Acta Chromatographica 21(2009)3, 499–512
DOI: 10.1556/AChrom.21.2009.3.12

A Simple and Reliable Preparative High-Performance Liquid Chromatographic Technique for Isolation of a Bioactive Flavone Diglycoside from and Extract of *Cuminum cyminum* seeds

N.K. SATTI[1,*], M. AMINA[1], P. DUTT[1], V.K. SHARMA[1], P. SHARMA[1], I. KHAN[1], B.D. GUPTA[1], K.A. SURI[1], S.C. SHARMA[2], R.K. JOHRI[2], AND S.N. SHARMA[3]

[1]Natural Products Chemistry Division, Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu Tawi-180001, India
[2]Pharmacology Division, Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu Tawi-180001, India
[3]Plant Survey Division, Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu Tawi-180001, India
E-mail: nksatti@rediffmail.com

Summary. In this paper we describe a sensitive and reproducible reversed-phase high-performance liquid chromatography (HPLC) method with photodiode-array detection for isolation and quantification of the bioactive hydrophilic constituent 7-(1-*O*-β-D-galacturonide-4′-(1-*O*-β-D-glucopyranosyl)-3′,4′,5,7-tetrahydroxyflavone, **1**, from the seeds of *Cuminum cyminum*. Compound **1** was separated isocratically on a C_{18} preparative column, in high purity, after removal of solvents. The purity and identity of the compound were established by use of LC–mass spectrometry and by spectroscopic techniques (^{1}H and ^{13}C NMR). The purity of **1** was also confirmed by HPTLC.

Key Words: *Cuminum cyminum* seeds, flavones diglycoside, Prep.HPLC, PDA, HPTLC, LCMS

Introduction

Herbs and spices have been used for generations by humans as food and to treat ailments. Scientific evidence is accumulating that many of these herbs and spices do have medicinal properties that alleviate symptoms or prevent disease [1]. Spices are well known appetizers and are regarded as essential in culinary art throughout the world. India is known as the home of spices and is an important exporter of this commodity. Besides their extensive use in food preparation to lend variety, they are also used in the traditional system of medicine in the treatment of many ailments. Cumin seeds, a popular spice regularly used as a flavouring agent in a number of ethnic dishes, are

0231–2522 © 2009 Akadémiai Kiadó, Budapest

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

widely used in Ayurvedic medicine for treatment of dyspepsia, diarrhoea, epilepsy, and jaundice [2]. They also have diuretic, carminative, emmanogogic, antispasmodic, anticarcinogenic [3], antidiabetic [4], estrogenic [5], anticonvulsant [6], anti-nociceptive, and anti-inflammatory properties [7] and it has been reported that the ether extract of *Cuminum cyminum* inhibits biosynthesis of eicosanoids [8] which are a pain mediators.
Compound **1** (*Fig. 1*) is a bioactive molecule present in hydrophilic extracts of *Cuminum cyminum.* It enhances the bioavailability of the anti-TB drug rifampicin [9–12]. Compound **1** had previously been isolated by a procedure described elsewhere [13]. The bioactivity of the molecule prompted us to seek an alternative method for isolating it from its natural source. In this paper we report a preparative HPLC method for obtaining pure compound **1** in quantitative yield.

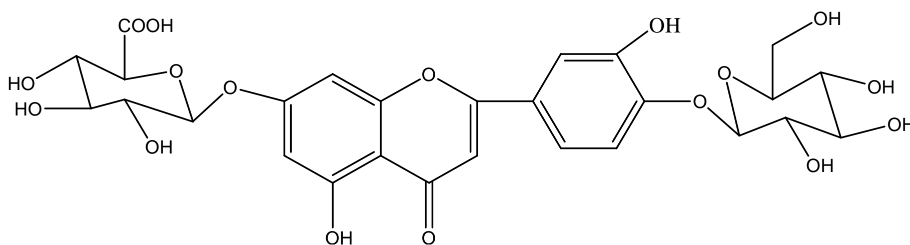

Fig. 1. Chemical structure of **7**-(1-*O*-*β*-D-galacturonide)-4′-(1-*O*- *β* -D-glucopyranosyl)-3′4′,5,7-tetrahydroxyflavone

Powdered seeds of *Cuminum cyminum* were treated with petroleum ether by percolation at room temperature (RT) for 16 h to remove oils and non-polar compounds. The defatted material was extracted with Millipore water on a steam bath. The extract was clarified by centrifugation then lyophilized. The lyophilized extract was partitioned between *n*-BuOH and water. The aqueous phase thus obtained was again lyophilized. The lyophilized extract was analyzed by analytical high-performance liquid chromatography (HPLC) (*Fig.* 2*A*). Separation of the molecule of interest by preparative HPLC was performed isocratically using different mobile phases. The solvent was removed by rotary evaporation under reduced pressure at 50 ± 5°C. The isolated molecule was dissolved in water and its purity ascertained by liquid chromatography coupled with positive-ion electrospray ionization tandem mass spectrometry. The identity was finally confirmed by ^{1}H and ^{13}C NMR and by HPTLC. The compound **1** thus obtained was stored at low temperature under dry conditions.

