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ARTICLES

Determination of Phenolic Acids and a Flavonoid in *Eleusine coracana* (L.) by Semi-Preparative HPLC Photo Diode Array Detector[1]

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Abstract—Semi preparative HPLC procedure is proposed for the determination of six phenolic acids and one flavonoid in *Eleusine coracana (L.)* with gradient elution and photodiode array detection. The influence of composition of the mobile phase concentration of the mix modifier and temperature on the separation of gallic acid, *p*-hydroxybenzoic, vanillic, caffeic, ferulic, and cinnamic acids and quercetin for 90 min is studied. The procedure was applied for the separation of phenolic compounds in methanolic extracts of *Eleusine coracana (*L.*)*. The lower limits of quantification of phenolic compounds and the flavonoid are 0.050–0.150 μg/mL.

Keywords: phenolic compounds, flavonoids, medicinal herbs, semi-preparative HPLC

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Eleusine coracana (L.), native to East African highland, India and China, is considered as one of the richest sources of phenolic compounds [1]. It contains flavonoids, which have high biological activities [2]. Many synthetic antioxidants are available but their long term or frequent use can cause potential hazard to human metabolism [3–4]. Therefore, new interest has been developed as purifying and characterizing safe antioxidants from natural sources. The predominant flavonoids and phenolic acids are almost exclusively present in glycosylated forms in *Eleusine coracana* (L.) [5]. Attempts were made to determine the contents and physiological activity of phenolic compounds in *Eleusine coracana* (L.) due to the apparent relationship of phenolics in minor millet with prevention of chronic diseases. Along with other antioxidant compounds, phenolics as natural secondary metabolites have been reported the drugs for hypertension, diabetes, heart diseases, osteoporosis, some forms of cancer and avian flu. Endometriosis, chronic fatigue syndrome, tetanus, different types of cancers, Lyme disease, chronic ear infection and even obesity are considered chronic diseases and natural phenolic acids are found to exhibit good therapeutic activities with minimum or no side effect [6]. There are many factors influencing the phenolics and their antioxidant capacity of *Eleusine coracana* (L.).

All the procedures listed above provide the determination of total phenolic substances; however, in most of cases in pharmacology, it is important to know the composition of the components. Chromatographic methods are employed in such cases however, they also have some disadvantages [7–10]. Thin layer chromatography is not sufficiently precise, while gas chromatography does not provide the determination of the majority of phenolic compounds. Taking into account the properties of phenolic compounds, HPLC can be considered as the most preferable method [11–12].

The aim of the present study is to develop a procedure for the determination of individual phenolic compounds in groups of phenolcarbonic, cinnamic acids and flavonoids in medicinal herbs by semi-preparative HPLC with photodiode-array (**PDA**) detector.

EXPERIMEMTAL

Reagents. The following phenolcarbonic acids were used in the study: gallic acid, *p*-hydroxybenzoic acid, vanilic acid, caffiec acid, ferulic acid and cinnamic acid were procured as standards from Himedia Chemicals, M/s Spectrochem Pvt. Ltd. and quercetin

[1] The article is published in the original.

Table 1. Retention factor (k) and selectivity coefficient (α) in the separation of phenolic compounds and one flavonoid in a model mixture using Gradient 4 ($P = 0.95$, $n = 3$)

Compound	k	α
Gallic acid	0.47 ± 0.01	–
p-Hydroxybenzoic acid	5.1 ± 0.01	3.34 ± 0.04
Vanillic acid	6.9 ± 0.2	1.62 ± 0.05
Caffeic acid	9.2 ± 0.2	1.28 ± 0.001
Ferulic acid	11.2 ± 0.3	1.19 ± 0.001
Cinnamic acid	12.6 ± 0.3	1.11 ± 0.001
Quercetin	13.6 ± 0.3	1.07 ± 0.001

from M/s Sigma Aldrich Chemical Co. Gradient grade methanol was procured from Qualigens Fine Chemicals and Ammonium acetate (A R grade) from Ranbaxy Fine Chemicals Limited and was used for preparation of the mobile phase.

The solutions of the phenolic compounds and flavonoid were prepared by the dissolution of precisely weighed portions of individual substances in methanol. The concentration of the stock solutions of phenolic acids was kept between 4 to 5 mg/mL depending upon solubility of compound.

