International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(1): 328-332

Received: 17-11-2017
Accepted: 25-12-2017

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Evaluation of hydroxycinnamic acids in finger millet (*Eleusine coracana*) grain cultivars by HPTLC

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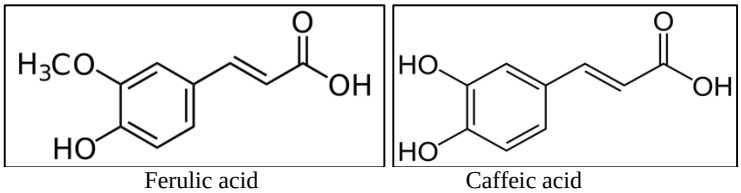
Abstract
Finger millet (*Eleucine coracana*) grain cultivars were analyzed for the phenolic components (hydroxycinnamic acids) which have beneficial effects on human health. The present study was conducted to evaluate the phenolic acids *viz*., ferulic acid and caffeic acid in four different varieties of finger millet. Phenolic compounds were extracted in grain samples and were primarily evaluated for total phenolic content (TPC), total flavonoid content (TFC) and antioxidant property. TPC was analyzed by Folin-Ciocalteu method, TFC by aluminium chloride method and antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Further, the sample extracts were subjected to High Performance Thin Layer Chromatography (HPTLC) analysis and the method was standardized for the evaluation of ferulic acid and caffeic acid, identification was done by comparing obtained R_f values of the reference standards with the sample extracts and quantification was done using the peak areas of the obtained chromatograms. Results revealed that TPC (949.57 GAEµg/g) was high in GPU28, whereas TFC (805.16 REµg/g) and antioxidant activity (53.38 I%) was found to be high in GPU48 variety.

Keywords: Finger millet, phenolic, ferulic acid, caffeic acid, HPTLC

1. Introduction
Millet grain phenolics possess bioactivities against several pathophysiological conditions and may serve as potential natural sources of antioxidants in food and biological systems [18]. Dietary polyphenols exert beneficial biochemical properties such as free radical scavenging, metal chelation and inhibition of lipid peroxidation [3]. These grains 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 [20].
Finger millet (*Eleusine coracana*), is an important staple food for thousands of year which is a drought resistant crop and form staple food for people belonging to low-income groups. It is commonly known as 'Ragi' and is one of the important minor millets of Indian subcontinent and some of the African countries, it is a rich source of several phytochemicals, dietary fiber and minerals especially calcium [9]. Finger millet stands unique among the minor cereals because of its superior nutritional contents. Since finger millet form good source of macro and micro nutrients, this could serve as promising staple grain for therapeutic diets [18]. It also contains substantial proportion of polyphenols, phytates and dietary fibre [4]. Finger millet seed coat is a reserve of several phenolic compounds such as phenolic acids, flavonoids, polymeric tannins and anthocyanins, and is an effective inhibitor of pancreatic amylase and intestinal a-glucosidase [4, 19].



Ferulic acid Caffeic acid

Fig 1: Chemical structures of phenolic acids

Caffeic acid, one of the most prominent naturally occurring cinnamic acids, is known to selectively block the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma, and allergic reactions [11]. Phenolic compound ferulic acid has added health benefits as anti-cancer agent [5]. The interest for these compounds increased in relation to their antioxidant activity [7, 15]. Caffeic, chlorogenic, and ferulic acids protect human LDL from oxidation [12]. Ferulic acid dehydrodimers or bran hemicellulose fragments containing ferulic acid also exhibit good antioxidant activities [2, 6, 13].

The general growing demand for novel tasty and healthy foods together with the increasing number of people suffering from celiac disease and wheat intolerances, has driven a new market, consisting of cereal products made from grains alternative to wheat and rye, in which oat, sorghum and millet have gained a special position [1].

As proper utilization of finger millet variety rich in antioxidants will help the consumer to avail its maximum nutritional benefits by incorporating them into several convenience foods. Hence, the present study was undertaken to determine the major phenolic acids which are commonly found in cereal grains *i.e*., ferulic acid and caffeic acid in different varieties of finger millet grain samples. A new method of analyzing these phenolic acids by High Performance Thin Layer Chromatography (HPTLC) is developed for the first time in the study, which is reliable, fast and accurate.

2. Materials and Methods

2.1 Plant Materials

Finger millet grain varieties (GPU28 and GPU48) were procured from University of Agricultural Sciences, Dharwad, Karnataka for the study. Finger millet local variety was purchased from the local market. White variety was procured from farmer field in the Dharwad district.

