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RP-LC determination of oleane derivatives in *Terminalia arjuna*

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Abstract

A rapid, sensitive and reproducible reversed phase high performance liquid chromatographic method with photo diode array detection is described for the simultaneous quantification of major oleane derivatives: arjunic acid **(4)**, arjunolic acid **(3)**, arjungenin **(2)** and arjunetin **(1)** in *Terminalia arjuna* extract. The method involves the use of a Waters Spherisorb S10 ODS2 column (250 × 4.6 mm, I.D., 10 μm) and binary gradient mobile phase profile. The various other aspects of analysis viz. Extraction efficiency, peak purity and similarity were validated using a photo diode array detector.

Keywords: *T. arjuna*; Bark; Oleane derivative; Insecticidal; Antibacterial; Triterpene; HPLC; PDA

1. Introduction

The fruits and bark of different species of Terminalia trees have been used since the Vedic period (1500–500 BC) for the treatment of various heart diseases. The pharmacognosy, phytochemistry, pharmacology and clinical use of one of the species, *Terminalia arjuna* (Roxburgh) Wight and Arnott, was reviewed by Dwivedi and Udupa [1]. Recently, the plant was found useful in the treatment [2] of cancer also. The plant exhibits fungicidal [3], antimicrobial [4], antibacterial [5], antifertility [6] and antihuman immuno-deficiency virus [7] induced diseases. Clinically, the bark stem powder/alcoholic extract was found effective in the treatment of cardiovascular diseases, including coronary artery diseases [8–11].

Tannins and oleane type triterpenes are the major chemicals of *T. arjuna* [12–17]. Although many triterpenes are reported in the plant, no work was carried out on the biological significance of these compounds. Recently, we have found many oleane derivatives of the bark of *T. arjuna* possessing potent insecticidal [18,19] and antibacterial activities (unpublished data). HPLC is the most commonly used analytical technique [20–26] for the accurate quantification of natural products. It is important to have a suitable analytical procedure, using HPLC for the quantification of important oleane derivatives in *T. arjuna*, in order to evaluate different accessions of the

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plants for their genetic improvement. A normal phase HPLC method [27] is reported on other oleane derivatives of *Chenopodium quinoa*. In this paper, we report a reverse phase HPLC method, suitable for separating and quantifying the four major oleanes: arjunic acid (**4**), arjunolic acid (**3**), arjungenin (**2**) and arjunetin (**1**) from a single run of chromatogram using photo diode array detector. To the best of our knowledge, the HPLC method reported here is new for the simultaneous quantification of compounds **1**–**4** in *T. arjuna*.

2. Experimental

T. arjuna stem bark was collected locally and a voucher specimen of the plant material has been deposited in the Gene Bank of this Institute.

2.1. Extraction

Compounds **1**–**4** from the stem bark of *T. arjuna* were isolated in the laboratory. A total of 4.5 kg of *T. arjuna* powdered bark was defatted using hexane. The defatted plant material was extracted using ethanol, concentrated in vacuo and reextracted by diethyl ether. Diethylether extract (100 g) was column chromatographed over silica gel (60–120 mesh; Spectrochem, India) using varying concentrations of ethyl acetate in hexane as eluent. Fractions (100 ml) were collected and monitored by TLC.

Fraction Nos. 325–442 afforded compound **4**, identified as arjunic acid, m.p. >280 °C, on the basis of spectral analysis [13,14,28], by using hexane–ethyl acetate as eluent in the ratio (50:50/v:v) and crystallized by using methanol.

Fraction Nos. 447–527 afforded compound **3**, identified as arjunolic acid, m.p. 296–297 °C, by spectral analysis [13,29], by using hexane–ethyl acetate as eluent in the ratio (50:50/v:v) and crystallized by using methanol.

Fraction Nos. 538–800 afforded compound **2**, identified as arjungenin, m.p. 293–294 °C, by spectral analysis [13,15], by using hexane–ethyl acetate as eluent in the ratio (50:50/v:v) and crystallized by using methanol.

