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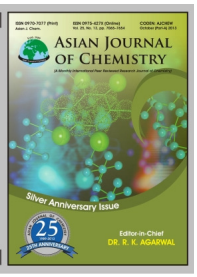

Screening of Polyphenolic Compounds in *Echinochloa crusgalli* Roxb Extracts by Various Analytical Techniques

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In the present study, the phenolic content of the selected extracts of *Echinochloa crusgalli Roxb* plants were determined with chromatographic techniques like thin layer chromatography, high performance thin layer chromatography and high performance liquid chromatography while the total phenol was determined by UV method. All the results of analytical methods were compared with standard phenolic compounds. The methanol macerated extract contained maximum total phenolic content (0.719 ± 0.67 mg GAE/g) than other extract by soxhlation. Eleven compounds identified by chromatographic techniques in the plant. The dominant phenolic compounds representing the main groups in *Echinochloa crusgalli* are flavonoids and phenol carboxylic acids. From LCMSMS results suggested that the myricetin, quercetin, artemisinin, cyanidin, kaempferol, luteolin, bilobol and its derivatives may be present in methanolic extracts of *Echinochloa crusgalli Roxb*. It may assign a new potential role of *Echinochloa crusgalli* Roxb extract in human health care.

Key Words: *Echinochloa crusgalli* Roxb, Phenols, HPLC, HPTLC, LCMS/MS.

INTRODUCTION

Phenolic compounds have been the subject of intense research in recent years due to their potential beneficial effects on human health. The phenolic compounds are secondary plant metabolites that are contained within the skin, seed and flesh of grapes and even are extracted into wines (especially red) during the process of vinification[1]. *Echinochloa crusgalli Roxb.* (Syn: panicum cursgalli linn.) belongs to Gramineae, Poaceae family, distributed throughout tropical Asia and Africa in fields and commonly present in rice fields and moist localities[2]. The plant extract is used in diseases of the spleen. Young shoots are eaten as a vegetable in Java. Reported to be preventative and tonic, barnyard grass is a folk remedy in India for carbuncles, stiffness of lower limbs, ascities, diabetes, sores, spleen trouble, cancer and wounds[3-6]. It is useful for reclamation of saline and alkaline areas used in diseases of spleen and for checking haemorrhage[7]. The shoots and/or the roots are applied as a styptic to wounds. The plant is a tonic, acting on the spleen[8,9]. High performance thin layer chromatography (HPTLC), high-performance liquid chromatography (HPLC) and LCMSMS are commonly used to determine qualitatively and quantitatively single phenols. All these analytical techniques are often preferred due to sufficient sensitivity and precision. However, since HPLC methods generally use UV detection and many phenols show UV spectra with λ_{max} in a narrow range (280-320 nm), different and often some treatments are required prior to the HPLC analysis, in order to prevent interferences. However, LCMSMS detection provides useful structural information and allows for tentative compound identification when standard reference compounds are unavailable and when peaks have similar retention time. No report concerning the phenolic content of the *Echinochloa Crusgalli* Roxb is available in literature. In the present investigation, petroleum ether, chloroform, ethanol and water extracts of air dried *Echinochloa crusgalli* Roxb plant using soxhlation and methanol extract by maceration were used to determine their phenolic content using UV spectra. Selected extracts were on further chromatographic analysis to determine the presence of constituents.

EXPERIMENTAL

Collection and authentification: Plant material of *Echinochloa crusgalli Roxb* was collected from local areas of Talakona, Andhra Pradesh and plant was authentified by Dr. A. Lakshma Reddy, Retired Professor, Dept. of Botany, Nagarjuna Govt. College (Autonomous) Nalgonda (Andhra Pradesh). Plant was dried in the shade and ground into uniform powder using milling machine.

Petroleum ether, chloroform, ethanol were purchased from SD Fine chemicals Ltd., (India). Chemicals used for determination of contents of total phenols using TLC and HPTLC such as toluene, acetone, formic acid, sodium carbonate and Folin-Ciocalteu reagent were obtained from Sigma, Merck and SD Fine chemicals Ltd. Chemicals employed for HPLC assays such as HPLC grade acetonitrile, water and concentrated phosphoric acid were purchased from SD Fine chemicals Ltd.