Apparatus. Separation of phenolics and flavonoid in crude extract of *Eleusine coracana* (L.) were achieved on Quaternary Gradient semi-Prep HPLC system with PDA Detector and 600e Multi Solvent Delivery System from Waters, and a Xterra MSC-18 column (7.8 × 100 mm, 5 μm) with octadecylsilane as solid support was used to separate the components. The data were analyzed and processed using the installed Empower 2 software. The column was maintained at room temperature and the flow rate of mobile phase was kept at 1.0 ml/min, which consisted of methanol, acetonitrile and phosphate buffer. Sample volume was kept 5000 μL with help of Rheodyne 77251 Injector and compounds were detected between 190–400 nm with 2996 Photodiode Array Detector. The separated phenolic acids and flavonoid were initially identified by direct comparison of their retention times with those of standards. Standard addition spike method was employed to identify each fractions of crude extract with aid of increase in respective peak area. This procedure was performed separately for each standard. The phenolic acids and flavonoid contents were calculated from the peak areas of HPLC chromatograms from the eight replicate samples.

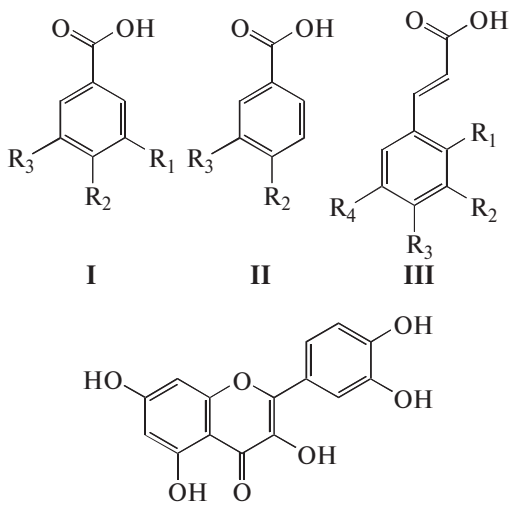
Gradient programme: The gradient programme, initiated with 5% methanol (eluent A) and 95% acetate buffer (eluent B), continued for first 30 min, then ramped linearly to 50% eluent A and 50% eluent B within 15 min. This proportion (50 : 50) was maintained for the next 15 min and, subsequently, the eluent gradient was reverted to the initial conditions (5 : 95) within the next 5 min and allowed to stabilize the baseline for 25 min before injecting the sample. (Separation of 8 compounds was accomplished in 60 min, while total run time was kept 90 min to ensure the complete elution of sample from the column.)

REUSLTS AND DISCUSSION

The selection of the conditions of chromatographic separation. Semi-preparative version of HPLC was used with column having chemically attached octadecylsilane groups and eluents of different compositions were usually employed in analytical practice for the separation of phenolic compounds of *Eleusine coracana* (L.). The main component of the eluent is usually isopropanol, methanol or acetonitrile. If isopropanol or methanol is used, the sufficient separation of the phenolic compounds is not achieved and therefore, it is preferable to introduce small amount of acetonitrile into the mobile phase.

Peak broadening is one of the possibilities due to dissociation of compounds and hence, pH 4.14 was maintained throughout the experiment by adding glacial acetic acid in eluent B (10 mM ammonium acetate). The gradient programme was employed to elute active ingredients from crude extract of *Eleusine coracana* (L.).

The gradient programme was initiated with 5% of eluent A and 95% of eluent B (v/v) (Gradient 1) and it was allowed to travel up to 30 min. The composition 50 : 50 (v/v) of eluent A and eluent B (Gradient 5) was achieved with linear ramped in the both solvents and flow rate within 15 min. The gradient was reverted back to original conditions within next 5 min. The baseline was allowed to stabilize for 25 min before injecting the actual samples. Separation of seven compounds was achieved successfully within total run time of 90 min. Retention factor (k) and selectivity coefficient (α) for all seven components were calculated and summarized in the Table 1. The resulting chromatogram showed that all seven components were separated with mobile phase of Gradient 1 to Gradient 4 in isocratic manner. The structures of separated components are depicted below:



I **II** **III**

IV

Scheme

Structures of separated phenolic acid and flavonoid are given with positions of hydroxyl group in the basic skeleton. **I**: Gallic acid ($R_1 = H$, $R_2 = OH$, $R_3 = OH$), PHBA ($R_1 = H$, $R_2 = H$, $R_3 = OH$), salicylic acid ($R_1 = OH$, $R_2 = H$, $R_3 = H$), vanillic acid ($R_1 = H$, $R_2 = OH$, $R_3 = OH$); **II**: caffeic acid ($R_1 = OH$, $R_2 = OH$, $R_3 = H$), ferulic acid ($R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$); **III**: cinnamic acid ($R_1 = R_2 = R_3 = R_4 = H$); **IV**: Quercetin.

The eluted each component was nicely depicted in the chromatogram (figure). Gallic and vanillic acids were found poorly retained on the solid adsorbent, which indicates that it requires lower content of methanol. The content of methanol was increased from 5 to 40% (Gradient 4) with decreasing content of eluent B. This was done to improve the smoothness of separation of each component. This procedure leads to significant improvement of the selectivity of the chromatographic system.

