012). A decrease in TPC was observed as the ethanol concentration increased above 55% (v/v) since it can cause protein denaturation, preventing dissolution of polyphenols. Such an observation was made from the studies in the ultrasonic extraction of polyphenols from black choke berry using 50% ethanol (d'Alessandro et al. 2012). These results are in accordance with the findings of Virot et al. (2010) who observed that the highest concentrations of total polyphenols in apple pomace by ultrasonication were obtained from 1:1 (v/v) water: ethanol mixture. This is because a wide range of phenols are soluble in aqueous ethanol which also has the advantage of being food grade as opposed to methanol (Ajila et al. 2011).

As a factor of time, the variance of extraction yields reached a maximum at 30 min and decreased further (Fig. 1c). Prolonged duration of extraction at high temperatures could have decreased the diffusion area, diffusion rate and led to the polymerization or degradation of heat sensitive polyphenols thereby decreasing the yield (Singh et al. 2017). The observations made by Chavan and Singhal (2013) are in tune with the results, reporting an increase in the yield of arecanut polyphenols with an increased sonication time up to 35 min, after which a reduction was observed. Similarly, various antioxidants from star fruits

Fig. 1 Three dimensional plots for quadratic model in Ultrasonic assisted extraction (UA), **a** interaction between temperature and time, **b** interaction between temperature and solvent, **c** interaction between time and solvent

were enhanced by ultrasonication in the methanolic extracts up to 30 min beyond which the yield and activity of polyphenols were lower (Annegowda et al. 2011). Teh and Brich (2014) inferred that ultrasonic extraction for 20 min resulted in high yields of phenolics and flavonoid contents from canola seed cakes after which there was a reduction in the content with respect to time.

Therefore, the optimal UA conditions for polyphenol from FMSC (250 W) were: temperature of 55 °C, time of 25 min and ethanol concentration of 50% to yield 16.06 ± 0.53 g GAE/100 g FMSC.

Enhancement of UA yield

That ultrasonic power has a positive effect on the yield of polyphenols has been reported in literature. This might be due to the collision of molecules when the ultrasonic power increases, facilitating the release of phenolic compounds. An increase in the yield (30%) from 16.06 ± 0.53 to 21.05 ± 0.47 g GAE/100 g FMSC was noted when power was enhanced from 250 to 500 W under the optimized

conditions (55 °C, 25 min, 50% ethanol). The results are in accordance with the reports of Lingzhu et al. (2015) in maize filament polyphenols, where yield increased with extraction power from 400 to 520.01 W, and decreased after 520.01 W. The high power probably resulted in increased temperature together with the degradation of the phenols due to oxidation (Spigno and De Favari 2009). This proves that ultrasonic power and frequency play a dynamic role in polyphenol extraction.

Effect of enzyme pretreatment

The aim of a pretreatment is to disrupt the plant cell wall structure by weakening/degrading the cellulosic-phenolic network so as to enable release and recovery of phenolic bioactives.

Figure 2a shows the effect of xylanase treatment and xylanase pretreated ultrasonication (EX, XUA) and Fig. 2b of the cellulase treatment (EC, CUA). The dual action of hydrolysis by enzymes and disruption by ultrasonication produced extracts with higher contents of total phenols

Fig. 2 **a** Effect of different concentrations of xylanase on TPC; **b** different concentrations of cellulase on TPC; **c** impact of conventional and non conventional extraction methods on polyphenols; **d** flavonoids and tannin content in different extracts; **e** radical scavenging activity of extracts

relative to EX and EC due to the liberation of the bound and unbound phenolics (Acosta-Estrada et al. 2014). In both the cases, the total polyphenol content increased dramatically with an increase in enzyme concentration up to 1500 U after which the phenolic levels decreased, as the substrate becomes limited at higher concentrations of enzymes (Barman et al. 2015). A 20% higher yield of polyphenols was observed with xylanase pretreatment (XUA)— 47.9 ± 1.22 g/100 g of FMSC (Fig. 2a) compared to cellulase pretreated sample (CUA)— 37.7 ± 1.23 g/100 g of FMSC (Fig. 2b). XUA yielding high polyphenols could be due to the presence of high content of hemicelluloses such as xylan-type polysaccharides covalently bound with the ferulic acid bridges, as revealed from our earlier studies in finger millet seed coat (Ayyappan et al. 2017).

Different enzymes have been shown to be suitable for different phenolic matrices. Lu et al. (2017) found that mixed/complex enzymes improved the extraction of phenolics from guava leaves than single cellulase enzyme. Also, Mushtaq et al. (2015), showed that mixed enzyme assisted (cellulase, pectinase and protease) supercritical extraction resulted in two-fold increase of polyphenols extracted from pomegranate peels. Till date, extraction of polyphenols from finger millet by methods other than heat reflux has not been reported.