Preparation of extracts: The extracts of *Echinochloa crusgalli* Roxb were prepared by successive soxhlation with various solvents. The shade dried whole plant powder was packed in thimble kept in the Soxhlet apparatus and extraction was allowed to run successively using the solvents, petroleum ether (60 ± 80ºC), chloroform and ethanol. Finally, the marc was dried and macerated with chloroform-water for 24 h to obtain the aqueous extract. Petroleum ether and chloroform were used for defat the final extract. Only chloroform and aqueous extract was concentrated by evaporating the solvent on the water-bath and the obtained extracts were weighed.

Even using maceration technique, the extract of *Echinochloa crusgalli* Roxb was prepared as follows. 50 g of shade dried whole plant powder was suspended and extracted with 10 volumes of methanol by shaking at room temperature for 15 h. The extracts were filtered through filter paper and the supernatants were pooled. The residue was re-extracted under the same conditions. Pooled extracts were condensed and methanol was removed with a rotary evaporator at 50 ºC.

The physical characteristics and percentage yield of all extracts were reported. The dried extracts of all solvent were in desiccator prior to analysis. All the extracts were subjected to preliminary phytochemical screening for the detection of various chemical constituents.

Thin layer chromatography: Extracts were dissolved their respective solvents and spotted on TLC plates (silica gel GF plates). The plates were developed in toluene-acetone-formic acid (4.5:4.5:1) for the determination of phenolic compound. After developing the plate they were dried and the resolution of components of extracts were studied by locating various spots on chromatogram using Folin-Ciocalteu reagent. Measure and record the distance of each spot from the point of its application and calculate the R_f value[10,11].

Total phenolic contents: Total phenolic content was analyzed spectrophotometrically by a modified Folin-Ciocalteu colorimetric method. 0.125 mL of all the extracts (1:10 g/mL) was taken in each test tube. 1.5 mL of water and 0.125 mL of Folin-ciocalteu reagent were added and allowed to stand for 6 min, 1.25 mL of 7 % sodium carbonate and 3 mL of water were added in to each mixture then allowed to stand for 90 min at room temperature. After the colour formation, the absorbance was measured at 769 nm using Elico UV-visible spectrophotometer. Gallic acid was used to prepare a standard curve (0.2-10 µg/mL; $y = 0.06218x + 0.131$; $r^2 = 0.9850$; y is the absorbance; x is the solution concentration). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of powdered crude drug[12].

HPLC (isocratic) method for phenolic compounds

Instrumental specification: An isocratic HPLC system (Analyical Technologies Ltd.) consisting of a model ALC 2010 high pressure pump and a model ASPD 2600 variable wavelength detector (UV-visible) was used. Manual injections were made using a rheodyne injectable valve (20 µL loop). The detector wavelength was set at 254 nm. The chromatographic separations were performed at ambient temperature on a Gracesmart RP18, 5 µ (250 mm × 4.6 mm). The mobile phase was a mixture of acetonitrile, water and concentrated phosphoric acid (400:600:5), filtered and degassed prior to use and flowing at the rate of 0.8 mL/min. The measurements were performed at room temperature. The duration of each analysis was 37.5 min. The data were collected and analyzed with analyst and crystal software in a computer system.

Sample preparation: About 2 g of selected extracts of *Echinochloa crusgalli* Roxb based on spectrophotometric and TLC report were placed into an individual 50 mL flask and extracted with 70 % aqueous methanol solution (50 mL) by boiling with reflux for 2 h on a water bath. After cooling, the extracts were filtered through a paper filter in to a 100 mL measuring flask and were subsequently made up to the mark with the same solvent and sonicated for 15 min. Then the solutions were filtered through 0.45 µ filter porosity membrane filter prior to injection. The sample components were identified by comparison of their retention times to those observed in the chromatograms of reference solutions which were collected from the library data. The relative content of each component was determined by measuring the area under the corresponding peak and using the method of internal normalization[13].

HPTLC analysis of plant samples for phenolic profile: The selected extracts for HPLC were dissolved in 1 mL of appropriate solvents and centrifuged at 3000 rpm for 5 min. The same procedure was followed for the reference standards such as quercetin, rutin, gallic acid and kaempferol. These solutions were used as test solution for HPTLC analysis. 2 µL of the above test solutions were loaded as 5mm band length in the 5 cm × 10 cm Silica gel 60 F_{254} TLC plate using Hamilton syringe and Camag Linomat 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with the mobile phase of toluene-acetone-formic acid (4.5:4.5:1) and the plate was developed in the same mobile phase up to 90 mm. The deve-loped plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (Camag Reprostar 3) and captured the images at white light, UV 254 nm and UV 366 nm. Before derivatization, the plate was fixed in scanner stage (Camag TLC Scanner 3) and scanning was done at UV 254 nm. The peak table, peak display and peak densitogram were noted. Then the developed plate was sprayed with 20 % sodium carbonate solution sprayed and brief dried followed by Folin Ciocalteu reagent and dried at 100 ºC in hot air oven. The plate was photo-documented at day light using photo-documentation (Camag Reprostar 3) chamber[10,11].

