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Hydroxycinnamic acid bound arabinoxylans from millet brans-structural features and antioxidant activity

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ABSTRACT

Hydroxycinnamic acid bound arabinoxylans (HCA-AXs) were extracted from brans of five Indian millet varieties and response surface methodology was used to optimize the extraction conditions. The optimal condition to obtain highest yield of millet HCA-AXs was determined as follows: time 61 min, temperature 66 °C, ratio of solvent to sample 12 ml/g. Linkage analysis indicated that hydroxycinnamic acid bound arabinoxylan from kodo millet (KM-HCA-AX) contained comparatively low branched arabinoxylan consisting of 14.6% mono-substituted, 1.2% di-substituted and 41.2% un-substituted Xyl*p* residues. The HPLC analysis of millet HCA-AXs showed significant variation in the content of three major bound hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic acid). The antioxidant activity of millet HCA-AXs were evaluated using three *in vitro* assay methods (DPPH, FRAP and β-carotene linoleate emulsion assays) which suggested both phenolic acid composition and structural characteristics of arabinoxylans could be correlated to their antioxidant potential, the detailed structural analysis revealed that low substituted KM-HCA-AX exhibited relatively higher antioxidant activity compared to other medium and highly substituted HCA-AXs from finger (FM), proso (PM), barnyard (BM) and foxtail (FOXM) millet.

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1. Introduction

Millets are small seeded cereal crops belonging to the family poaceae. Millets can grow well in dry zones as rain-fed crops under marginal conditions of soil fertility and moisture. In many parts of India, especially in rural areas millets are considered as staple diet for large low income population. India is the largest producer of many kinds of millets which are often referred as “nutri-cereals” [1]. Besides, millets being rich in fibers, antioxidants and complex carbohydrates are the potential candidates for having several health benefits as epidemiological studies have clearly demonstrated that consumption of whole grain and soluble dietary fibers have beneficial effects against cardiovascular diseases, cancer and diabetes [2,3]. Many of the life style disorders and chronic diseases are associated with oxidative stress which is combined with free radical formations such as superoxide anions, hydroxyl radicals and nitric oxide radicals. Phenolic acids such as ferulic acid and other hydroxycinnamic acid derivatives exhibit very strong antioxidant activity; since they form a resonance stabilized phenoxy radicals and terminates free radical chain reactions. Cereal grains are rich in both free and bound phenolic compounds, majority of these phenolic compounds are concentrated in the bran of cereal kernels. However, in cereals significant level of phenolic acids in the bound form are esterified to 5′-hydroxyl group of arabinose of cell wall arabinoxylan polysaccharides and influences their physicochemical and functional properties [4]. More interestingly, hydroxycinnamic acid bound arabinoxylans as cell wall component of the cereal grain exhibit stronger antioxidant activities than free acids [5–7]. Several studies on the antioxidant activity of phenolic acid bound arabinoxylans from wheat bran were reported [8–10]. A recent study demonstrated the antioxidant capacity of arabinoxylan oligosaccharides from wheat aleurone [11], it was also documented that two heteroxylans from wheat bran exhibited strong antioxidant and anti-complementary activities [12]. Many studies regarding the antioxidant potential of whole millet grains have been reported [13,14]. However, there is limited information on structural characteristics and biological activities of non-starch polysaccharides from different species of millet grains, the previous investigations were mainly on non-starch polysaccha-

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rides from finger millet. Earlier study on water soluble non-starch polysaccharides from native and malted finger millet revealed that feruloyl arabinoxylans exhibited very strong antioxidant activity which could be several folds (4.9-1400) higher than expected activity due to ferulic acid content and 1300-5000 folds higher than activity of sulphated polysaccharides [7,15]. The study also suggested processing methods such as malting and cooking affected antioxidant content and activity [7,16]. Furthermore, a comparative study on *in vitro* antioxidant potential of non-starch oligosaccharides (xylo-oligosaccharides) from rice, ragi (finger millet), wheat and maize revealed relatively higher antioxidant capacity of finger millet xylo-oligosaccharide mixtures compared to other cereal xylo-oligosaccharide mixtures [17]. A recent study also reported the immunomodulatory activity of purified arabinoxylans from finger millet [18]. To best of our knowledge, there is no data documented in the literature about the comparative study of structural features and antioxidant activity of the HCA bound arabinoxylans (HCA-AXs) from Indian millet varieties. Hence, the aim of the present study is to evaluate the *in vitro* antioxidant activity of HCA bound arabinoxylans from five small seeded Indian millet varieties (KM: kodo, FM: finger, PM: proso, BM: barnyard and FOXM: foxtail millet) in relation to their structural characteristics. The present study can be exploited in preparing nutraceutical health foods based on dietary fibers enriched with HCA-AXs from Indian millet brans.