2.2 Chemicals

Analytical grade reagent chemicals aluminium chloride, ethyl acetate, ethyl alcohol, hydrochloric acid, methanol, sodium hydroxide, sodium nitrite, sodium sulphate and sodium carbonate were procured from Rankem, Bangalore, India. Folin-Ciocalteu reagent and HPTLC plates, Silica gel 60 F_{254} were procured from Merck KGaA, Darmstadt, Germany. Gallic acid, rutin, butylayed hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA) and 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co., St.Louis, USA.

2.3 Experimental protocols

2.3.1 Extraction of bioactive compounds

Phenolic compounds were isolated according to the method [8]. Flours of the grain samples were extracted using 70% (v/v) ethyl alcohol (4 x 50 mL, 3 h each) with agitation; the supernatants were obtained by centrifugation at 15,000 rpm for 15 min at 4˚C (Sigma 3K30, Germany) and concentrated under vacuum with rotary evaporator at 50˚C, 45 rpm (Hahnvapor, Hahnshin Scientific Co., Korea) and the pH was adjusted to 2.00 with 4 M HCl. Phenolic compounds were separated by ethyl acetate phase separation (4 x 50 mL), and the pooled fractions were treated with anhydrous sodium sulphate to remove moisture; filtered, and evaporated to dryness. The residue obtained is redissolved in methanol (1ml) and stored at -20 ˚C till further analysis.

2.3.2 Total Phenolic Content (TPC)

TPC was determined by the method described by Singleton and Rossi [21]. Briefly, the appropriate dilutions of extracts were reacted with the Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate. Incubated at room temperature under dark for 60minutes and the absorbance were measured at 765nm using UV-Visible Spectrophotometer (Cary 50, Varian, Middelburg, Netherlands). Gallic acid was used as reference standard, and TPC of the samples were expressed as gallic acid equivalent microgram per gm (GAE µg/g) of sample on dry basis.

2.3.3 Total Flavonoid Content (TFC)

TFC was determined by aluminium chloride colorimetric method [22] with minor modification explained by Hemalatha and Giridhar [10]. In brief, aliquots (1ml) of appropriately diluted extracts or standard solutions were pipette into 15ml polypropylene conical tubes containing 2 ml double distilled H_2O and mixed with 0.15ml of 5% $NaNO_2$. After 5min, 0.15ml of 10% $AlCl_3.6H_2O$ solution was added and the mixture was allowed to stand for another 5min, and then 1ml of 1M NaOH was added. The reaction solution was well mixed, incubated at room temperature for 15min and the absorbance was determined at 415nm using the UV-Visible Spectrophotometer (Cary 50, Varian, Middelburg, Netherlands). Rutin was used as reference standard, and TFC of the samples were expressed as rutin equivalent microgram per gm (RE µg/g) of sample on dry basis.

2.3.4 Evaluation of Antioxidant activity

Free radical-scavenging capacity in phenolic extracts of finger millet cultivars were estimated according to the previously reported procedure using the DPPH radical [16]. In brief, appropriately diluted aliquot (1 mL) of phenolic extracts were mixed with the freshly prepared 1mL of (200 µM) DPPH in ethyl alcohol. The control contained all the reagents except phenolic extracts. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured using UV-Visible spectrophotometer (Cary 50, Varian, Middelburg, Netherlands) at 517 nm. Absorbance data were collected and transformed to radical scavenging activity (I%) according to equation:

$$I\% = (Ac-As) \times 100/Ac$$

where 'Ac' is the absorbance of control (without extract) and 'As' is the absorbance of sample. Synthetic antioxidants such as BHA and BHT were used for comparison.

2.3.5 HPTLC conditions and analysis

HPTLC analysis was performed on precoated silica gel 60 F_{254} aluminium back HPTLC plates (Merck, Darmstadt, Germany) of size 20 x 10cm. The standard solutions along with sample extracts were applied onto the plates as 8mm length bands with automatic sample applicator (Linomat V, CAMAG, Switzerland) with the aid of Hamilton syringe (10µL). Development of the plate was done at room temperature (27±2 °C) in the vertical glass twin-trough glass chamber (20 cm × 10 cm, with metal lids; CAMAG, Switzerland) which was previously saturated with mobile phase (20mL) for 20 minutes. Mobile phase was composed of solvents toluene, ethyl acetate, formic acid and methanol in the ratio 6:6:1.6:0.4 (v/v) respectively. The plate was

developed up to a distance of about 75 mm and was air-dried at room temperature for 20 min and then heated at 105±2 °C for 5 minutes in hot air oven. The plate was observed for the compact bands inside UV cabinet (CAMAG, Switzerland). Scanning was performed in TLC Scanner III (CAMAG, Switzerland) and quantification of phenolic acids was done at wavelength 254nm in absorbance mode with winCATS software (Version 1.2.0). A deuterium (D2) lamp was used to scan the plates with the remission absorption mode having the slit width 6.0 × 0.30 mm (micro). The densitograms were further scanned for their in situ UV spectra from 200 to 700 nm and overlaid with the UV absorption spectra of phenolic acid standard in all the sample extracts (Fig.2).