Determination of phenolic compounds by LCMSMS

Instrument specification: The phenolic extracts were analyzed using a Waters UPLC-Triple Quadrupole (LC-MS/

MS) mass spectrometer equipped with ESI and APCI for MSMS and UV6000LP photodiode array detector for retention time. Separation was achieved on a Waters ODS-2 (250 × 4.6, 5 μ). The operating conditions were: column oven at 35 ºC; injection volume, 10 μL; eluent flow rate, 0.8 mL/min. The elution solvents were A (100 % acetonitrile) and B (0.1 % v/v formic acid in water). Spectral data from 200-700 nm were recorded. Separation of phenolic compounds by linear gradient was evaluated. For the linear gradient method, the program started with 90 % B from 0 to 30 min, 90-30 % B from 30 to 35 min, 30-20 % B from 35 to 45 min, 20-90 % B from 45 to 90 min and a post-run with 90 % B for 10 min to equilibrate the column for the next injection. The sheath gas (nitrogen) was set to 65 arbitrary units and the auxiliary gas (He) was set to 10 arbitrary units. The capillary voltage was -26 V and the temperature was set at 250 °C. The source was operated in a positive ion mode. The data were scanned in a range of 100-1000 amu. Identification of phenolic compounds was carried out by comparison of their retention time and MS spectra with data from the database of SDBS (Spectral database system, National Institute of Advanced Industrial Science and Technology (AIST), Japan)[14] and Mass Bank[15]. The name, molecular weight and structure of the components of the test materials were ascertained.

Sample preparation: 2 g of the extracted were dissolved in methanol. The extracts were then filtered through Whatmann filter paper No. 41 along with 2 g sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper along with sodium sulphate was wetted with methanol. The filtrate was then concentrated by bubbling nitrogen gas into the solution.

RESULTS AND DISCUSSION

The extraction yield of all extracts was found to be 3.2, 2.4, 4.8 and 6.4 % w/w for petroleum ether, chloroform, ethanol and aqueous extract, respectively. The natures of extracts were dark green for petroleum ether; green for chloroform; brown for ethanol and brown for aqueous extract. Macerated methanol extract was found to be 8.976 % with greenish brown in nature. The results of preliminary phytochemical investigation of the extracts of *Echinochloa crusgalli* Roxb showed the presence of glycoside for petroleum ether and ethanol; phenolic compound and tannins for chloroform, ethanol and aqueous; steroids for chloroform and ethanol; flavonoids and carbohydrates for ethanol and saponins for aqueous. Macerated methanol extract showed the presence of flavonoids, phenolic compounds, tannins and saponins. Then *Echinochloa crusgalli* Roxb extracts were investigated for their total phenolic compounds using TLC and UV. The report of TLC was shown that R_f values of petroleum ether, chloroform, ethanol and water were 0.86, 0.92, 0.74 and 0.7, respectively. Macerated methanol extract consists R_f values of 0.83. It is the most basic method of confirming the presence of phenolic compound. The results of phenolic content by UV visible spectroscopy was expressed by mean ± standard deviation. The milligrams of gallic acid equivalents (GAE) per gram of extracts were found to be 0.066 ± 0.02, 0.646 ± 0.113, 0.511 ± 0.06, 0.660 ± 0.07 and 0.719 ± 0.67 for petroleum ether, chloroform, ethanol, aqueous and macerated methanol extracts, respectively. The macerated methanol extract contained maximum total phenolic content (0.719 mg GAE/g) than other extract. From the results of TLC and UV, the levels of these components in the various solvent extracts of the *Echinochloa crusgalli* Roxb also showed differences. Shahidi and Naczk[16] reported that the usage of Folin-Ciocalteu reagent also was measured based on the colour measurement which was non-specific on phenol. Perhaps there were other components that can react with the reagent such as ascorbic acid. Besides, various phenolic compounds have different response to this assay[17]. However, the measurement of colour changes after two hours storage could be used to determine the existence of phenol in samples. Based on the report of TLC and UV, ethanol, aqueous and macerated methanol extracts contains remarkable levels of phenols.

