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Short Communication

High-performance thin-layer chromatographic method for quantitative determination of 4α-methyl-24β-ethyl-5α-cholesta-14,25-dien-3β-ol, 24β-ethylcholesta-5,9(11),22*E*-trien-3β-ol, and betulinic acid in *Clerodendrum inerme*

A sensitive, selective, precise, and robust high-performance thin-layer chromatography method was developed and validated for analysis of two new recently isolated sterols, 4α-methyl-24β-ethyl-5α-cholesta-14,25-dien-3β-ol (**1**) and 24β-ethylcholesta-5,9(11),22*E*-trien-3β-ol (**2**), and a triterpene, betulinic acid (**3**), in *Clerodendrum inerme* extract. The method employed HPTLC plates precoated with silica gel $60F_{254}$ as the stationary phase. To achieve good separation, an optimised mobile phase consisting of toluene-acetone (94:06, v/v) was used (R_f 0.48, 0.34, and 0.22 for compounds **1**, **2**, and **3**, respectively). Densitometric determination of the above compounds was carried out in reflection/absorption mode at 620 nm. Optimised chromatographic conditions provide well separated compact spots for the compounds **1**, **2**, and **3**. The calibration curves were linear in the concentration range of 100 – 2500 ng/spot. The method was validated for precision, robustness, and recovery. The limits of detection and quantitation were 5, 6, and 10 μg/mL and 14, 18, and 29 μg/mL, respectively, for **1**, **2**, and **3**. The method reported here is reproducible and convenient for quantitative analysis of these compounds in the aerial parts of *C. inerme*.

Keywords: *Clerodendrum inerme* / HPTLC / Sterols / Triterpene

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1 Introduction

Sterols are known to possess a wide range of biological activities and physical properties. Plant sterols (*i.e.* phytosterols), in particular, are important agricultural products for the health and nutrition industries. They act as emulsifiers in the cosmetics industry and constitute the majority of steroidal intermediates and precursors for the production of hormone pharmaceuticals [1]. Many animal and human studies have shown phytosterols to reduce serum/plasma total cholesterol and low density lipoprotein (LDL) cholesterol levels [2]. In several countries commercial margarines formulated with certain levels of phytosterols are currently available [3]. Phytosterols have shown inhibition of tumours induced by chemicals in animals. A decreased risk of colon cancer in populations consuming high levels of phytosterols has been indicated by epidemiological studies [2]. 4-Methylated sterols have been reported to activate meiosis in mouse oocytes [4]. Betulinic acid (**3**) possesses a wide spectrum of biological activities such as antimalarial, anti-inflammatory, antitumour, and anti-HIV [5, 6]. It has also shown potential allelopathic activity [7]. The genus *Clerodendrum* is the largest genus of the family Verbenaceae and comprises *ca.* 560 species and varieties [8]. It is known for its insect antifeeding activity. The various species of the genus are used in traditional medicine as antimalarials and against intestinal parasites [9]. The potential use of species of *Clerodendrum* as a crude drug (antihypotensive, diuretic, anthelmintic, *etc*.) has been reported [10]. The genus *Clerodendrum* is known to contain rare 4-methylated as well as triene sterols [11 – 13]. *C. inerme* G. like many other species of the genus is a well known medicinal plant having alterative and febrifugal properties. An alcoholic extract of the plant has also been reported to exhibit uterine stimulant activity [14]. Recently we have isolated two new sterols *viz*. 4α-methyl-24β-ethyl-5α-cholesta-14,25-dien-3β-ol (**1**) and 24β-ethylcholesta-5,9(11),22*E*-trien-3β-ol (**2**) [15] together with betu-