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

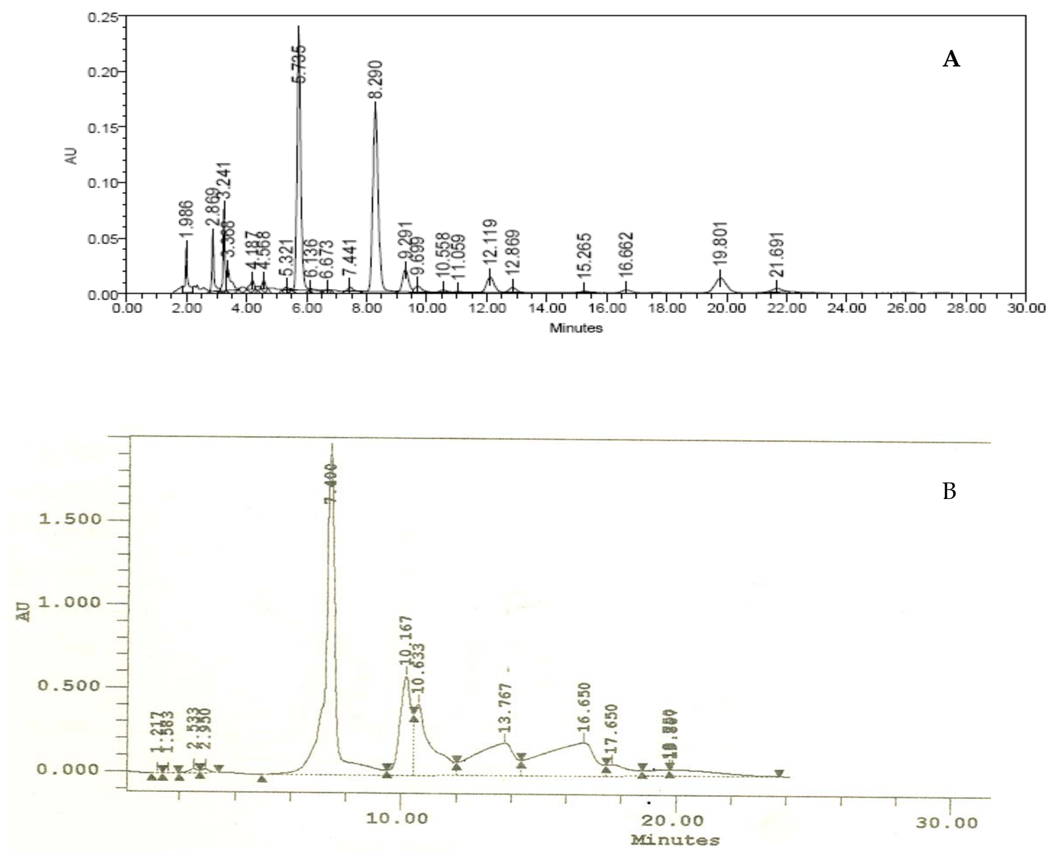

Fig. 2. Analytical (A) and preparative (B) chromatograms obtained by HPLC of lyophilized extract of *Cuminum cyminum*

Experimental

Chemicals and Material

HPLC-grade acetonitrile was from Ranbaxy India (Rankem City, India) and water was Millipore HPLC grade. Chemicals and reagents used for TLC were analytical grade from Merck Chemicals (Mumbai, India).

Seeds of *Cuminum cyminum* were supplied by plant survey division of IIIM Jammu Tawi (India). A voucher specimen of the material has been deposited in the herbarium of IIIM Jammu.

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

Extraction of Hydrophilic Compounds

Seeds of *Cuminum cyminum* were crushed and 100 g of this material was loaded for extraction with *n*-hexane (200 mL) by percolation for 16 h. The defatted material (94 g) was extracted with Millipore HPLC-grade water (300 mL) in a water bath at 98 ± 1°C for 2 h. The extract was squeezed through muslin cloth. The marc so obtained was extracted three more times under same conditions using Millipore water (200 mL each time). All four extracts were combined. The total aqueous decoction was clarified by centrifugation. The clear filtrate was lyophilized to furnish a pale yellow amorphous powder (16 g). This was dissolved in Millipore water (160 mL) and extracted with *n*-BuOH (5 × 160 mL). The aqueous part remaining was lyophilized to yield a residue (13.6 g) which was used for isolation of compound **1**.

Analytical HPLC

Analytical HPLC was performed with Shimadzu HPLC equipment comprising an LC-10 ATVP pump, SPD-M.OA AVP photodiode-array (PDA) detector set at 271 nm, and a Rheodyne 7010 SIL-10 ADVP injector with 50-µL sample loop. Compounds were separated on a 250 mm × 4.0 mm, 5 µm particle size LiChrospher 100 RP-18 column from Merck (Darmstadt, Germany). The mobile phase was a 17:83 mixture of acetonitrile (ACN) and 1.5% aqueous acetic acid (pH 4.9) at a flow rate of 1.0 mL min^{-1}. Before use the mobile phase was filtered and degassed.

Extract (23 mg) was dissolved in 5 mL Millipore water (HPLC grade) and filtered through a 0.45 µm Millipore filter. Compound **1** was detected at a retention time (t_R) of 5.735 min (*Fig. 2A*).