Fraction Nos. 949–1377 afforded compound **1**, identified as arjunetin, m.p. 232–234 °C (dec.), by spectral analysis [12,13], by using hexane–ethyl acetate as eluent in the ratio (20:80/v:v) and crystallized by using methanol.

Solvents used were HPLC grade (JT Baker, USA).

2.2. Chromatographic instrument and conditions

HPLC analysis was carried out on a Shimadzu (Japan) LC-10A gradient high-performance liquid chromatographic instrument, equipped with two LC-10AD pumps controlled by a CBM-10 interface module, a model SIL-10ADvp auto injector, an in-line degasser DGU-14A and a multidimensional UV-VIS detector SPD-10A. Photo diode array detector SPD-M10Avp (Shimadzu) was used for the peak purity and similarity test of all four terpenoids (**1**–**4**). Solvents were prefiltered by using a Millipore system and analysis was performed on a Waters Spherisorb S10 ODS2 reversed-phase column, 10 μm (250 × 4.6 mm, ID). The analytical parameters were selected after screening a number of solvent systems and gradient profiles. Separation was achieved with a two-pump gradient program for pump A (acetonitrile:water, 30:70) and pump B (acetonitrile:water, 70:30) as follows: initially 30% B, flow rate 0.8 ml/min; then increased gradually to 50% B until 10.0 min, flow rate 0.8 ml/min; again increased gradually 50–70% B up to 30 min, flow rate 1.2 ml/min; washed the column for 20 min, 30% B, flow rate 0.8 ml/min. The detection wavelength was 220 nm, the absorption maxima close to all the compounds. Injection size for standard and sample was 20 μl. Column temperature was 26 °C.

2.3. Sample preparation

Air dried bark of *T. arjuna* (1 g) was extracted in ethanol for 10 h (3 × 10 ml), filtered, evaporated and reextracted using diethyl ether (3 × 10 ml), dried and again redissolved in 10 ml of methanol for HPLC analysis.

Stock solutions of compounds **1**–**4** were prepared in methanol (1 mg/ml) separately and dif-

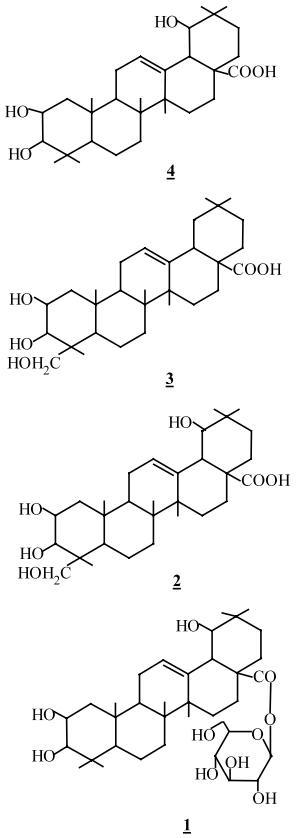

Fig. 1. Substances studied.

ferent amounts of these were injected in HPLC for the preparation of calibration graphs and LC analysis. Calibration graphs were plotted by using area count of each peak (X) and corresponding concentration (Y). Percent content of oleane derivatives (**1**–**4**) were calculated by using calibration graphs.

3. Results and discussion

3.1. Selection of mobile phase

To optimize the mobile phase for binary gradient profile, different compositions of acetonitrile in water were used. Fig. 1 and Fig. 2 illustrates the structure and separation of oleane derivatives (**1**–**4**) in a standard mixture (A) and a plant sample extract (B) respectively. Peaks corresponding to compounds (**1**–**4**) were base line separated and symmetrical. Retention times were 5.40, 9.03, 16.02 and 18.96 for compounds **1**–**4**, respectively. Recoveries of compounds **1**–**4** were 98, 97, 97, 96%, respectively. For the examination of recovery, known amounts of stock solution of pure compounds (**1**–**4**) were added in the *T. arjuna* bark extract and the quantification repeated three times. Selected wavelength (220 nm) was closed to absorption maxima of all four compounds (**1**–**4**). Column performance report for *T. arjuna* plant extract is presented in Table 1. As a measure of column performance, the number of theoretical plate counts (N) for compounds **1**–**4** were 5723, 11208, 16683 and 16071, respectively. Concentra-