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linic acid from the aerial parts of the plant *C. inerme*. Compound **2** possesses antibacterial activity against three gram positive bacteria [submitted for publication]. Mainly GLC and HPLC methods are used for sterol analysis [3, 16] but they suffer from the drawback that for GLC sometimes derivatisation of the particular compound becomes essential whereas in HPLC choice of a suitable detector sometimes becomes difficult as sterols usually lack a chromophore for UV absorption, thus precluding their detection by a UV detector, the most commonly used kind of detector. Recently, use of high-performance thin-layer chromatography has gained momentum in plant analysis as it is a simple, low cost, and rapid analytical tool [17–20]. Moreover, progress in instrumentation has made this technique more efficient and reliable. To the best of our knowledge no analytical procedure for identification or quantitation of any group of chemical markers from this plant has been reported. Here, we report a new HPTLC method for simultaneous quantification of sterols **1** and **2** together with the triterpene betulinic acid (**3**).

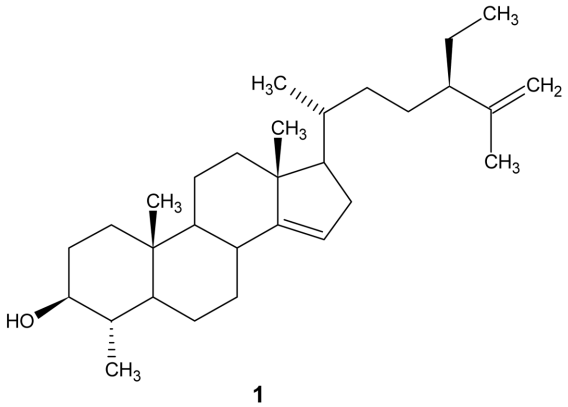
2 Experimental

2.1 Materials

The plant material of *C. inerme* was collected from ten different locations around Lucknow, India, in the month of September, 2005. The samples were authenticated and identified by our Botany and Pharmacognosy Department (voucher specimen no. CIMAP 9055). All the three chemical markers (Fig. 1) were isolated and identified in our laboratory. Precoated silica gel $60F_{254}$ HPTLC glass plates (10 × 10 cm; 20 × 10 cm; E. Merck, Darmstadt, Germany) were used. All chemicals and solvents used were either of analytical or of HPLC grade (E. Merck Ltd., Mumbai, India).

2.2 Extraction procedure

Air dried and finely powdered aerial parts of the plant (8.2 kg) were exhaustively extracted at room temperature (25–28°C) with ethanol (10 L × 3 times) and the ethanolic extract was concentrated *in vacuo* to give a residue. Water (500 mL) was added and it was then partitioned with *n*-hexane, chloroform, and *n*-butanol. The *n*-hexane extract (80.46 g) was subjected to column chromatography on silica gel, 1.5 kg (60–120 mesh). Elution was carried out with varying percentages of ethyl acetate in hexane. Fractions eluted with hexane–ethyl acetate in the ratio 95:5, v/v, afforded compounds **1** (0.06 g) and **2** (0.35 g) while those eluted with hexane–ethyl acetate in the ratio 85:15, v/v, resulted in isolation of compound **3** (0.03 g). For crystallisation, respective fractions were dissolved in a minimum quantity of chloroform and then acetone



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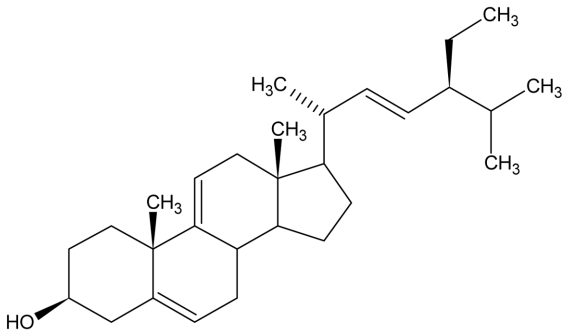

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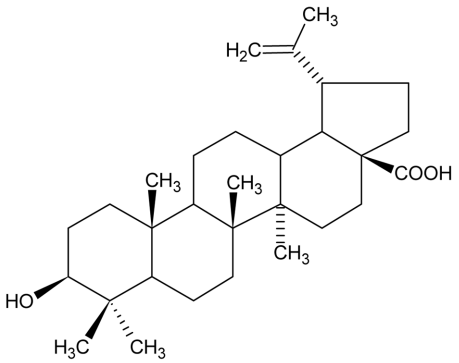

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Figure 1. Chemical structures of the compounds.