Preparative HPLC

Preparative HPLC was performed with a Waters LC 4000 system with Rheodyne 7010 injector with 4-mL sample loop and a Waters PDA detector 996, set at 271 nm. Compounds were separated on a 25 mm × 100 mm, 15–20-µm particle size with 125-Å pores, C-18 Prep Pak cartridge combined with a 25 mm × 100 mm C-18 Guard Pak insert in a 25 mm × 100 mm radially compressed module (RCM). The overall length of the column was increased to 200 mm by adding an extension tube containing another 25-mm × 100 mm Prep Pak cartridge to the RCM module. The analysis was

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

supported by Millennium Chromatography Manager software version 2.15. Isolated fractions were collected by Waters fraction collector.

After confirmation of the composition by analytical HPLC, 4 mL extract of concentration 15 mg mL^{-1} in 1:9 ACN–H_2O was injected with the Rheodyne 7010 injector. Standardization of the method was achieved by using ACN–H_2O and MeOH–H_2O in the ratios 1:1, 2:3, 3:7, 4:1, and 9:1 to asses their suitability as mobile phase. Each was isocratically pumped at a flow rate of 10 mL min^{-1}. ACN–H_2O 1:9 resulted in good resolution of **1**. In the chromatogram the peak of interest was observed at t_R 13.767 min; detection was at 271 nm (*Fig. 2B*). Compound **1**-rich solution (35 mL) was collected in a clean, weighed flask from 12 to 14.2 min. at room temperature under a normal atmosphere.

Processing of the Collected Fraction

The solvent was removed at under reduced pressure by rotary evaporation on a Buchi Rotavapor at 30 ± 5°C in the weighed flask. Complete removal of water resulted in a yellow compound sticking to the walls of the flask. The flask was again weighed, and the weight of compound **1** collected was determined (0.8 mg).

HPLC Method Validation

Calibration Plot

A calibration plot was constructed using the standard previously isolated by the method reported in Ref. 13. Five different concentrations were analysed in triplicate and peak area was plotted against concentration. Good linearity ($r^2 > 0.997$) was obtained in the range 740 to 3700 ng. The concentration of target samples was calculated by use of the regression equation.

Accuracy and Recovery

Intra- and inter-day precision were calculated for analysis of the compound **1** in the extract by triplicate analysis on the same day and over a three-day period. The results of these investigations are summarized in *Table I*. Intra- and inter-day precision, as relative standard deviation (RSD), ranged from 0.61 to 1.72%, indicative of satisfactory precision of the method for quantification of the compound **1** extracted from *Cuminum cyminum*.

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

Table I. Intra- and inter-day repeatability of analysis of compound **1** in *Cuminum cyminum* seed extract

Intra-day (n = 5)						Inter-day (n = 3)	
Day 1		Day 2		Day 3		Mean ± SD[a]	RSD (%)
Mean ± SD[a]	RSD (%)	Mean ± SD[a]	RSD (%)	Mean ± SD[a]	RSD (%)		
0.732 ± 0.004	0.61	0.748 ± 0.012	1.72	0.7480 ± 0.008	1.19	0.7449 ± 0.007	1.049

[a] Data are mean ± standard deviation from triplicate analysis

Accuracy was determined by adding three known quantities (370, 740, and 1110 μg mL^{-1} solution) of the standard to the extract solution. Three sample replicates were prepared for each concentration and percentage recovery was calculated. The results are given in *Table II*. The mean recovery of the method was 100 ± 4.8% and RSD was <0.51. On the basis of these results the method was deemed to be accurate.

Table II. Recovery of the standard from *Cuminum cyminum* seed extract (n = 3)

Amount added (μg mL^{-1})	Amount detected (μg mL^{-1})	Recovery (%)	RSD (%)
370	368 ± 2[a]	104.86	0.51
740	665.6 ± 0.57	90.08	0.08
1110	1030 ± 0.57	92.79	0.055

[a] Data are mean ± standard deviation from triplicate analysis

Liquid Chromatographic–Mass Spectrometric Analysis of the Purity of the Collected Fraction

Liquid chromatography–mass spectrometry (LC–MS) was performed with Agilent (Palo Alto, CA, USA) 1100 HPLC equipment, comprising binary pump, photodiode-array detector, automatic sample-injection module, and thermostatic column oven, linked to a Bruker Daltonics (Germany) Esquire 3000 ion-trap mass spectrometer with electrospray ionization (ESI) source (LC–ESI-MS–MS). Compounds were separated on a 250 mm × 4.0 mm, 5-μm particle size, Merck LiChrosphere 100 Rp-18 column with 17:83 ACN–1.5% aqueous acetic acid as mobile phase at a flow rate of 0.8 mL min^{-1}.

To check the purity of the compound **1,** 2.3 mg was dissolved in 5 mL HPLC-grade water in a volumetric flask and 20 μL of this solution of **1** was injected with an auto injector. Compound **1** peak eluted at t_R 6.832 min

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

(*Fig. 3A*). By use of a T-joint, part of LC eluent was fed into the mass spectrometer, and mass spectra of all the peaks observed in the chromatogram were recorded (*Fig. 3*). The mass spectrum contained a molecular ion peak at m/z 625 in positive-ion mode (*Fig. 3*).

