Asian Journal of Chemistry; Vol. 23, No. 8 (2011), 3663-3666

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Microwave-Assisted Extraction of *Cynodon dactylon* Linn. Whole Plant and Simultaneous Analysis of Four Phenolics by Diode Array Detection with RP-HPLC

SUBASH CHANDRA VERMA[1,*], CHHETEN LAL JAIN[2] and MADAN MOHAN PADHI[1]

[1]Central Council for Research in Ayurveda and Siddha, 61-56, Institutional Area, Opp.-D-Block, Janakpuri, New Delhi-110 058, India
[2]Department of Chemistry, M.M.H. College, Ghaziabad-201 001, India

*Corresponding author: Fax: +91 11 28524810; E-mail: scvpharma@rediffmail.com

(*Received*: 4 December 2010; *Accepted*: 27 April 2011) AJC-9872

Economical and simple microwave-assisted extraction of *Cynodon dactylon* whole plant and simultaneously quantitative analysis of four phenolics *viz*., 4-hydroxy benzoic acid (**1**), vanillic acid (**2**), 4-hydroxy benzaldehyde (**3**) and ferulic acid (**4**) have been carried out by diode array detection with RP-HPLC. Ethanol as a solvent and microwave's irradiation time 120 s were found to be most favourable for maximum extraction of desired compounds **1-4**. The samples were separated on Eclipse XDB-C18 column with gradient elution of acetonitrile and water: trifluoroacetic acid (99.5:0.05, v/v, pH 2.9) at a flow rate 1.0 mL/min and detected at 280 nm. All calibration curves showed good linearity ($r^2 > 0.999$). The LOD and LOQ were found in the range 0.12-0.27 and 0.40-0.89 μg/mL, respectively. Good recoveries were achieved (92.56-104.89 %) within range. Compound **1** was found in maximum quantity (= 10.312 μg/mL) while compound **4** was found in least quantity (3.621 μg/mL). This method will be useful for quality control and assessment of *C. dactylon* containing product.

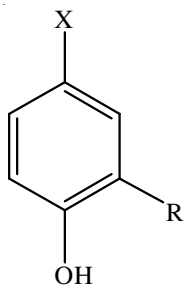
Key Words: *Cynodon dactylon* (Linn), Microwave-assisted extraction, RP-HPLC Diode array detection, Four phenolics, Validation.

INTRODUCTION

Cynodon dactylon (Linn.) Pers. belongs to the family Poaceae, the English name of this plant is Bermuda grass and in Hindi, it is known as Doob[1,2]. It grows throughout India and ascending to 2440 m and is commonly cultivated as lawns for decorative purpose. It is a perennial creeping grass rooting at every node forming matted tufts[1]. Traditionally, it is used in diabetes[3], jaundice[4], kidney problems[5], urinary diseases, gastro-intestinal disorders, constipation and abdominal pain[6]. It has been reported to possess antimicrobial[7], wound healing[8], anti-oxidant[9] and anticancer[10] activities. The constituents reported in this plant are cynidin, hydrocynic acid, triticin, protein[11], carbohydrates, β-carotene and minerals like calcium, phosphorus, iron and potassium[12], phenolics phytoconstituents (ferulic acid, vanillic acid, *p*-hydroxy benzoic acid, syringic acid, *p*-coumaric acid and *o*-hydroxy phenyl acetic acid)[1], β-sitosterol and stigmasterol acetate[2]. Secondary metabolites of the plant especially phenolics have attracted researcher's interest because of their multiple activities[3-10].

Extraction is the first step in the recovery of desired phyto-constituents from plants. Various extraction methods like conventional extraction, solvent extraction, Soxhlet extraction and heat reflux extraction have been used. But these methods take long extraction time and consumption of large amount of solvents[11]. Microwave assisted extraction (MAE) is a rapid and efficient technique for the extraction of compounds from plant herbs. In this technique, microwave energy is used to heat solvent in contact with a sample in order to partition of phyto-constituents from the sample matrix into the solvent[12]. The main virtue of microwave assisted extraction technique is the reduction of time and solvent used[13]. The quality evaluation of *C. dactylon* is achieved by few methods such as thin-layer chromatography (TLC)[1], high-performance thin-layer chromatography (HPTLC) using β-sitosterol as a marker for quantitation[2] and high-performance liquid chromatography (HPLC)[14,15] used for fingerprint development[14] and separation of carotenoids[15]. HPLC is a widely exploited tool for the quality assessment of *C. dactylon* and its products. However, there is major need for development of a cost effective, rapid and precise method for the quality assessment of *C. dactylon* and its products.