(for **1** and **2**)/methanol (for **3**) was added dropwise until the onset of turbidity in the solution. Colourless crystals were obtained after crystallisation at room temperature. The structures of compounds **1**, **2,** and **3** (Fig. 1) were elucidated on the basis of IR, mass, and ^{1}H and ^{13}C NMR spectral analysis [7, 15, 21].

2.3 Standard stock solution and sample preparation

Standard stock solutions containing 0.5 mg/mL of pure compounds **1**–**3** were prepared in a mixture of methanol

and chloroform (1:1, v/v) and filtered through 0.45-μm filters (Millipore, Billerica, MA) for calibration studies.

Samples were prepared from dried and powdered aerial parts (1.000 g each) of *C. inerme* collected from different locations. The powder was extracted with ethanol (3 × 10 mL), concentrated, and fractionated with *n*-hexane (3 × 10 mL). The vacuum-dried hexane extract was treated with charcoal using a chloroform–methanol (1:1, v/v) mixture to obtain chlorophyll-free hexane extracts. Known amounts of extracts (5 mg) were taken and dissolved in chloroform–methanol (1 mL; 1:1, v/v) and filtered through a 0.45-μm filter for HPTLC analysis.

The effect of the extracting solvent on the recovery (or percentage content) of the compounds was also studied. For this purpose in one instance the sample was prepared using the same procedure as above except that charcoal was not used. In another case extraction was carried out directly with hexane with subsequent charcoal treatment. Use of methanol in place of ethanol was also tried.

2.4 Apparatus

Linomat IV, Vario System, Immersion device III, TLC plate heater, TLC scanner III, Reprostar 3 (all Camag, Muttenz, Switzerland).

2.5 HPTLC procedure

Precoated silica gel $60F_{254}$ HPTLC glass plates were prewashed with methanol and activated at 100°C for 5 min prior to chromatography. Standard and sample solutions were spotted in the form of bands of 5-mm width at 15 mm from both the lower and left edge, and with a space of 10 mm between two bands, with a microlitre syringe using the Linomat IV, under a stream of nitrogen gas. A constant application rate of 5 μL/s was employed. Linear ascending development was carried out in 20 × 20-cm twin trough glass chambers saturated with the mobile phase. Mobile phase selection was carried out using the Vario System, wherein different compositions consisting of different ratios of toluene, acetone, hexane, and ethyl acetate were tried. Finally, a mobile phase consisting of toluene–acetone (94:06, v/v) was selected for chromatography. Other mobile solvent compositions resulted in a poor separation of compounds **1**–**3**. The length of the chromatographic run was 8 cm. The mobile phase saturation time of the TLC chamber was optimised to 5 min for better resolution of the three tested compounds. Detection of the spots was carried out by dipping the chromatoplate in freshly prepared vanillin–sulphuric acid–ethanol (1 g:5 mL:95 mL) reagent using the Camag Immersion device and subsequent heating at 110°C for 15 min on TLC plate heater. Quantitative evaluation of the plate was carried out with a TLC Scanner running winCATS 1.3.3 software. The selection of the scanning wavelength was made by scanning the plate at different wavelengths and the wavelength at which all the three compounds showed maximum absorption was selected. Densitometric scanning was performed in the reflectance/absorbance mode at 620 nm, slit width 6.00 × 0.40 mm, scanning speed 20 mm/s, and data resolution 10 μm/step. The radiation source utilised was a tungsten lamp. Both deuterium and tungsten lamps were used to record the spectra of compounds **1**, **2**, and **3** in the range 200–800 nm. The Reprostar 3 with cabinet cover and mounted digital camera was used for imaging and archiving the thin-layer chromatograms. Concentrations of the compounds chromatographed were determined from the intensity of the reflected light. Evaluation was via peak areas with linear regression.