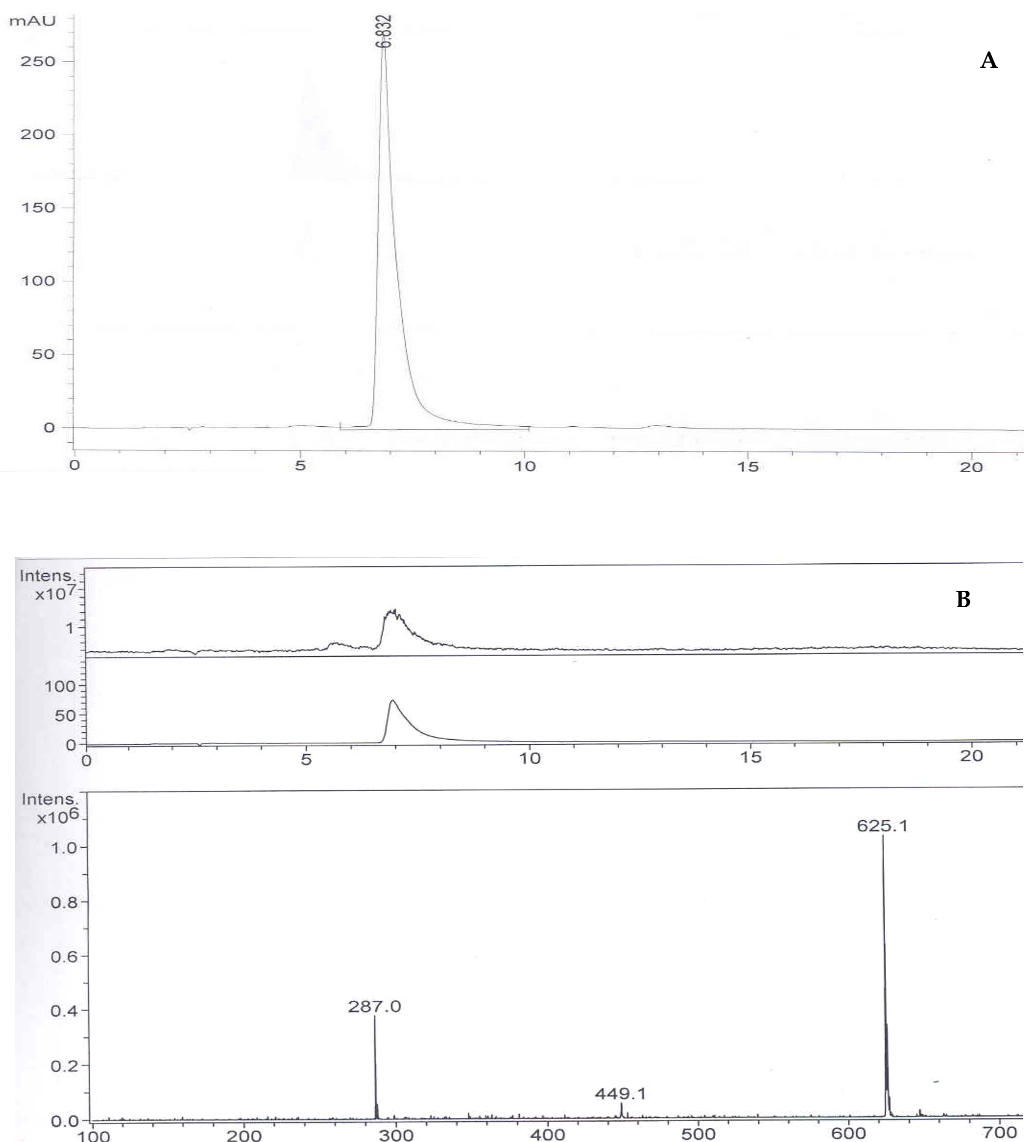

Fig. 3.