Therefore, pretreatment with xylanase followed by ultrasonication at 500 W enhanced yields twofold compared to ultrasonication alone. Pretreatment with cellulase was marginally less effective with enhanced yield of 1.7 fold as seen from Fig. 2c.

Conventional HR method

Conventional process employs refluxing the seed coat with the acidic methanol solvent, raising the temperature and duration of the process. The total phenolic content by heat reflux was 20.92 ± 0.50 g GAE/100 g FMSC. Banerjee et al. (2012) have optimized the extraction by conventional method in the same variety (GPU 28) and reported a total phenolic content of 14.1 ± 0.02 g %. However, such extracts maybe limited by the presence of harmful residues and thus not conducive for food applications. Figure 2c explains the comparative analysis of polyphenol yield by different methods graphically. The phenolic content achieved by UA was similar to HR, but was advantageous in terms of utilizing a non-hazardous solvent, shorter extraction time (60 min/cycle to 30 min) and lower temperature (60–50 °C). Singh et al. (2017) had demonstrated that polyphenol content of mung bean hull increased during ultrasonication compare to conventional process. Pascal Agbangnan et al. (2012) also observed high polyphenol

content extracted from sorghum by ultrasonication which reduced the extraction time over 70% compared to conventional magnetic stirring (4–6 h). Further, an enzyme pretreatment followed by ultrasonication (XUA, CUA) enhanced the yield 2.2 fold in XUA and 1.8 fold in CUA against the conventional HR process. Results of Liu et al. (2018) also showed the efficiency of ultrasonication and enzyme treatment in effectively extracting the polyphenols from *Trapa quadrispinosa* (floating leaved macrophyte) three-fold than the conventional process heat extraction. The study clearly demonstrated that xylanase pretreatment followed by ultrasonication increases the polyphenol extracted from FMSC.

Characterization of polyphenol extracts

The characteristics of polyphenol extracts obtained from (1) xylanase pretreated ultrasonicated (XUA), (2) ultrasonicated at 500 W (UA) and (3) heat reflux (HR) are discussed below.

Total flavonoids and total tannins

The flavonoid and tannin content in the extracts are represented graphically in Fig. 2d from the different processes. UA (492 ± 0.57 CE mg/100 g FMSC) and XUA (483 ± 0.57 CE mg/100 g FMSC) showed similar flavonoid content, while HR resulted in lower values (350 ± 0.50 CE g/100 g FMSC). With respect to tannins, XUA gave maximum yield (915 ± 0.86 CE mg/100 g FMSC) followed by UA (850 ± 1.0 CE mg/100 g FMSC) and HR (725 ± 1.1 CE mg/100 g FMSC). The results are in line with studies of Singh et al. (2017) who recorded high flavonoid and tannins contents using ultrasound treatment in comparison to traditional process. The factors contributing to differences may be explained by the solubility of flavonoids and tannins with respect to polarity of solvent used as well as thermal degradation of phenolics during HR (Carrera et al. 2012).

Radical scavenging activity by DPPH

Plant phenolics are receiving great research interest due their impressive antioxidant properties that helps fight oxidative stress and related health disorders. This makes it important to define the efficacy of the recovery process in terms of its extracted antioxidants.

A dose dependent inhibition of DPPH activity (Fig. 2e) was observed due to the redox properties of the polyphenols in the extracts. The hydrolysates from XUA showed the highest scavenging of 75%; UA had a similar effect with antioxidant activity of up to 70%, whereas HR showed a lesser effect of 65%. The higher radical scavenging

potential of enzyme treated samples may be due to the hydrolytic release of bound phenolics (Mushtaq et al. 2015). Liu et al. (2018) also observed highest antioxidant activity by *T. quadrispinosa* stem residues which were attributed to the polyphenols extracted efficiently by enzyme pretreated ultrasonication. A significantly high correlation ($p < 0.05$) between different extracted concentrations of polyphenols and the corresponding antioxidant activity was observed ($r = 0.981$ for HR, 0.986 for UA, 0.988 for XUA) in FMSC. Such a positive correlation was also observed by Liu et al. (2018) in sorghum polyphenols and d'Alessandro et al. (2012) in ultrasonic extracted black chokeberry polyphenols.