3 Results and discussion

3.1 Sample preparation

The effect of extracting solvents or sample pre-treatment with charcoal was studied with respect to percentage content of compounds **1**–**3**. Though the use of charcoal-treated methanolic extract fractionated with hexane or only hexane gave results comparable to those for charcoal-treated hexane fraction of ethanolic extract, the percentage content of compounds **1**–**3** was found to be slightly less (Table 1) in both cases. Samples untreated with charcoal resulted in poorer recovery (Table 2) of the studied compounds as there was partial merging of peaks due to chlorophyll and standards. This also affected the peak purity of the compounds under study.

3.2 Development of the optimum mobile phase

The TLC procedure was optimised with a view to accomplishing simultaneous identification of these three marker compounds in the plant extract. Charcoal treatment of the extract was necessary to eliminate the possibility of merging of compound spots with chlorophyll present in the extract. Different compositions of solvent systems, *viz.* toluene–acetone (94:06), toluene–acetone (90:10), hexane–ethyl acetate (95:05) were tried. The

Table 1. Percent content of compounds **1**–**3** found in extract of *C. inerme* using different extracting solvents.

Solvent	% Content		
	1	**2**	**3**
Hexane[a)]	0.033	0.005	0.005
Alcohol then hexane[a)]	0.035	0.006	0.006
Methanol then hexane[a)]	0.031	0.005	0.004

[a)] Each extract was treated with charcoal.

Table 2. Effect of sample pre treatment with charcoal on recovery of compounds **1**–**3.**

Compound	Charcoal treated sample		Charcoal untreated sample	
	Recovery (%)	Average recovery (%)	Recovery (%)	Average recovery (%)
1	98.98	99.82	90.22	89.98
	100.10		89.50	
	100.39		90.23	
2	97.48	98.43	88.77	88.31
	98.74		88.11	
	99.08		88.04	
3	98.08	98.15	84.51	83.84
	98.43		83.22	
	97.93		83.78	

mobile solvent system consisting of toluene–acetone (94:06, v/v) gave sharp, symmetrical well resolved separation of test compounds (Figs. 2 and 3). The spot at R_f 0.48 was identified as **1**, the spot at R_f 0.34 as **2**, and that at R_f 0.22 as **3** by comparison of chromatograms of samples with individual standard chromatograms.

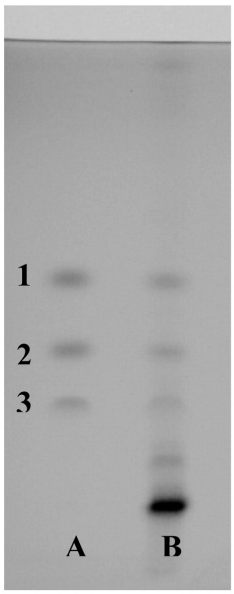

Figure 2. Photograph of the derivatised HPTLC plate depicting the separation of a mixture of standards **1**, **2**, and **3**, on track A (0.5 mg/mL) and a hexane extract (charcoal treated; 5 mg/mL) on track B.

3.3 Method validation

3.3.1 Linearity and quantification

The stock solutions of each standard **1, 2**, and **3** (0.5 mg/mL) were prepared in chloroform–methanol (1:1, v/v) solution. Stock working solutions were prepared by dilution of the stock solution with chloroform–methanol (1:1, v/v) to give solutions containing compounds **1, 2**, and **3** in the concentrations of 20, 25, 50, 100, and 500 μg/mL. Five microlitres from each standard solution was spotted on the TLC plate to obtain final concentration of 100, 125, 250, 500, and 2500 ng/spot. Each concentration was spotted three times on the TLC plate.