The separation of caffeic, ferulic, and cinnamic acids was achieved by increasing the composition of eluent A from 5 to 10% (Gradient 2). The optimal separation of compounds was achieved when composition of eluent A reached 20% (Gradient 3) with 1 mL/min flow rate. Resolution of each separated component was calculated from the chromatogram of crude extract of *Eleusine coracana* (L.) and was depicted in Table 2.

Optimization of experimental conditions in liquid chromatography is major task for an analyst: pH of gradient eluent, organic subtract, nature of mobile phase and temperature must be taken into account. To increase the selectivity and resolution of the tested substance, a methanol and a phosphate buffer solution were used. The temperature of chromatographic process was varied from 25 to 60°C at 5 grad/min to separate each elute with better resolution. The optimum temperature was found at 30°C, judging by the resolution of each peak of phenolic acid and flavonoid. The chromatogram of standards and actual sample was represented in figure a and b. The separation parameters along with wavelength maximum were summarized in Table 3, which suggests that all seven components exhibit UV absorption band via $\pi \longrightarrow \pi^*$ and $n \longrightarrow \pi^*$ transitions.

Table 2. The value of resolution (R_s) for individual component at pH 4.14 in the phosphate buffer solution

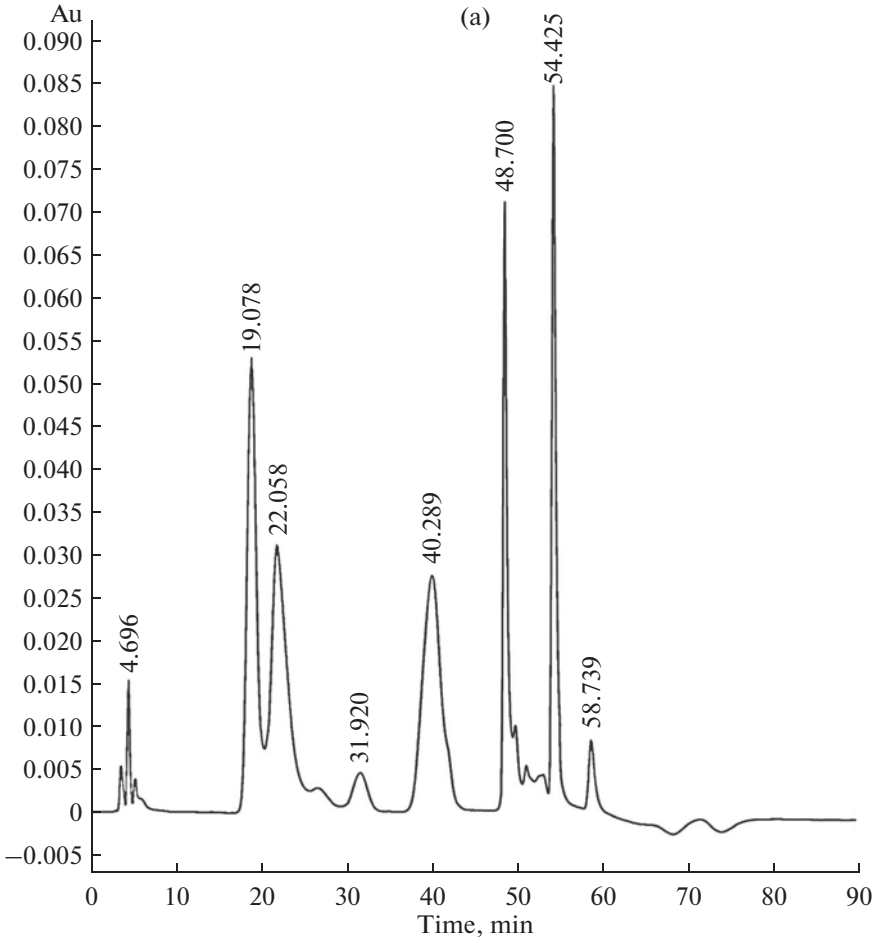
Compound	R_s, at pH 4.14
Gallic acid	–
p-Hydroxybenzoic acid	11.0
Vanillic acid	9.4
Caffeic acid	6.4
Ferulic acid	6.8
Cinnamic acid	5.6
Quercetin	3.8