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

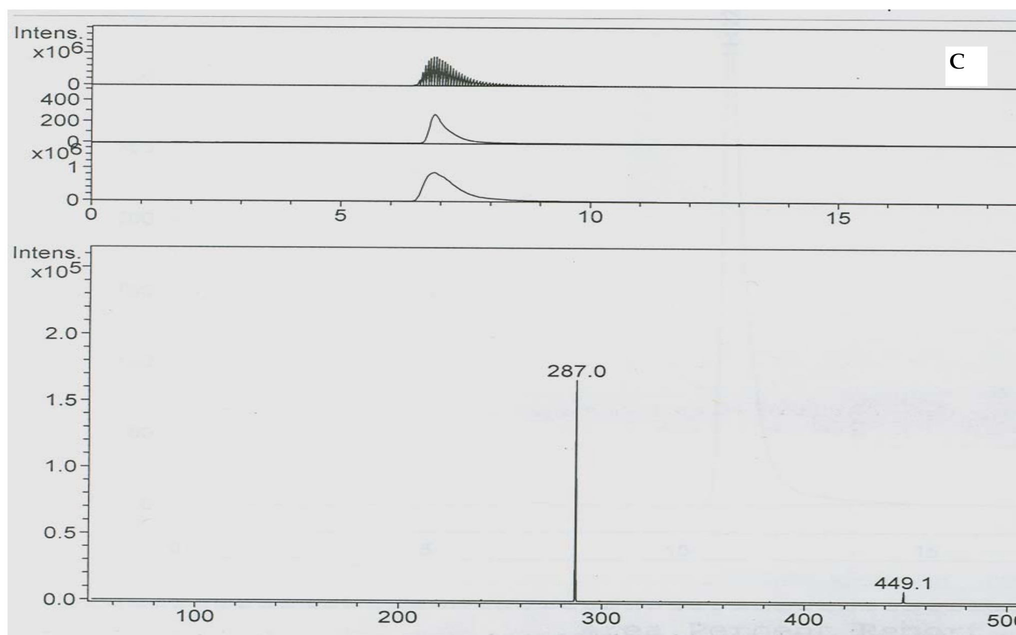

Fig. 3. A. LC–MS chromatogram of compound **1**. B. LC–MS chromatogram of compound **1** showing the molecular ion peak. C. LC–MS chromatogram of compound **1** showing daughter ion peak

Analytical HPLC Analysis of the Purity of Compound 1

Compound **1** (2.3 mg) was dissolved in 5 mL Millipore water (HPLC grade) and the solution was filtered through 0.45 µm Millipore filter. Analytical HPLC was performed using the conditions described above (section "Analytical HPLC") and compound **1** was detected at t_R 5.682 min (*Fig. 4*).

Thin-Layer Chromatography

TLC was performed on 20 cm × 20 cm aluminium-backed plates precoated with 0.2-mm layers of silica gel 60 F_{254} (E. Merck). *n*-Butanol–acetic acid–water and ethyl acetate–acetic acid–formic acid–water mixtures in different proportions were investigated for their suitability as mobile phases. The upper layer from the mixture *n*-butanol–acetic acid–water 4:1:5 resulted in good resolution of the extract, so this mobile phase was selected for further study.

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

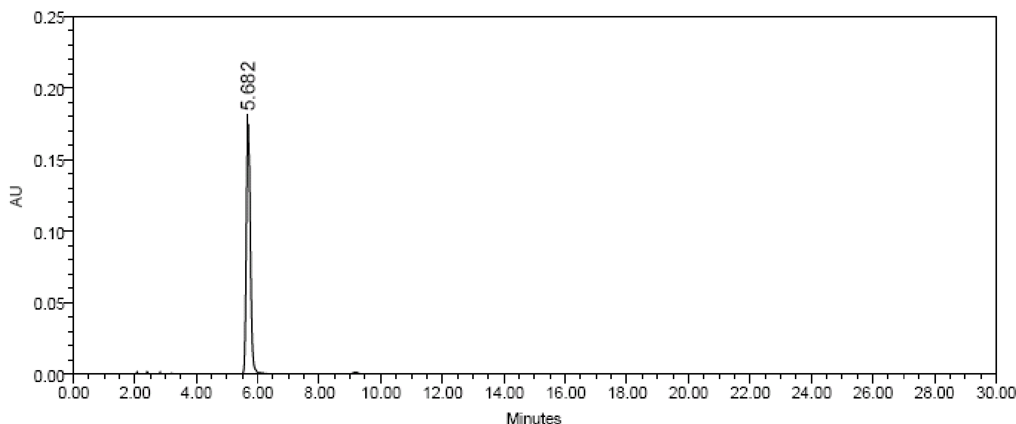

Fig. 4. Analytical HPLC of compound **1**

Samples were applied to the plates as bands 6 mm wide and 10.6 mm apart by means of a Camag (Muttenz, Switzerland) ATS4 automatic spray-on sample applicator fitted with a 25-µL syringe (Hamilton, Bonaduz, Switzerland). A constant application rate of 90 nL s^{-1} was used. Linear ascending development was performed in a twin-trough glass chamber previously saturated with mobile phase vapour for 30 min at 18°C. The development distance was 67 mm and the development time 50 min. The plate was then dried in a current of hot air using an air drier.

After drying, the plates were scanned at 265 nm with a Camag TLC Scanner 3 in absorbance mode and controlled by winCATS v.1.4.1 software. The slit dimensions were 5.0 mm × 0.45 mm, micro, and the scanning speed 20 mm s^{-1}. A Camag video documentation system in conjunction with the Reprostar 3 was also used for imaging and archiving the thin-layer chromatograms. The image was captured by means of a highly sensitive digital camera with a 4.0 megapixel CCD sensor and 3× optical zoom, model power short G2 (Cann, Singapore).

Calibration

For calibration, a stock solution (350 µg mL^{-1}) of the standard was prepared and different volumes (1, 2, 4, and 6 µL) of this solution were applied to the plate to furnish 350, 700, 1400, and 2100 ng per band. Calibration data obtained by linear least squares regression of peak area and amount applied were indicative of a good linear relationship (r^2 = 0.99650, and Sdv 5.68%) in

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

the range 350–2100 ng per band. The calibration plot for the standard is shown in *Fig. 5*. The regression equation was $y = 1208.934 + 4.551x$.