To the best of our knowledge, microwave assisted extraction of *C. dactylon* whole plant and simultaneous quantitative analysis of four compounds **1-4** by diode array detection with RP-HPLC is not reported in literature. Therefore, in the present communication an economical, precise and rapid method for extraction and quantitative analysis of compounds **1-4** (Fig. 1) in *C. dactylon* whole plant are reported.



Compound	X	R
4-Hydroxy benzoic acid (**1**)	-COOH	-H
Vanillic acid (**2**)	-COOH	$-OCH_3$
4-Hydroxy benzaldehyde (**3**)	-CHO	-H
Ferulic acid (**4**)	-CH=CHCOOH	$-OCH_3$

Fig. 1. Structure of four phenolics

EXPERIMENTAL

Authentic whole plant of *C. dactylon* was collected during August 2008 from herbal garden of Central Research Institute, Gwalior, India. Whole plant was dried under a gentle stream of air in the laboratory (temp. 25 ± 2 ºC and relative humidity 60 ± 5 %) and powdered in an electric grinder. Standards **1-4** (purity > 98 %) and all HPLC grade solvents were purchased from E. Merck, Mumbai, India. All samples and solvents were filtered through 0.45 µm membrane filters (Millipore, Germany).

Microwave assisted extraction (MAE) and liquid chromatography (LC) condition: MAE experiments were performed with a domestic Kenstar microwave (900 W, frequency 2450 MHz). 5 g dried and powdered whole plant of *C. dactylon* was kept in a flask. It was extracted thrice with 15 mL ethanol (99.9 %) and irradiated with microwave for 120 s. Extract obtained by MAE was filtered and solvent was removed under vacuum with the help of vacuum rotatory evaporator (Heidolph, laborata 4003 control, water bath temp. 50 ºC).

The quaternary HPLC system (Agilent 1200) equipped with Zorbax Eclipse XDB-C_{18} column (250 mm × 4.6 mm i.d., 5 µm, Agilent, USA), a built-in degasser, an auto-sampler, a DAD detector and Agilent 2D and 3D Chemstation software. Acetonitrile (solvent A) and water: trifluoroacetic acid (99.5: 0.05, v/v, pH 2.9) (solvent B) were used as a mobile phase with a linear gradient elution as follows: 0-5 min, 5 % A; 5-30 min, 30 % A; 30-35 min, 100 % A; 35-40 min, 5 % A, (equilibration time); at a flow rate of 1.0 mL/min. The detection wavelength, column temperature and injection volume were set 280 nm, 40 ºC and 2 µL, respectively, for the analysis of compounds **1-4**.

Linearity, limit of detection (LOD) and limit of quantification (LOQ): Linearity curve, limit of detection and limit of quantification were done according to the ICH guidelines and published research papers[16-19].

Standard stock solutions of individual compounds **1-4** were prepared separately by dissolving 10 mg of each in 10 mL of methanol (1 mg/mL). 5 mL of each solution (1 mg/mL) was diluted separately to 50 mL with methanol to get 100 µg/mL stock solutions. The desired concentration range was obtained by serial dilution for standard curve preparation (Table-1). For preparing a calibration curve of compounds **1-4**, each working standards solution was injected in to the HPLC system separately. Six point calibration curves of compounds **1-4** were obtained by plotting the concentration of standards **1-4** *versus* peak area. LODs and LOQs were measured following the standard methods[16].

Precision, accuracy and recovery: Precision, accuracy and recovery of the method were done by reported methods[17,18]. The precision of the proposed method was expressed as a RSD and it calculated by six replicate injections of each compound (intra-day and inter-day). Percentage RSD for retention times and obtained peak areas were calculated. The standard solutions used for repeatability experiments were same as used in the calibration curve experiment.

The accuracy of the method was determined by analyzing the percentage of recovery of main constituents in ethanolic extract (6 mg/mL) of whole plant of *C. dactylon*. The samples were spiked with three different amounts of standard compounds **1-4** (2, 8 and 16 µg/mL), before MAE extraction. The spiked samples were extracted by triplicate and analyzed under the above-mentioned conditions. The standard solutions used for recovery studies were same as used in the preparation of calibration curves. The obtained average contents of the target compounds were used as the real values to calculate the percentage recovery.

Preparation of sample solutions: Dried MAE extract (60 mg) of *C. dactylon* was (obtained from section 2.2) re-dissolved in 10 mL of methanol (HPLC grade) to get the sample solution of 6 mg mL^{-1} and filtered through 0.45 µm membrane filters. 2 µL sample solution was injected for HPLC analysis.