3. Results and Discussion

3.1 Phenolic compounds

TPC and TFC in the analyzed finger millet grain cultivars is presented in Table 1. TPC was in the range from 482.92 to 949.57 GAE µg/g, it was in the sequence GPU28 (949.57)>GPU48 (926.11)>Local (840.51)>White (482.92). Whereas, TFC was comparatively less than TPC which was ranging from 283.44 to 805.16 RE µg/g. Wherein GPU48 got high TFC (805.16 RE µg/g) compared to other varieties. A similar study reported that, the polyphenols extracted in finger millet exhibited a very high degree of sensitivity to changes in pH [4].

Table 1: TPC, TFC & antioxidant activity in finger millet varieties

Variety	Total phenolic content (GAE µg/g*)	Total flavonoid content (RE µg/g*)	Antioxidant activity (I%)
GPU28	949.57	786.99	49.52
GPU48	926.11	805.16	53.38
White	482.92	283.44	28.44
Local	840.51	756.70	45.69

*GAE-Gallic acid equivalent; RE-Rutin equivalent; All values are means of three replicate experiments

3.2 Antioxidant activity

The antioxidant activity analyzed by radical scavenging activity method using 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) in the grain samples is presented in Table 1. It was in the range from 28.44 to 53.38 I%. Amoung the varieties, GPU48 variety had the highest antioxidant property i.e., 53.38 I%, whereas White variety had the least antioxidant property i.e., 28.44 I%. The antioxidant activity was found to be significantly lower than that of synthetic antioxidants such as butylated hydroxyanisole (BHA, 65.03) and butylated hydroxytoluene (BHT, 70.28) for the concentration 10 µg/ml.

3.3 HPTLC analysis

Thin layer chromatography, in particular its high-performance application, is still a widely used analytical technique in analysis of pharmaceuticals, botanicals, foodstuff, environmental and clinical samples [17].

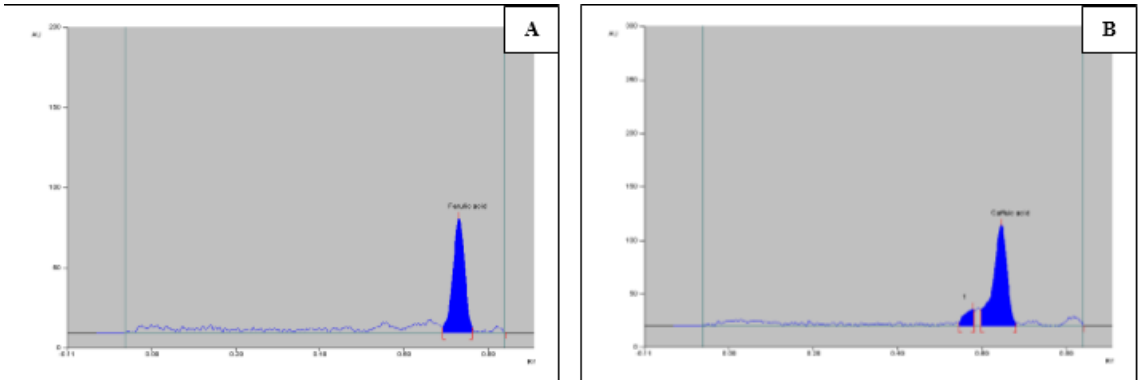
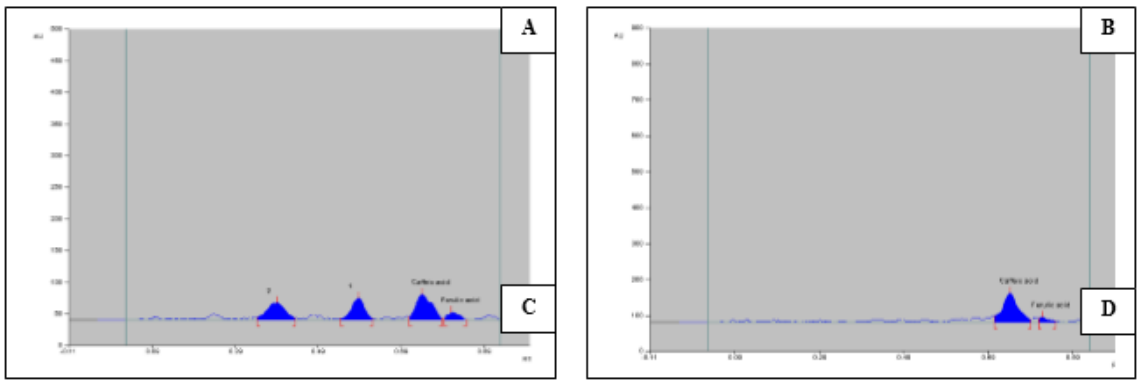


Fig 2: HPTLC chromatogram of reference standards (A) Ferulic acid (B) Caffeic acid

A new method of HPTLC analysis of phenolic acids is introduced in the present study, which is fast, reliable and accurate for quantification of phenolic compounds such as ferulic acid and caffeic acid which are commonly found in cereal grains. The phenolic compounds were analyzed in all the finger millet varieties used in this study. Ferulic acid and Caffeic acid were identified in the sample extracts, and were confirmed by comparing the R_f values with the reference standard. The R_f values and area under peak of the sample extracts is given in Table 2.