2. Material and methods

2.1. Materials

Authenticated varieties of five Indian millets namely kodo (JK48), finger (RA14), proso millet (TNAV-145), barnyard (CO-KV-2) and foxtail (CO-Te-7) were procured from Tamil Nadu agricultural university. All the analytical grade chemicals, reagents and solvents were procured from Sigma-Aldrich chemicals (USA). Acetic anhydride was procured from Fisher scientific (India). Amylase, amyloglucosidase and protease were procured from Megazyme (Ireland).

2.2. Extraction of millet HCA-AXs

Five Indian millet varieties were milled to prepare the powdered materials. 100 g of the each powdered material was washed with de-ionized water (5 × 200 ml) and filtered through sieve cloth to partially remove starchy endosperm from bran. The collected bran was washed with chloroform: methanol (9:1, 200 ml) to remove lipophilic and colored extractive compounds, the bran obtained was treated with α-amylase, amyloglucosidase and protease to remove the residual starch and protein respectively [17]. Finally, the resulting water unextractable polysaccharides (WUP) was treated with 0.5% NaOH [12]. The alkali extracted material was centrifuged and neutralized with glacial acetic acid and dialyzed against deionized water (8 × 5 l) and finally lyophilized to prepare hydroxycinnamic acid bound arabinoxylans (HCA-AXs).

2.3. Experimental design for optimization of FM-HCA-AX extraction and statistical analysis

Single factor experiments were carried out to determine preliminary range of extraction variables such as extraction time (A), extraction temperature (B), ratio of solvent to sample (0.5% NaOH to WUP, C). Then, 17 runs based on Box-Behken (BBD) were carried out in random order to statistically optimize the yield of finger millet HCA-AX as shown in Table 1. The three factors were designated as +1, 0, and −1 for high, intermediate and low levels. All the tests were performed in triplicate. Analysis of variance (ANOVA) was performed using RSM and the test for significance was conducted at $P < 0.05$. Statistical analysis was conducted using the software of Design-Expert 9 (Stat-Ease, Inc.; Minneapolis, MN, USA)

Table 1
BBD with observed responses for the extraction yield of FM-HCA-AX.

Run	Time (min) A	Temperature (°C) B	Ratio (ml/g) C	Yield (%) Y
1	60	60	10	5.43
2	60	60	10	5.38
3	60	60	10	5.29
4	90	60	15	4.88
5	30	60	15	4.04
6	90	60	5	3.72
7	30	75	10	5.33
8	90	75	10	5.83
9	60	75	5	4.36
10	90	45	10	3.79
11	60	45	5	2.54
12	30	45	10	4.04
13	60	45	15	3.02
14	30	60	5	2.81
15	60	60	10	5.58
16	60	60	10	5.68
17	60	75	15	5.43

2.4. Isolation and characterization of bound phenolic acids

The bound phenolic acids were isolated from millet HCA-AXs upon alkaline hydrolysis [19,20]. Isolated phenolic acids dissolved in methanol were quantified by HPLC on a reverse phase C_{18} column (Waters Spherisorb, 5 μm, particle size, 46 mm × 250 mm) using a solvent system of water/acetic acid/methanol (83:2:15) with diode array detection at 320 nm. The elution from the HPLC was infused into the mass spectrometer (Q-TOF MS) (ABSCIEX 5600) using electrospray ionization (ESI) in negative mode. The MS was set as follows, source temperature: 100 °C, voltage applied to the capillary tip was 4.5 kV, nitrogen was used as the nebulising gas at a pressure of 20 psi, a flow rate of 10 l/min; capillary heater was set to 275 °C, syringe rate 3 μl/min.

2.5. Glycosyl composition analysis

2.5.1. Neutral sugar composition analysis

The sugar compositions of the millet HCA-AXs were determined by GC–MS as alditol acetate derivatives [21]. Briefly, HCA-AXs (~1 mg) were hydrolyzed with 2 M TFA at 120 °C for 2 h, each of the hydrolyzed material was reduced with $NaBD_4$ at room temperature for 2 h and acetylated with acetic anhydride-pyridine (1:1) at 50 °C for 20 min to prepare alditol acetate derivatives.

2.5.2. Estimation of uronic acid

The content of uronic acid in the millet HCA-AXs was estimated by carbazole method [22]. Briefly, to 0.5 ml solution of each HCA-AX (100–500 μg/ml) kept in ice bath, concentrated sulphuric acid was added. Samples were incubated in boiling water bath for 20 min; purified carbazole solution (0.1 ml, 0.1% in ethanol) was added to each sample and kept in dark for 2 h. Absorbance of each sample solution was measured at 530 nm. Standard solution of D-glucuronic acid (20–100 μg/ml) was used to prepare standard graph.

2.6. Linkage analysis

Millet HCA-AXs were methylated as described by Ciucanu and Kerek [23]. Each lyophilized sample (~2–3 mg) was dissolved in DMSO (2 ml) and freshly prepared powdered NaOH (~15 mg) was added. The reaction mixture was allowed to stand at 21 °C for 20 min, iodomethane (1 ml) was added and sample was stirred at

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