RESULTS AND DISCUSSION

Optimization of microwave assisted extraction method: MAE is an alternative to conventional techniques, as seen by number of scientific papers published during the last years[20]. The extraction of desired phyto-constituents **1-4** depends on solvent choice, solvent volume and irradiation time, when using microwave energy. In this study, MAE extraction parameters such as ethanol concentration and irradiation time for extraction of compounds **1-4** were chosen[21,22]. 15 mL of ethanol (99.9 %) and 120 s microwave's irradiation time were found

TABLE-1
PARAMETERS OF THE LINEARITY, LOD AND LOQ FOR THE COMPOUNDS **1-4**

Compound	Linearity range (µg/mL)	Linear equation	r^2	LOD (µg/mL^1)	LOQ (µg/mL)
4-Hydroxy benzoic acid (**1**)	2-20	y = 3.2651x + 0.1278	0.9996	0.12	0.40
Vanillic acid (**2**)	2-20	y = 3.3701x + 0.2894	0.9993	0.23	0.76
4-Hydroxy benzaldehyde (**3**)	2-20	y = 14.395x + 1.2235	0.9993	0.16	0.53
Ferulic acid (**4**)	2-20	y = 5.92x + 0.2321	0.9997	0.27	0.89

r^2 = correlation coefficient.

to be most favourable for maximum extraction of desired compounds **1-4** from *C. dactylon* whole plant.

Optimization of RP-HPLC method: During LC method optimization, different mobile phases, a number of different columns and elution modes were tested. When acetonitrile-water or methanol-water combination were used as the mobile phases, there was severe tailing of the peaks was observed and the separation efficiency was found unsatisfactory. Thus, trifluroacetic acid was used as a modifier with different pH along with different mobile phases. Due to addition of acid in the mobile phase, it improves the peak tailing of solute by lowering the pH and thus suppressing the ionization of acidic functional groups[18,23]. In addition, an elution gradient system was selected due to the closeness in R_f of vanillic acid and *p*-hydroxy benzaldehyde and other constituents which are present in ethanolic extract. Three chromatographic columns were tested, including an Eclipse XDB- C8 (250 mm × 4.6 mm, 5 µm), Eclipse XDB-C8 (150 mm × 4.6 mm, 5 µm) and Eclipse XDB -C18 (250 mm × 4.6 mm, 5 µm). The detection wavelength was set at 280 nm, according to the UV spectra obtained by diode array detection, where all the compounds could be detected with adequate adsorption. Finally, we chosen acetonitrile (solvent A) and water:trifluoroacetic acid (99.5: 0.05, v/v, pH 2.9) (solvent B) as a mobile phase with a linear gradient elution as follows 0-5 min, 5 % A; 5-30 min, 30 % A; 30-35 min, 100 % A; 35-40 min, 5 % A (equilibration time) at a flow rate of 1.0 mL/min. This proved successful as it allowed the separation of desired compounds **1-4** in a single run with a C18 column along with shortened analysis time of 0.5 h. The separation was performed under the optimized analytical conditions and the resulting chromatograms of standards and sample are shown in Figs. 2 and 3, respectively with baseline separation.

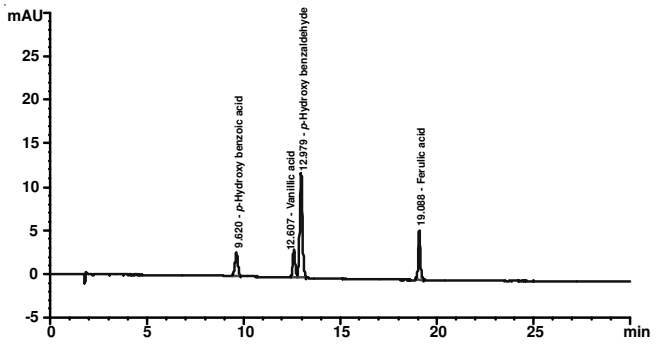


Fig. 2. HPLC chromatogram of standard compounds (**1-4**)

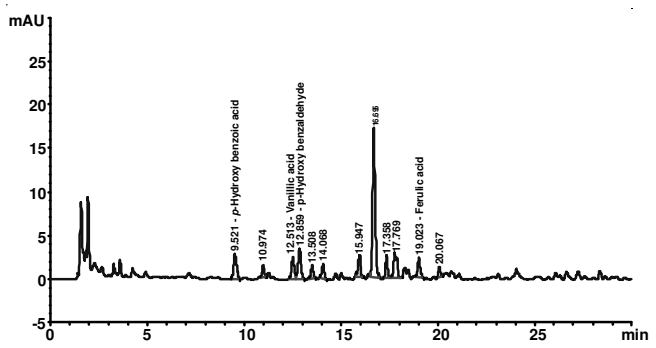


Fig. 3. HPLC chromatogram of MAE ethanolic extract of whole plant of *C. dactylon*

Method validation: The selectivity of the method was determined by comparing the chromatographic profile and the data obtained for the standards and samples, considering the following parameters like retention time, maximum wavelength of absorption and UV spectrum overlay[24]. The peaks of compounds **1-4** in sample were identified by comparing their retention times and UV-spectra obtained from the peaks with those of standards. Peak purity of compounds were assessed by comparing the spectra at three different points *i.e*., peak start, peak apex and peak end positions.