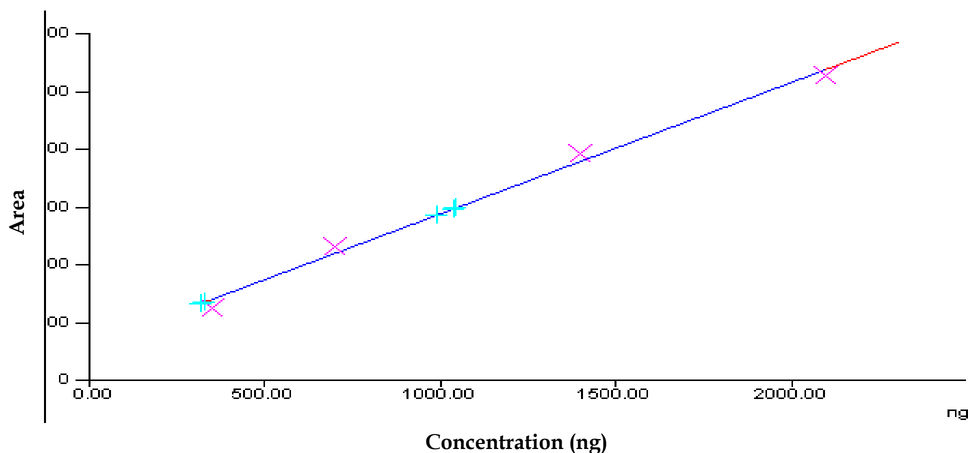

Fig. 5. Calibration plot of peak area against concentration for the flavone diglycoside standard

Analysis of Extract and Compound 1

Stock solutions of the extract (4600 µg mL^{-1}) and compound **1** (350 µg mL^{-1}) were prepared and 10 µL extract solution and 2 µL compound **1** were applied five times each to a TLC plate followed by development and scanning (*Fig. 6*). The spectra acquired *in situ* from standard and compound **1** could be superimposed (*Fig. 7A*).

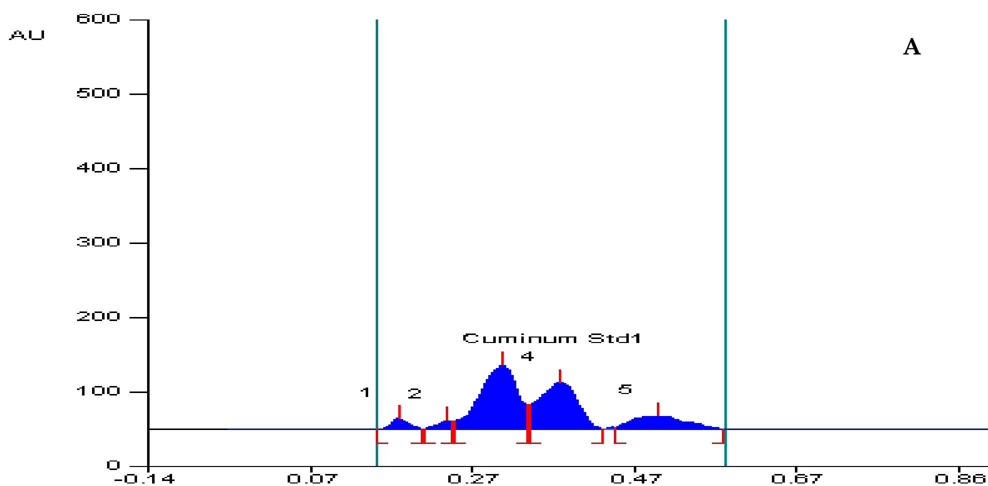

Fig. 6.