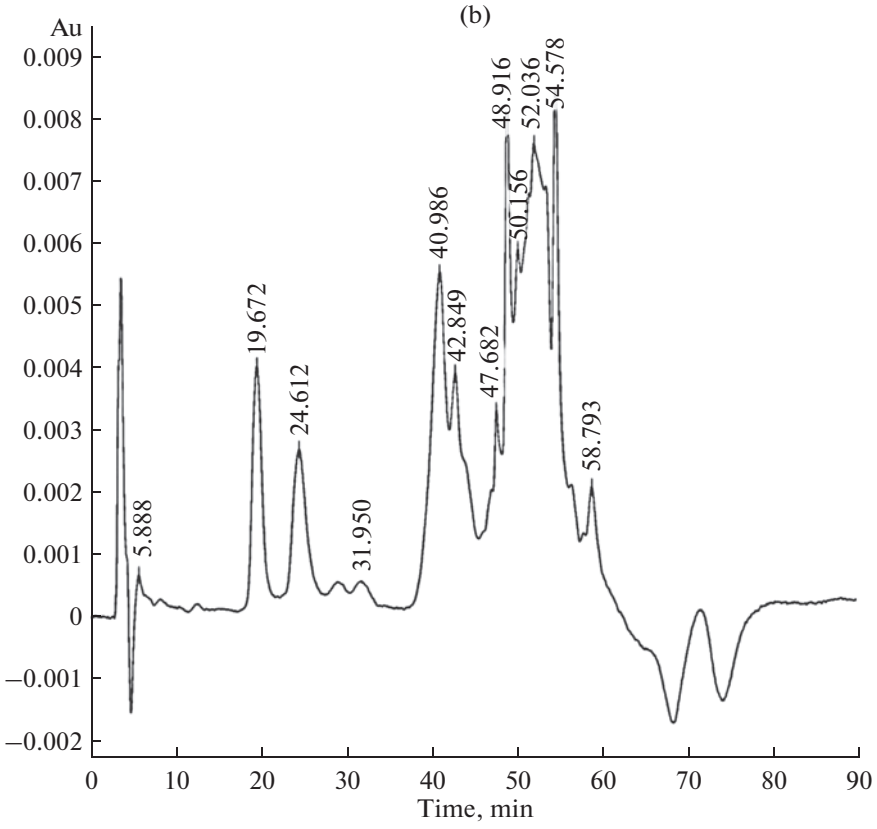
Table 3. Parameters of the identification of phenolic and flavonoid compounds

Compound	t_R, min	λ_{max}, nm
Gallic acid	4.696	213/262
p-Hydroxybenzoic acid	19.075	251
Vanillic acid	22.058	230/296/372
Caffeic acid	31.924	255/290/365/382
Ferulic acid	40.290	215/317
Cinnamic acid	48.701	216/317
Quercetin	54.425	255/368

Finally, optimum conditions for separation of seven components, six phenolic acids and one flavonoid were achieved and summarized here. Gradient 3: eluent A methanol-acetonitrile; eluent B, 10 mM ammonium acetate with glacial acetic acid to maintain pH 4.14, with flow rate 1 mL/min and column thermosetting at 30°C. Analytical characteristics for HPLC method were calculated using least square equation method and an excellent relationship was obtained for regression analysis and correlation coefficient. The data was summarized in the Table 4.

The retention time was found to be dependent on the presence of hydroxyl and methoxy groups in the structure of phenolic acids. It was observed that retention time of elute increased with less number of hydroxyl groups and increased with addition of methoxy group. The retention time of phenolcarbonic acids


(a)
Au
0.090
0.085
0.080
0.075
0.070
0.065
0.060
0.055
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0
−0.005
4.696
19.078
22.058
31.920
40.289
48.700
54.425
58.739
0
10
20
30
40
50
60
70
80
90
Time, min


(b)
Au
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
−0.001
−0.002
5.888
19.672
24.612
31.950
40.986
42.849
47.682
48.916
50.156
52.036
54.578
58.793
0
10
20
30
40
50
60
70
80
90
Time, min

Chromatograms of mix standard of seven phenolic compounds and one flavonoid (a) and chromatograms of *Eleusine coracana* crude mixture of a seven known compounds for gallic acid (5.888 min), *p*–hydroxybenzoic acid (19.672 min), vanillic acid (22.058 min), caffeic acid (31.924 min), ferulic acid (40.290 min), cinnamic acid (48.701 min), quercetin (54.738 min) (b). The detection wavelength was 236 nm.

Table 4. Analytical characteristics of the procedure for the determination of phenolic and flavonoid compounds $P = 0.95$, $n = 6$)

Compound	c, mg/mL	Regression equation	Correlation coefficient (R^2)
Gallic acid	5–100	$y = 52165x - 37084$	0.9961
p-Hydroxybenzoic acid	5–100	$y = 4E+06x + 23754$	0.9982
Vanillic acid	5–100	$y = 25751x - 45338$	0.9962
Caffeic acid	4–100	$y = 6E+06x - 2E+06$	0.9721
Ferulic acid	5–100	$y = 2E+06x + 35628$	0.9736
Cinnamic acid	5–100	$y = 3E+06x + 1E+06$	0.9081
Quercetin	5–100	$y = 39194x - 57752$	0.9817

showed following pattern for separation under the specified experimental conditions: ferulic acid < *p*-hydroxybenzoic acid < vanillic acid < gallic acid < cinnamic acid < caffeic acid < quercetin.

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