HPLC method: RP-HPLC coupled with UV-visible detector was employed to separate, identify and quantify phenolic compounds in the ethanol and aqueous extracts of *Echinochloa crusgalli* Roxb. The ethanol, aqueous and macerated methanol extract under study showed little variations in their content of the different phenolic compounds and nearly 11 compounds were identified. In general, arbutin, ferulic acid, resorcinol for three selected extracts; vitexin, umbelliferone, herniarin for aqueous and macerated methanol extracts; 4-oxycoumarin for macerated methanol extract; salicylic acid, caffeic acid and robinin for aqueous extract were major phenolic compounds. Other than these peaks, some other peaks were also observed in all extracts. Retention time (relative content) of unidentified peaks were 0.297 (18.2), 3.573 (11.7) and 6.743 (40.9) for ethanolic extract; 1.81 (33.1), 3.173 (5.3), 3.393 (13.9), 4.66 (5.4), 6.693 (1.4) and 7.94 (4.0) for aqueous extract and 1.177 (17.3), 3.403 (28.5), 4.797(1.4), 4.963 (1.9), 6.803 (4.4) and 8.430 (0.7) for macerated methanol extracts. Table-1 showed the retention time and relative content of identified phenolic compounds by HPLC. Thus, we have established that *Echinochloa crusgalli* Roxb contains a rich complex of biologically active compounds of phenolic nature. HPLC analyses showed that these phenolic compounds belong for the most part to flavonoids and derivatives of phenol carboxylic acid.

HPTLC Method: HPTLC coupled with CAMAG TLC SCANNER 3 was employed to separate, identify and quantify phenolic compounds in the selected three extracts of *Echinochloa crusgalli* Roxb. Blue coloured zone at day light mode present in the given standard and sample tracks observed in the chromatogram after derivatization, which may be the presence of phenolics in the given samples. The concentrations were determined by calculating the spot areas which are proportional to the amount of analyte in a peak and presented. In aqueous extract total 7 spots were appeared among those two were found to be phenols and between two, one spot was coincided with the R_f value of kaempferol. In ethanol extract, total 11 spots were appeared among those three were found to be phenols and among threee, two spots were coincided with the R_f value of rutin and quercetin. In macerated methanol extract, total 6 spots were appeared among those one spot was found to be phenol which was coincided with the R_f value of quercetin. Table-2 summarized the results of HPTLC for the

TABLE-1
RETENTION TIME AND RELATIVE CONTENT OF *Echinochloa Crusgalli* ROXB. EXTRACTS BY HPLC

Compounds	Standard	Ethanolic extract		Aqueous extract		Methanolic extract	
Minutes	Rt	Rt	RC %	Rt	RC %	Rt	RC %
Arbutin	3.73	3.820	9.9	3.850	7.5	4.107	8.1
Ferulic acid	4.37	4.350	6.7	4.257	5.6	4.260	9.4
Resorcinol	5.230	5.227	5.5	5.133	4.7	5.263	14.4
Salicylic acid	5.953	–	–	5.940	1.5	–	–
Phenol	5.987	6.007	7.1	–	–	6.053	10.9
Caffeic acid	6.123	–	–	6.190	1.9	–	–
Vitexin	8.221	–	–	8.240	1.2	8.140	0.9
Umbelliferone	8.65	–	–	8.633	1.2	8.780	1.1
Herniarin	9.31	–	–	9.063	1.0	9.243	0.9
4-oxycoumarin	15.20	–	–	–	–	15.407	0.1
Robinin	22.55	–	–	22.280	7.2	–	–
Peaks other than above compounds	–	0.297	18.2	1.81	33.1	1.177	17.3
	–	3.573	11.7	3.173	5.3	3.403	28.5
	–	6.743	40.9	3.393	13.9	4.797	1.4
	–	–	–	4.660	5.4	4.963	1.9
	–	–	–	6.693	1.4	6.803	4.4
	–	–	–	7.940	4.0	8.430	0.7

Rt = retention time; RC % = percentage of relative content.