Five microlitres of each sample solution (5 mg/mL) from different locations was applied on the HPTLC plate in triplicate with a similar band pattern. The experimental parameters were identical for all the above analyses. The percentage contents of analytes in the sample were calculated on the basis of calibration curves.

Linearity was found over the concentration range 100–2500 ng/spot for **1, 2**, and **3** with correlation coefficient r = 0.9996, 0.9995, and 0.9993, respectively. The regression data obtained showed a good linear relationship (Table 3).

3.3.2 Limit of detection and quantification

The LOD defined as three times the baseline noise was found to be 5, 6, and 10 μg/mL for compounds **1, 2**, and **3**, respectively. The LOQ defined as ten times the baseline noise was calculated to be 14, 18, and 29 μg/mL for the analytes **1, 2** and **3,** present in sample solution, respectively.

3.3.3 Specificity

The specificity of the method was ascertained by analysing standards and sample. The spots for the three compounds **1, 2**, and **3** in samples were confirmed by comparing the R_f and spectra of the spot with those of standard. The peak purity of individual **1**, **2**, and **3** spots was assessed by comparing the spectra at peak start, peak apex, and peak end positions of the spot, *i.e.* r(start, middle) = 0.9999, 0.9998, 0.9999 and r(middle, end) = 0.9999,

Table 3. Linearity data for compounds **1**–**3.**

	1	**2**	**3**
Linearity range	100–2500 ng/spot	100–2500 ng/spot	100–2500 ng/spot
Regression equation	Y = 1027.185 + 869.109X	Y = 1461.628 + 540.871X	Y = 1140.226 + 535.291X
	r = 0.9996	r = 0.9995	r = 0.9993
	%CV = 1.42	%CV = 1.32	%CV = 1.69

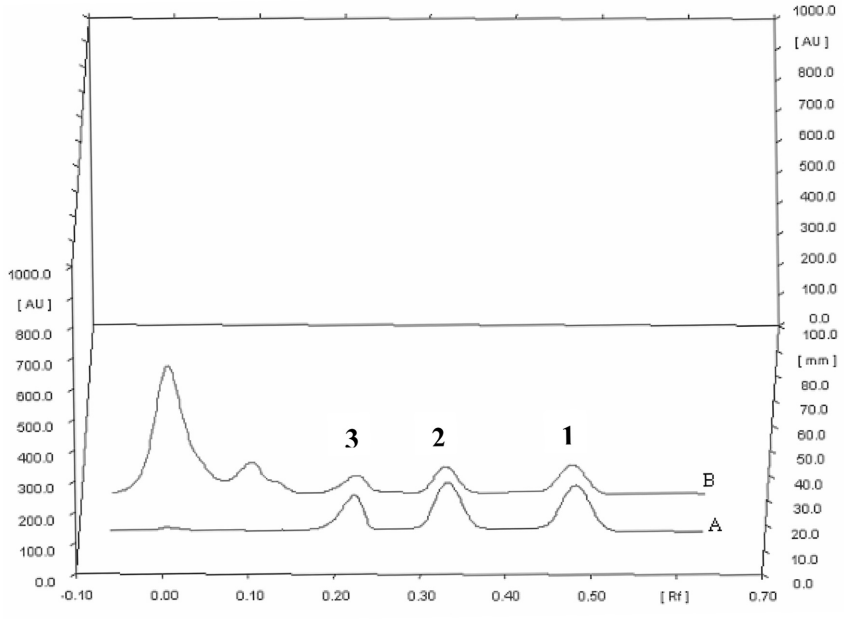

Figure 3. Representative HPTLC chromatogram showing separation of the compounds. Track A: Artificial mixture of compounds **1**, **2**, and **3**. Track B: Charcoal-treated hexane extract of *C. inerme*.