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

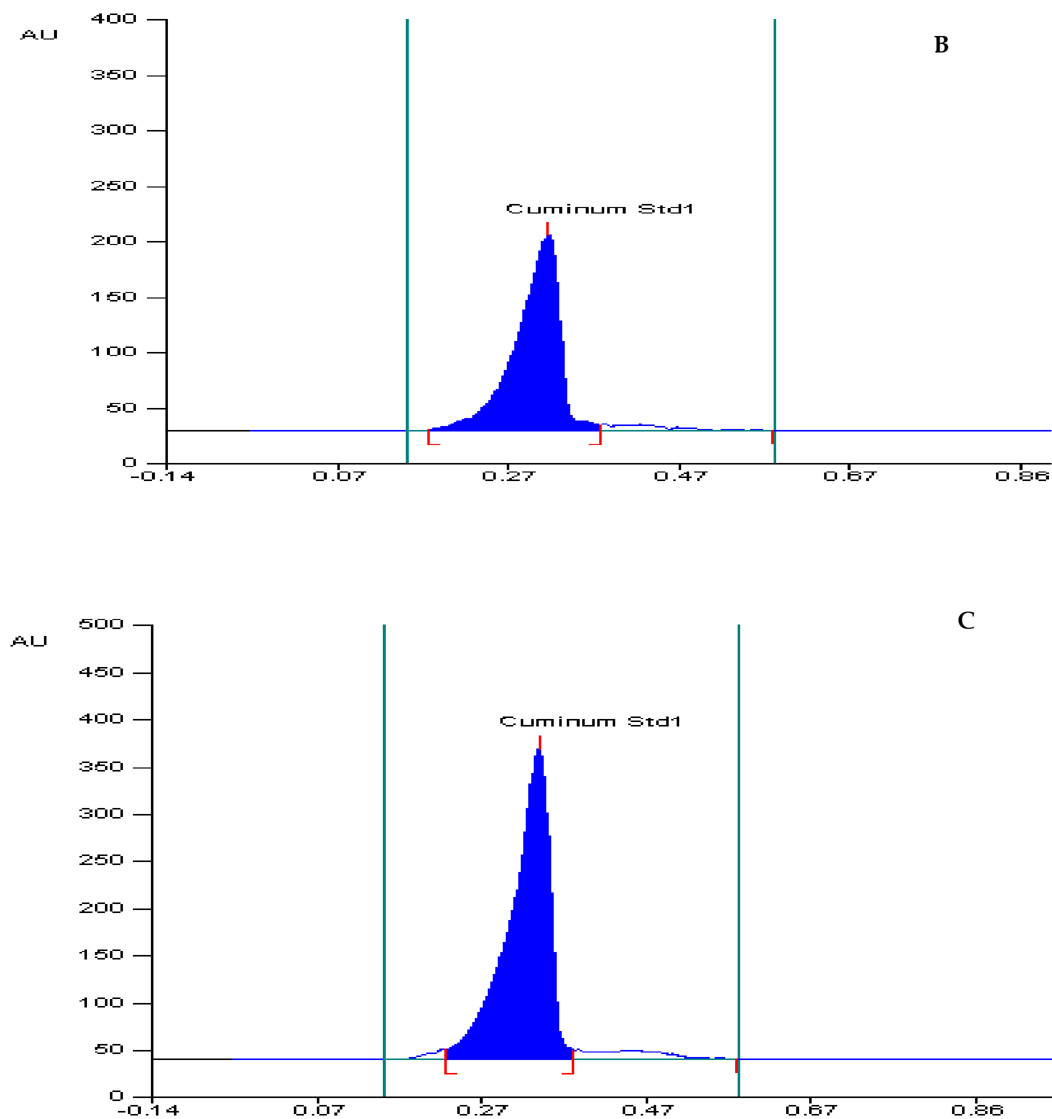

Fig. 6. A. Peaks obtained from compound **1** (R_F 0.32) and other constituents of *Cuminum cyminum* seed extract. B. Typical chromatogram obtained from isolated compound **1** (R_F 0.32). Typical chromatogram obtained from *Cuminum cyminum* standard (R_F 0.32)