TABLE-2
RESULT OF HPTLC CHROMATOGRAM OF *Echinochloa Crusgalli* ROXB EXTRACTS

Peak	R_f	Height	Area	Assigned substance	Peak	R_f	Height	Area	Assigned substance
	Ethanol extract					Aqueous extract			
1	0.05	29.9	367.6	Unknown	1	0.01	172.2	1018.8	Unknown
2	0.07	38.3	561.2	Unknown	2	0.07	97.4	2640.8	Phenolic 1
3	0.13	10.9	156.5	Phenolic 1 (may be rutin)	3	0.22	41.3	2076.0	Unknown
4	0.16	10.2	97.5	Unknown	4	0.62	12.9	180.1	Unknown
5	0.23	18.0	397.3	Unknown	5	0.70	123.1	4717.3	Phenolic 2 (may be kaempferol)
6	0.58	17.0	518.1	Phenolic 2	6	0.77	55.5	2420.9	Unknown
7	0.67	288.8	10170.4	Unknown	7	0.97	159.0	4615.1	Unknown
8	0.69	295.8	5415.7	Phenolic 3 (may be quercetin)	Methanol extract	–	–	–	–
9	0.76	63.2	2962.0	Unknown	1	0.23	19.0	577.1	Unknown
10	0.88	33.5	1946.3	Unknown	2	0.47	345.6	17124.2	Unknown
11	0.97	136.3	4231.9	Unknown	3	0.69	469.4	28637.0	Phenolic 1 (may be quercetin)
Quercetin	0.68	548.0	11711.0	–	4	0.76	44.5	1242.0	Phenolic 2
Rutin	0.12	331.3	8282.3	–	5	0.84	10.4	288.7	Unknown
Gallic acid	0.51	421.4	19467.8	–	6	0.97	162.9	5076.1	Unknown
Kaempferol	0.71	346.6	8957.7	–	–	–	–	–	–

TABLE-3
LCMSMS REPORTS FOR METHANOLIC EXTRACTS OF *Echinochloa crusgalli* ROXB

Retention time	MS	Ion	Rt range for MS/MS	MS/MS	Tentative ID
4.62	279	+	4.6-4.76	279, 280, 242, 219, 205, 201	Myricetin
8.27	698,	+	8.27	698, 279	
8.75	698,	+	8.75	698, 279	
9.23	811	+	9.19-9.44	811, 698, 680, 584, 471, 435, 322	
18.13	282	+	18.16-18.32	282, 277, 254, 255 236	Quercetin (or) artemisinin
18.48	527,	+	18.45-18.57	527, 331, 332	
18.98	331	+	18.89-19.01	331, 332, 316, 315	
20.39	403	+	20.36	403, 385, 386	
27.29	286	+	27.26	286, 287, 279	Cyanidin (or) kaempferol (or) luteolin
28.46	445	+	28.46	445, 446, 286	
29.29	383	+	29.29	383, 384	Quercetin 3-sulphate
30.83	318	+	30.83	318, 279	Myricetin, (or) quercetagetin (or) bilobol
36.14	341	+	36.14	385, 341	
39.27	604	+	39.27	604, 605, 279	

determination of phenol in *Echinochloa crusgalli* Roxb extract compare with standards.

LCMSMS: LCMSMS analysis of the products of phenolic compounds showed the presence of 14 compounds in methanolic extracts of *Echinochloa Crusgalli* Roxb. Table-3 showed LCMSMS data of methanolic extracts of *Echinochloa crusgalli* Roxb. Identification of phenolic compounds was carried out by comparison of their MS spectra with data from the database of SDBS (Spectral database system, National Institute of Advanced Industrial Science and Technology (AIST), Japan) and Mass Bank (www.massbank.jp/jsp). The results suggested that myricetin (4.62 Rt &279MS), quercetin (or) artemisinin (18.13Rt & 282MS), cyanidin (or) kaempferol (or) luteolin (27.29 Rt &286MS), quercetin 3-sulfate (29.29 Rt & 383MS) and myricetin, (or) quercetagetin (or) bilobol (30.83 Rt & 318MS) may be present in methanolic extracts of *Echinochloa crusgalli* Roxb.

Conclusion

In this investigation, we concluded that methanol could extract the highest concentration of polyphenols from the *Echinochloa crusgalli* Roxb plant; these extracts may have a good pharmacological potency due to the presence of polyphenols. So further studies are required to confirm its pharmacological potency, by that we can assure its potential for exploitation to promote human and animal health.

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