Table 4. Peak purity test for compounds **1**–**3.**

Compound	*r* (s, m)[a]		*r* (m, e)[b]	
	Standard track	Sample track	Standard track	Sample track
1	0.9999	0.9983	0.9999	0.9994
2	0.9998	0.9999	0.9999	0.9997
3	0.9999	0.9996	0.9999	0.9988

[a] *r* (s, m) = Correlation of spectrum at start of peak with spectrum at the centre of peak.

[b] *r* (m, e) = Correlation of spectrum at centre of peak with spectrum at end of peak.

0.9999, 0.9999, respectively. Good correlation was also obtained between standards and sample overlaid spectra (Table 4).

3.3.4 Recovery

Recovery studies of the analytes in the sample were carried out to assess the accuracy of the method. For this purpose, three different spiking concentrations of the standard stock solutions of compounds **1, 2**, and **3** were prepared. Three sets were prepared from a pre-analysed sample, *i.e.* location 1 (1 mL each of concentration 5 mg/mL). Then these three sets were individually spiked with three different spiking concentrations of the individual standard solutions (in the range 50, 100, and 150% of the amount of the standard present in the sample). Chromatography of these spiked samples was as described in Section 2.5. The average recoveries for compound **1, 2**, and **3** were found to be 99.82, 98.43, and 98.15%, respectively (Table 5).

3.3.5 Precision

The repeatability of measurement (n = 5) of peak area of spots was expressed in terms of percent coefficient of variation (% CV) and found to be 0.54, 0.77, and 1.34 for compounds **1, 2**, and **3**, respectively. The results depicted in Table 6 showed that no significant intra- and inter-day variation was observed in the analysis of the compounds **1, 2,** and **3**.

3.3.6 Robustness

The robustness of the method was determined by introducing small changes in certain chromatographic parameters, *viz.* mobile phase composition, plate pretreatment, and the time from heating the plate after chromatography to scanning. Only one parameter was varied at a time while the rest were kept constant. The effects on the results, *i.e.* peak areas, were examined. The standard deviation of peak areas was calculated for each parameter and %CV was found to be less than 2% in each case. The low values of %CV as shown in Table 7 indicates the robustness of the method. Separation was not affected by changing the scanning wavelength by ± 5 nm.

3.4 Quantitative evaluation of extracts

Ten different samples of *C. inerme* were analysed for compounds **1**, **2**, and **3** and the results are presented in Table 8. The percentage contents of compounds **1, 2**, and **3** range from 0.012 to 0.035, 0.006 to 0.025, and 0.005 to 0.010, respectively.

Table 5. Recovery study to assess accuracy of the method.

Compound	Amount of compound in sample (μg/mL)	Spiked amount (μg/mL)	Theoretical value (μg/mL)	Mean experimental value (μg/mL) (n = 3)	Recovery (%)	Average recovery (%)	%CV
1	113.35	60	173.35	171.59 ± 0.75%	98.98	99.82	0.75
		100	213.35	213.56 ± 0.29%	100.10		
		150	263.35	264.37 ± 1.08%	100.39		
2	17.49	10	27.49	26.80 ± 0.42%	97.48	98.43	0.86
		20	37.49	37.02 ± 0.58%	98.74		
		25	42.49	42.10 ± 0.47%	99.08		
3	34.37	20	54.37	53.33 ± 0.98%	98.08	98.15	0.26
		30	64.37	63.37 ± 0.28%	98.43		
		45	79.37	77.23 ± 0.41%	97.93		

Table 6. Precision studies.

Precision (n = 5)	%CV		
	1	**2**	**3**
Instrumental	0.54	0.77	1.34
Intra-day	0.83	1.45	1.33
Inter-day	1.34	1.83	2.58

Table 7. Robustness testing.

Parameter	%CV of peak areas		
	1	**2**	**3**
Mobile phase composition	1.39	1.47	1.73
Plate pre-treatment	0.49	0.60	0.50
Time from detection of spots to scanning	1.06	1.19	1.32

Table 8. Percent content of compounds **1 – 3** found in extract of *C. inerme* by the proposed method.