Table 1
C_{18} column performance in the separation of oleane derivatives (**1**–**4**) from the extract of *T. arjuna* bark

Oleane derivatives	Rt	No. of theoretical plate counts (N)	Capacity factor (k)	Recovery (%)	Separation factor	Linear regression equation [$Y = A \pm$ S.D. $X \pm C \pm$ S.D.]
Arjunetin (**1**)	5.40 ± 0.02	5723	1.64	98 ± 2	1.77	$Y = 4.9 \pm 0.1 \times 10^{-6}$ $X - 0.13 \pm 0.02$ ($r = 0.9990$)
Arjungenin (**2**)	9.03 ± 0.02	11208	3.40	97 ± 1	2.08	$Y = 4.2 \pm 0.2 \times 10^{-6}$ $X - 0.21 \pm 0.03$ ($r = 0.9992$)
Arjunolic acid (**3**)	16.02 ± 0.03	16683	6.81	97 ± 2	1.42	$Y = 6.8 \pm 0.1 \times 10^{-6}$ $X + 0.06 \pm 0.01$ ($r = 0.9993$)
Arjunic acid (**4**)	18.96 ± 0.03	16071	8.25	96 ± 2	1.21	$Y = 4.9 \pm 0.1 \times 10^{-6}$ $X - 0.07 \pm 0.01$ ($r = 0.9992$)

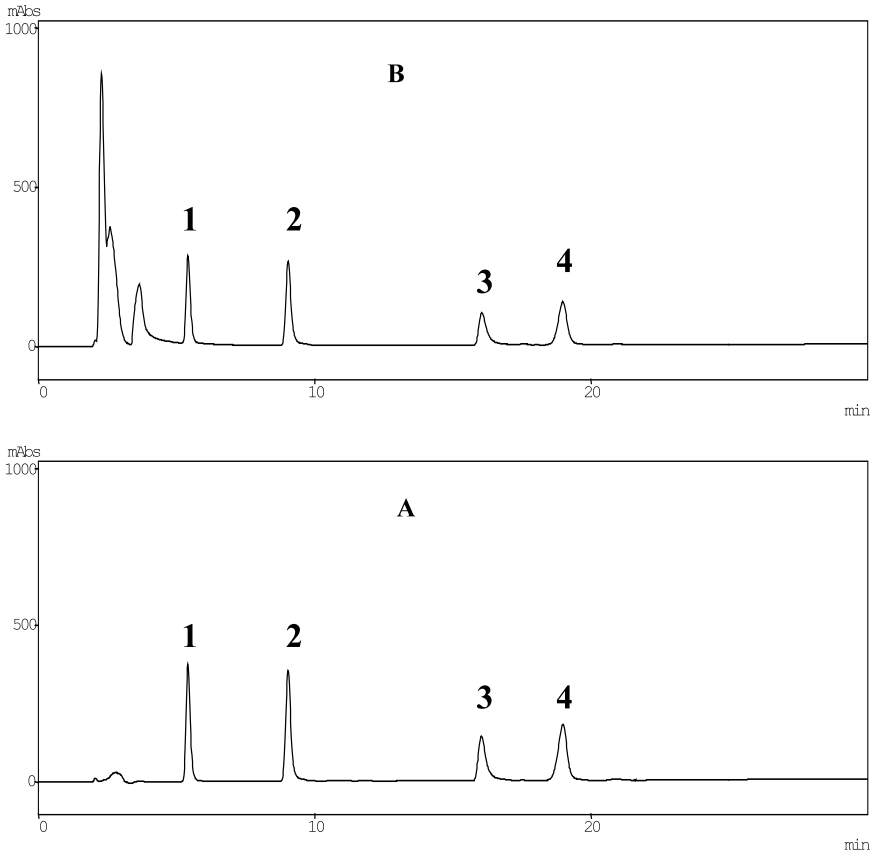

Fig. 2. RPLC separation of oleane derivatives (**1**–**4**) in an artificial mixture of pure compounds (A); 1 mg/ml and a *T. arjuna* bark extract (B). (**1**) Arjunetin; (**2**) arjungenin; (**3**) arjunolic acid and (**4**) arjunic acid.

tion of analytes was estimated at different intervals and no change was observed for the studied time, i.e. 24 h.