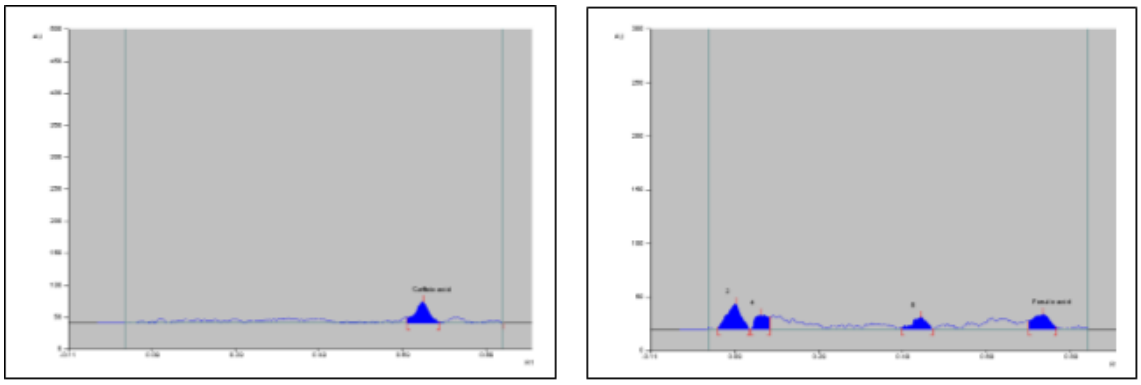


Fig 3: HPTLC chromatogram of finger millet sample extracts (A) GPU28 variety, (B) GPU48 variety, (C) Local variety, (D) White variety

The R_f values obtained for the reference standards was: Ferulic acid (0.82) and Caffeic acid (0.74), presented in Figure 2. Quantification of ferulic acid and caffeic acid in the detected samples was done using the calibration curve obtained with increasing concentrations (100-300ng) of standard solutions.

The results obtained for the finger millet grain varieties samples is presented in Table 2. Both the phenolic acids *viz*., Ferulic and Caffeic acid were only detected in GPU28 and GPU48 varieties, whereas Local variety showed presence for Caffeic acid, whereas Ferulic acid was not detected. However, White variety showed presence of Ferulic acid and Caffeic acid was absent in it (Fig.3). High concentration of Ferulic acid was obtained in White variety (128.55ng) and Caffeic acid in GPU28 variety (496.94ng). Since these both the varieties, GPU28 and GPU48 which had the presence of both the phenolic acids with varying concentrations can be utilized in developing functional foods with health beneficial effects.

Table 2: R_f value, Area & Concentration of Ferulic acid & Caffeic acid in different varieties

Finger millet sample extracts						
Variety	**Ferulic acid**			**Caffeic acid**		
	R_f value	**Area (AU)**	**Concentration (ng)**	**R_f value**	**Area (AU)**	**Concentration (ng)**
GPU28	0.82	375.60	112.83	0.74	2527.36	496.94
GPU48	0.82	260.98	78.29	0.74	1302.91	256.15
Local	ND*	ND*	ND*	0.74	906.50	178.21
White	0.82	428.53	128.55	ND*	ND*	ND*

ND*-Not detected

4. Conclusion

Due to their favorable nutritional properties and phytochemical associated health benefits, these millets offer an enormous potential for use as ingredients in functional food product development [14]. The present study revealed the total phenolic and flavonoid content in different varieties of finger millet, which will give an insight into the concentration of these health beneficial components. These phenolics are proved to have antioxidant property, which was studied in these finger millet varieties by DPPH radical scavenging method and revealed the antioxidant activity with varying concentrations. A new HPTLC method was developed in the study for analyzing the phenolic acids *viz.*, ferulic acid and caffeic acid in these grains. These phenolic compounds were present in the analyzed samples with varying concentrations, which have been previously researched to combat several health disorders. Hence the study will help in identifying a specific variety which is enriched with phenolic compounds and hence itcan be developed into functional food to act against several disorders. Also their usage in commercial food systems such as multigrain and gluten-free products would help in increasing the consumption of millets amongst non-millet consumers.

5. References

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