Location	% Contents		
	1	**2**	**3**
1	0.035	0.006	0.006
2	0.022	0.025	0.010
3	0.012	0.006	0.008
4	0.023	0.018	0.005
5	0.024	0.020	0.008
6	0.015	0.022	0.006
7	0.031	0.012	0.006
8	0.017	0.025	0.008
9	0.028	0.018	0.009
10	0.022	0.020	0.006

4 Concluding remarks

The present HPTLC method is precise, specific, accurate, and robust for the determination of these important markers of *C. inerme*. The method was found to be repeatable and selective. The standardised HPTLC chromatograms can be used effectively for the screening analysis or quality assessment of the plant or its derived products.

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5 References

[1] Clark, J., *Lipid Technol.* 1996, *5*, 111.

[2] Ling, W. H., Jones, P. J. H., *Life Sci.* 1995, *57*, 195 – 206.

[3] Abidi, S. L., *J. Chromatogr. A* 2001, *935*, 173 – 201.

[4] Byskov, A. G., Andersen, C. Y., Nordholm, L., Thogersen, H., Guoliang, X., Wassmann, O., Andersen, J. V., Guddal, E., Roed, T., *Nature* 1995, *374*, 559 – 562.

[5] Steele, J. C. P., Warhuret, D. C., Kirby, G. C., Simmonds, M. S. J., *Phytotherapy Res.* 1999, *13*, 115 – 119.

[6] Patocka, J., *J. Appl. Biomed.* 2003, *1*, 7 – 12.

[7] Macias, F. A., Simonet, A. M., Esteban, M. D., *Phytochemistry* 1994, *36*, 1369 – 1379.

[8] Jacke, G., Rimpler, H., *Phytochemistry* 1983, *22*, 1729 – 1734.

[9] Dorsaz, A. C., Marston, A., Stoeckli-Evans, H., Msonthi, J. D., Hostettmann, K., *Helv. Chim. Acta* 1985, *68*, 1605 – 1610.

[10] Lin, Y.-L., Kuo, Y.-H., Chen, Y.-L., *Chem. Pharm. Bull.* 1989, *37*, 2191 – 2193.

[11] Akihisa, T., Ghosh, P., Thakur, S., Oshikiri, S., Tamura, T., Matsumoto, T., *Phytochemistry* 1988, *27*, 241 – 244.

[12] Akihisa, T., Tamura, T., Matsumoto, T., Kokke, W. C. M. C., Ghosh, P., Thakur, S., *J. Chem. Soc. Perkin Trans. I* 1990, 2213 – 2218.

[13] Akihisa, T., Ghosh, P., Thakur, S., Nagata, H., Tamura, T., Matsumoto, T., *Phytochemistry* 1990, *29*, 1639 – 1641.

[14] Rao, L. J. M., Pereira, J., Gurudutt, K. N., *Phytochemistry* 1993, *34*, 572 – 574.

[15] Pandey, R., Verma, R. K., Singh, S. C., Gupta, M. M., *Phytochemistry* 2003, *63*, 415 – 420.

[16] Volin, P., *J. Chromatogr. A* 2001, *935*, 125 – 140.

[17] Singh, D. V., Verma, R. K., Gupta, M. M., Kumar, S., *Phytochem. Anal.* 2002, *13*, 207 – 210.

[18] Srivastava, A., Misra, H., Verma, R. K., Gupta, M. M., *Phytochem. Anal.* 2004, *15*, 280 – 285.

[19] Coran, S. A., Giannellini, V., Bambagiotti-Alberti, M., *J. Chromatogr. A* 2004, *1045*, 217 – 222.

[20] Singh, N. P., Gupta, A. P., Sinha, A. K., Ahuja, P. S., *J. Chromatogr. A* 2005, *1077*, 202 – 206.

[21] Galgon, T., Hoke, D., Drager, B., *Phytochem. Anal.* 1999, *10*, 187 – 190.