The linear equation between the concentration of the standards injected and the peak area can be expressed as y = mx + c, where y is the concentration and x is the peak area of the standard and m and c are constants. A good linearity was achieved in the range $r^2 = 0.9993$-0.9997 for all the compounds **1-4** (Table-1).

The LOD and LOQ were estimated in accordance with base line noise[16]. LOD is the lowest amount of analyte in a sample that can be detected, but not necessarily quantified. The lowest limit is usually evaluated as the signal to noise ratio that is equivalent to 3 times the standard deviation of the noise (S/N = 3). LODs for compounds **1-4** were found in the range 0.12-0.27 µg/mL Table-1. LOQ, which is defined as the lowest concentration that can be determined with acceptable accuracy and precision, can be established as a signal to noise ratio of 10. LOQ for compounds **1-4** were found in the range 0.40-0.89 µg/mL (Table-1).

The intra-day and inter-day precision (repeatability) of the method was calculated by six replicate injections of three different working concentrations of each compound **1-4**. The intra-day RSDs of retention time and peak area were observed in the range of 0.15-0.86 and 1.02-1.86 %, respectively and in case of inter-day RSDs of retention time and peak area were observed in the range of 0.14-0.88 and 2.16-3.02 %, respectively.

Recoveries of the experiment were performed in order to study the accuracy of the method. The optimized reverse phase HPLC-DAD conditions were applied for determination of percentage recovery of the compounds **1-4** in *C. dactylon*. The detected amounts of compounds **1-4** were calculated from the corresponding calibration curve and the percentage recovery was calculated by the given formula:

$$\text{Recovery } (\%) = \frac{\text{Component measured}}{\text{Component added in spiked extract}} \times 100$$

The blank samples did not show any peak interference with compounds **1-4**. The result of recoveries of compound **1-4** were found in the range between 92.56-104.89 %, which testifies the accuracy of the proposed method for determination of the compounds **1-4**.

In the present study, linearity, LOD, LOQ, precision and recovery results were in agreement with the required criteria. Therefore, the proposed method was found to be most suitable for rapid extraction and simultaneous quantitation of compounds **1-4** in *C. dactylon*.

Quantitative determination of compounds 1-4 in the MAE extract: The microwave assisted ethanolic extract of *C. dactylon* was examined under proposed RP-HPLC-DAD

method. The concentrations of the compounds **1-4** in *C. dactylon* were analyzed (Table-2). The results showed that the concentrations of the target compounds were quite different from each other. However, 4- hydroxybenzoic acid was found in highest concentration (10.312 µg/mL) and ferulic acid was found in lowest concentration (3.621 µg/mL). Some compounds, such as *p*-coumaric acid and vanillin were not detected, as their amounts were below the LOD. The observed variations due to the difference in geographical locations[17,25,26], in target compound concentrations might result from a variance in the preparations according to where and when the herbs were harvested[27]. In general, the concentration of compound **1** was found to be highest followed by other compounds in the order **1 > 2 > 3 > 4** as is evident in Table-2.

TABLE-2
QUANTITATIVE ANALYSIS OF COMPOUNDS **1-4**
IN THE MAE ETHANOLIC EXTRACT OF *C. dactylon*

Compounds	Measured concentration (µg/mL)
4-Hydroxy benzoic acid (**1**)	10.312
Vanillic acid (**2**)	8.515
4-Hydroxy benzaldehyde (**3**)	3.644
Ferulic acid (**4**)	3.621

Conclusion

An economical, precise and rapid method employing MAE for the extraction of compounds **1-4** from *C. dactylon* and simultaneously quantitative analysis of four phenolics **1-4** by diode array detection with RP-HPLC has been developed for the first time. High extraction efficiency, less labour cost, minimum uses of solvent, ease and rapidity is the advantage of performing the extraction in microwave from other conventional methods. RP-HPLC gradient mode helped to achieve the separation with good resolution, precision, accuracy and short analysis time. The fine repeatability and recovery of developed protocol makes it suitable for rapid quantitation and identification of raw drugs, extracts and formulations containing *C. dactylon*. This study will be helpful for food and perfumery industries, pharmaceutical industries, researchers, R and D Centre, academicians, rapid quality control and quality assessment of herbal products containing *C. dactylon*.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge to Director General, CCRAS, New Delhi and Principal, MMH College, Ghaziabad for providing necessary facilities during the study. Authors are also thankful to Dr. Abhijeet S. Khate, Application Chemist, Agilent Technologies, Bangalore for providing technical help.