FTIR spectra of extracted polyphenols

Fourier Transform Infrared (FT-IR) spectroscopy was used to obtain structural information and molecular features of the extractives (Fig. 3). The signals confirm the presence of various phenolic structural groups. The peak signals for HR, UA and XUA at 3436 , 3500 and 3120 cm^{-1} are due to OH wagging which is particular to phenolic groups, while the peaks at 2950 , 2940 and 2980 cm^{-1} are specific to the OH group again confirming the presence of phenolics (Oliveira et al. 2016). The peaks at 2380 and 2100 cm^{-1} in HR and UA extracts could not be identified. A spectral peak at 1680 cm^{-1} in both HR and XUA while at 1700 cm^{-1} in UA could be due to stretching vibration of C=C groups; stretching vibration of C=O due to presence of flavonoids and amino acids; or the C=O stretching vibration of caffeic acid and its derivatives (Oliveira et al. 2016). The band at 1480 , 1440 and 1320 cm^{-1} in HR, UA and XUA could be related to CH_3 , CH_2 , flavonoids and aromatic groups, with bending (δ) vibration of C–H and the stretching vibration of aromatics (Oliveira et al. 2016). A defined band at 1000 cm^{-1} in HR and 1050 cm^{-1} in UA and XUA could indicate the structural conformation of gallic acid (Kannan et al. 2011). A spectral band at 620 and 650 cm^{-1} in the extracts is attributed to the C-H aromatic bond formation (Bancuta et al. 2015). The confirmation of phenolic functional groups in the extract indicates that all three methods enable recovery of polyphenols.

Polyphenolic profile

Reverse phase HPLC analysis of the millet seed coat phenolics showed the presence of gallic acid, ferulic acid, trans-cinnamic acid, quercetin and caffeic acid along with some unidentified peaks in all the extracts. A similar range of 5–6 phenolic compounds was observed by Shobana et al. (2009) in the finger millet characterization studies of the same variety (GPU 28). A range of phenolic compounds from lower molecular weight simple phenols to higher

Fig. 3 FTIR spectra of polyphenols extracted by **a** heat reflux (HR) **b** ultrasonic assisted extraction (UA) **c** xylanase pretreated ultrasonication (XUA)

molecular weight tannins and anthocyanins are reported to be present in FMSC.

Since all peaks could not be identified by RP-HPLC, direct infusion of Electro Spray Ionization Mass Spectrometry (ESI-MS) was carried out. Phenolic metabolites

identified by their m/z values, from ESI-MS finger prints of the extracts were compared (given as supplementary data), and the spectra of ESI MS is illustrated in Fig. 4. Phenolic acids such as hydroxy benzoic and hydroxy cinnamic acids, flavonoids comprising about 5 different sub

families were detected in the extracts showing similarity up to 80% in all the extracts. These agree with the spectral analysis of extracted phenolics by HR in FMSC (v. GPU 28), carried out by Shobana et al. (2009).

HR of FMSC resulted in the recovery of phenolic acids (4-O-methyl gallic acid, syringic acid, 4-hydroxy benzoic

acid, apigenin, kaemferol, myrecitin, caffeoyl glucose, quercetin) and certain flavonoids while catechins and cyanidins were absent among them. A noticeable feature in UA and XUA samples was the presence of 3-(3''-malonyl) glucoside cyanidin; 6-C-pentosyl-8-C-pentosyl luteolin, a flavone; and trimers of catechins. These are known to

Fig. 4 ESI-MS of polyphenols from **a** heat reflux (HR) **b** ultrasonic assisted extraction (UA) **c** xylanase pretreated ultrasonication (XUA)

possess strong antioxidant activity which has also been documented in the present study. Certain caffeic acid derivatives such as caffeoyl shikimic and dicaffeoyl shikimic acid were found only in XUA extracts suggesting the role of xylanase in releasing these compounds. These differences may be explained by the extraction methods where different mechanisms operate. It is significant that the non-conventional extraction resulted in an altered profile which has scope for utilization in the development of food products enriched with specific polyphenols.

Conclusion

The process of ultrasound assisted extraction of finger millet polyphenols has been optimized. The method has shortened the extraction time threefold, decreased the requirement for solvent threefold and lowered the operating temperature by 10 °C compared to the conventional heat reflux method. The study underscores the effectiveness and advantages of ultrasonication despite similar yield of FMSC polyphenols. Pretreatment with xylanase followed by ultrasonication enhanced the yield twofold compared to the conventional process accompanied by a corresponding increase in antioxidant activity. Distinctive phenolics—caffeoyl, dicaffeoyl shikimic acid, luteolin and cyanidin were recovered by ultrasonication and enzyme assisted ultrasonication. This is the first study to report the enhancement of polyphenol extraction by a combination of enzyme treatment and ultrasonication in finger millet seed coat which has immense potential for food applications. The use of extracted polyphenols as alternatives to synthetic antioxidants can be explored in various food systems.

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Compliance with ethical standards

Conflict of interest There All authors declare that they have no conflict of interest.

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