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

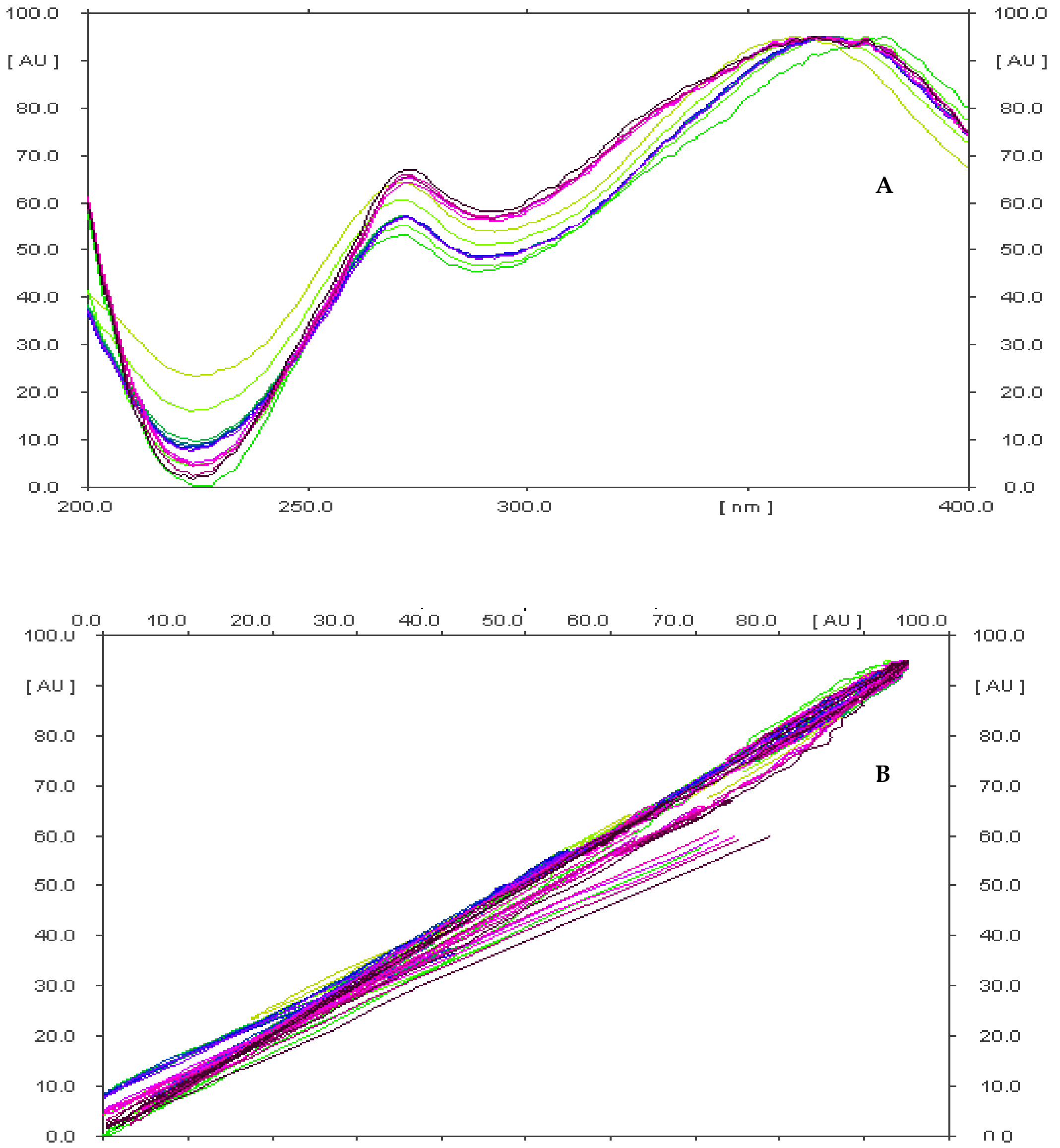

Fig. 7. A. Spectra obtained from flavone diglycoside standard and from compound 1 in the wavelength range 200–400 nm, showing maximum absorbance at 265 nm. B. Purity comparison spectra of flavone diglycoside standard and of compound **1**

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

Results and Discussion

The purity of the collected fraction was determined by LC. It was confirmed simultaneously by comparison of a total-ion chromatogram (TIC) with an LC–UV chromatogram. Complete overlap of the TIC and UV chromatogram was indicative of the purity of the collected standard. The possibility of the presence of impurities with no UV detectable chromophore was thus ruled out. MS–MS was also performed (*Fig. 3C*) to further check the purity of the collected fraction.

The peaks formed from m/z 625 were thus identified as daughter ion peaks of **1**. Based on the area under the curve of the LC chromatogram, **1** was determined to be sufficiently pure. Spiking of the hydrophilic extract with the standard also indicated enhancement in the area of the peak of **1**.

The collected compound compares well physicochemically with the reported compound obtained using polyamide, Whatman no. 3, and Sephadex LH-20 techniques as reported earlier [13]. A simple isocratic HPLC method has been described which yields **1** quantitatively in high purity. The purity of the compound **1** was also established by HPTLC (*Fig. 7B*). *Fig* 7B shows a correlation curve, which displays the intensities of the specta of compound **1** and the standard. The peak purity index was determined and ranged from 0.9932 to 0.9997.

Conclusion

A preparative HPLC method has been established for isolation of 7-(1-*O*-*β*-D-galacturonide-4′-(1-*O*-*β*-D-glucopyranosyl)-3′,4′,5,7-tetrahydroxyflavone rom *Cuminum cyminum* seed extract. HPLC and HPTLC methods were established for analysis of the isolated compound. It was apparent from the results obtained that both methods were precise, simple, and suitable for analysis of compound **1** in *Cuminum cyminum* extract.

References

[1] G. Charalambous, Spices, Herbs and Edible Fungi, Elsevier, Amsterdam, 1994
[2] A. Zargari, Medicinal Plants, Vol. II, Tehran University Press, Tehran, 1989, p. 519
[3] S.G. Joshi,. Medical Plants: Family Apicaceae, 1st edn, IBH Publishing, Oxford, 2000, p. 34
[4] S.A. Willatgamuwa, K. Platel, G. Sarawathi, and K. Srinivasan, Nutr. Res., **18**, 131 (1998)
[5] T. Malini and G. Vanithakumari, Indian J. Exp. Biol., **5**, 442 (1987)

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC

[6] M. Sayyah, A. Mahboubi, and M. Kamalinejad, Pharm. Biol., **40,** 47 (2002)
[7] M. Sayyah, A. Peirovi, and M. Kamalinejad, Iran Biomed. J., **6,** 141 (2002)
[8] K.C. Srivastava, Prostaglandins Leukotrienes Essential Fatty Acids, **37**, 57 (1989)
[9] G.N. Qazi, K.L. Bedi, R.K. Johri, M.K. Tikoo, A.K. Tikoo, S.C. Sharma, S.A. Tasduq, O.P. Suri, B.D. Gupta, K.A. Suri, N.K. Satti, A.K. Kalsotra, and R.K. Khajuria, US Patent 7,070,814 (2006)
[10] G.N. Qazi, O.P. Suri, K.L. Bedi, I. A. Khan, V. Verma, R.K. Johri, K.A. Suri, B.D. Gupta, N.K. Satti, M.K. Tikoo, S.C. Sharma, and A.K. Tikoo, US Patent 7119075 (2006)
[11] G.N. Qazi, K.L. Bedi, R.K. Johri, S.C. Sharma, M.K. Tikoo, A.K. Tikoo, S.A. Tasduq, K. Singh, R. Pandita, O.P. Suri, B.D. Gupta, K.A. Suri, N.K. Satti, R.K. Khajuria, B.K. Kapahi, and A.K. Kalsotra, US Patent filed; Indian Patent NF-292/2002
[12] B.S. Sachin, S.C. Sharma, S. Sethi, S.A. Tasduq, M.K. Tikoo, A.K. Tikoo, N.K. Satti, B.D. Gupta, KA. Suri, R.K. Johri, and G.N. Qazi, Phytother Res., **21,** 157 (2007)
[13] S.I. El-Negoumy and R.M.A. Mansour, Grasas Aceites (Seville), **40,** 87 (1989)

Accepted by MWH

Unauthenticated | Downloaded 12/10/23 11:47 PM UTC