REFERENCES

1. Ayurvedic Pharmacopoeia of India; Durva (whole plant) API, Part. 1, Vol. 4, The Controller of publication, Civil Line, New Delhi, edn. 1, pp. 28-30 (2004).
2. A.K. Gupta, N. Tandon and M. Sharma, Quality Standards of Indian Medicinal Plants, Indian Council of Medical Research, New Delhi, Vol. 1, pp. 116-122 (2006).
3. K.K. Kirtikar and B.D. Basu, Indian Medicinal Plants, Lalit Mohan Publications, India, p. 2650 (1980).
4. P.K. Borah, P. Gagoi, A.C. Phukan and J. Mahanta, *Indian J. Trad. Knowled.*, **5**, 510 (2006).
5. A.L. Cheryl, *J. Ethnobiol. Ethnomed.*, **2**, 45 (2006).
6. M.G. Paolo, *Fitoterapia*, **76**, 1 (2005).
7. V.G. Premkumar and D. Shyamsundar, *Indian Drugs*, **41**, 748 (2004).
8. T.K. Biswas and B. Mukherjee, *Int. J. Low Extrem. Wounds*, **2**, 25 (2003).
9. B. Auddy, M. Ferreira, F. Blasina, L. Lafon, F. Arredondo, F. Dajas, P. C. Tripathi, T. Seal and B. Mukherjee, *J. Ethnopharmacol.*, **84**, 131 (2003).
10. A. Baskar and S. Ignacimuthu, *Exp. Toxicol. Pathol.*, **62**, 423 (2010).
11. M. Gao, B.Z. Song and C.Z. Liu, *Biochem. Eng. J.*, **32**, 79 (2006).
12. C.S. Eskilsson and E. Bjorklund, *J. Chromatogr. A*, **902**, 227 (2000).
13. H.B. Wan and M.K. Wong, *J. Chromatogr. A*, **754**, 43 (1996).
14. A. Garjani, A. Afrooziyan, H. Nazemiyeh, M. Nijafi, A. Kharazmkia and N.M. Dizaji, *BMC Comp. Alter. Med.*, **9**, 28 (2009).
15. B.H. Chen and C.A. Bailey, *J. Chromatogr.*, **393**, 297 (1987).
16. International Conference on Harmonization Q2B: Validation of Analysis Procedures, US FDA Federal Register, Vol. 62, pp. 27463-27467 (1997).
17. M. Ribani, C.H. Collins and C.B.G. Bottoli, *J. Chromatogr. A*, **1156**, 201 (2007).
18. S.C. Verma, N.P. Singh and A.K. Sinha, *J. Chromatogr. A*, **1097**, 59 (2005).
19. A.K. Sinha, S.C. Verma and U.K. Sharma, *J. Sep. Sci.*, **30**, 15 (2007).
20. S. Bonny, E. Hitti, J. Boustie, A. Bernard and S. Tomasi, *J. Chromatogr. A*, **1216**, 7651 (2009).
21. A. Sharma, S.C. Verma, N. Saxena, N. Chadda, N.P. Singh and A.K. Sinha, *J. Sep. Sci.*, **29**, 613 (2006).
22. F. Zhang, Y. Yang, P. Su and Z. Guo, *Phytochem. Anal.*, **20**, 33 (2009).
23. R. Mateos, J.L. Espartero, M. Trujillo, J.J. Rios, M. Leon-Camacho and F. Alcudia, *J. Agric. Food Chem.*, **49**, 2185 (2001).
24. P. Bhandari, N. Kumar, A.P. Gupta, B. Singh and V.K. Kaul, *Chromatographia*, **64**, 599 (2006).
25. W.H. Gerwick, W. Fenical and J.N. Norris, *Phytochemistry*, **24**, 1279 (1985).
26. T.T.X. Dong, X.M. Cui, Z.H. Song, K.J. Zhao, Z.N. Ji, C.K. Lo and K.W.K. Tsim, *J. Agric. Food Chem.*, **51**, 4617 (2003).
27. B. Lu, Y. Liu, L. Yin, X. Wang and J. Peng, *Phytochem. Anal.*, **20**, 385 (2009).