3.2. Evaluation of peak purity

Peak purity test of compounds **1**–**4** was performed using a photo diode array detector. All peaks were found pure, both up and down the peaks (Table 2). A similarity test of compounds **1**–**4** in a sample extract was performed by comparing the similarity of peaks in a sample track to that of a library maintained for oleane derivatives **1**–**4**. Similarity of all the compounds were >0.99 (Table 2). The peak homogeneity was tested by examining the UV spectrum for different points of the resolving peaks.

3.3. Linearity

To determine the linearity, five different concentrations of each compound (**1**–**4**) were used in a working range of 1–20 μg. Linear regression

Table 2
Peak purity test results of oleane derivatives (**1**–**4**) using photo diode array detector

Oleane derivatives	Peak purity		Similarity
	Up	Down	
Arjunetin (**1**)	1.00	1.00	1.00
Arjungenin (**2**)	1.00	0.99	1.00
Arjunolic acid (**3**)	0.99	1.00	0.99
Arjunic acid (**4**)	1.00	0.99	1.00

Table 3
Effect of solvent in the extraction of oleane derivatives (**1**–**4**) from *T. arjuna* bark

Solvent used for extraction	% Content ± S.D. of oleane derivatives (**1**–**4**)			
	Compound			
	1	**2**	**3**	**4**
Hexane	ND	ND	ND	ND
Chloroform	0.002 ± 0.0002	0.016 ± 0.004	0.012 ± 0.002	0.045 ± 0.003
Ethyl acetate	0.008 ± 0.0003	0.031 ± 0.002	0.017 ± 0.003	0.062 ± 0.003
Acetone	0.160 ± 0.0040	0.074 ± 0.002	0.020 ± 0.004	0.086 ± 0.004
Methanol	0.188 ± 0.0040	0.056 ± 0.003	0.014 ± 0.003	0.075 ± 0.003
Ethanol	0.216 ± 0.0030	0.084 ± 0.004	0.022 ± 0.002	0.099 ± 0.004

ND, not detectable.

equations and correlation coefficient (r) for compounds **1**–**4** have been given in Table 1. Calibration plots of peak area versus concentration are linear with r values in between 0.9990 and 0.9993. The values show good linearity in the examined concentration range.

3.4. Detection limits

Detection limits, a measure of minimal mass of compounds **1**–**4** that can be quantified were 0.04, 0.04, 0.08, 0.06 μg per injection, respectively.

3.5. Extractive value in different organic solvents

Experiments were performed to select the suitable solvent for the maximum extraction of oleane derivatives (**1**–**4**). Different solvents, viz. hexane, chloroform, ethyl acetate, acetone, methanol and ethanol, were used for the extraction of plant and the results of compounds **1**–**4** content are presented in Table 3. Ethanol was found a suitable solvent for extraction of maximum oleane derivatives (**1**–**4**).

3.6. Precision

Precision of the method was measured by repeating each experiment four times. Mean and S.D. values for the retention times and recoveries for the compounds **1**–**4** were ± 0.02 to ± 0.03 and ± 1.0 to ± 2.0, which are presented in Table 1.

4. Conclusions

The reported reversed phase LC method, using photo diode array detector, is suitable for the analysis of oleane derivatives **1**–**4** (possessing potent insecticidal and antibacterial activities. These are new findings on these compounds and detailed results will be published elsewhere) in *T. arjuna* extract. The method is simple, rapid and precise. A base line separation of all four oleane derivative has been achieved and could be used for rapid screening of *T. arjuna* plant for genotypic quality assessment, drug analysis, etc.

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References

[1] S. Dwivedi, N. Udupa, Fitoterapia 60 (1989) 413–420.
[2] G.R. Pettit, M.S. Hoard, D.L. Doubek, J.M. Schmidt, R.K. Pettit, L.P. Tackett, J.C. Chapuis, J. Ethanopharm. 53 (1996) 57–63.
[3] R. Kumar, R.K. Verma, Ind. Phytopathol. 40 (1987) 274.
[4] P.G. Ray, S.K. Majumdar, Econ. Bot. 30 (1976) 317–320.
[5] Y.N. Shukla, A. Srivastava, T.R. Santha Kumar, S.P.S. Khanuja, S. Kumar, Ind. Drugs 37 (2000) 60–61.

[6] C. Seshadri, S.R. Pillai, Venkataraghawan, J. Sci. Res. Plants Med. 2 (1/2) (1981) 1–3.
[7] I.T. Kusumoto, T. Nakabayashi, H. Kida, H. Miyashiro, M. Hattori, T. Namba, K. Shimotohno, Phytother. Res. 9 (1995) 180–184.
[8] S. Dwivedi, R. Jauhari, Ind. Heart J. 49 (1997) 507–510.
[9] A. Bharani, A. Ganguly, K.D. Bhargava, Int. J. Cardiol. 49 (1995) 191–199.
[10] A. Ram, P. Lauria, R. Gupta, P. Kumar, V.N. Sharma, J. Ethanopharm. 55 (1997) 165–169.
[11] N. Singh, K.K. Kapur, S.P. Singh, K. Shanker, J.N. Sinha, R.P. Kohli, Planta Med. 45 (1982) 102–104.
[12] T. Tsuyuki, Y. Hamada, T. Honda, T. Takahashi, K. Matsushita, Bull. Chem. Soc. (Japan) 52 (1979) 3127–3128.
[13] T. Honda, T. Murae, T. Tsuyuki, T. Takahashi, M. Sawai, Bull. Chem. Soc. (Japan) 49 (1976) 3213–3218.
[14] A.S.R. Anjaneyulu, A.V. Rama Prasad, Phytochemistry 21 (1982) 2057–2060.
[15] T. Honda, T. Murae, T. Tsuyuki, T. Takahashi, Chem. Pharm. Bull. 24 (1976) 178–180.
[16] A.S.R. Anjaneyulu, A.V. Rama Prasad, Ind. J. Chem. 21B (1982) 530–533.
[17] V.K. Tripathi, V.B. Pandey, K.N. Udupa, G. Rucker, Phytochemistry 31 (1992) 349–351.
[18] D.V. Singh, M.M. Gupta, A.K. Tripathi, V. Prajapati, S. Kumar, Phytother. Res. (2001) (communicated).
[19] R.S. Bhakuni, Y.N. Shukla, A.K. Tripathi, V. Prajapati, S. Kumar, Phytother. Res. (2001) (in press).
[20] M.M. Gupta, R.K. Verma, G.C. Uniyal, S.P. Jain, J. Chromatogr. A 637 (1993) 209–212.
[21] R.K. Verma, K.G. Bhartariya, M.M. Gupta, S. Kumar, Phytochem. Anal. 10 (1999) 191–193.
[22] D.V. Singh, A. Maithy, R.K. Verma, M.M. Gupta, S. Kumar, J. Liq. Chromatogr. 23 (2000) 601–607.
[23] S. Srivastava, R.K. Verma, M.M. Gupta, S. Kumar, J. Chromatogr. A 841 (1999) 123–126.
[24] D.V. Singh, S. Prajapati, S. Bajpai, R.K. Verma, M.M. Gupta, S. Kumar, J. Liq. Chromatogr. 23 (2000) 1757–1764.
[25] S. Srivastava, R.K. Verma, M.M. Gupta, S.C. Singh, S. Kumar, J. Liq. Chromatogr. 24 (2001) 13–19.
[26] D.C. Jain, M.M. Gupta, S. Saxena, S. Kumar, J. Pharm. Biomed. Anal. 22 (2000) 705–709.
[27] M. Burnouf-Radosevich, N.E. Delfel, J. Chromatogr. 292 (1984) 403–409.
[28] L. Ramchandra Row, P.S. Murty, G.S.R. Subba Rao, C.S.P. Sastry, K.V.J. Rao, Ind. J. Chem. 8 (1970) 716–721.
[29] F.E. King, T.J. King, J.M. Ross, J. Chem. Soc. (1954) 3995–4003.