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Original article

X-ray crystallographic and validated HPTLC analysis of the biomarker chromone glucoside (schumanniofioside A) isolated from *Acalypha fruticosa* growing in Saudi Arabia

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ABSTRACT

A chromone glucoside 2-methyl-5,7-dihydroxychromone 5-*O*-β-D-glucopyranoside (schumanniofioside A, compound 1) was isolated from the methanol extract of *Acalypha fruticosa*. The structure of compound 1 was fully assigned based on nuclear magnetic resonance (NMR) (^{1}H, ^{13}C and 2D) spectra and electrospray ionization mass spectrum (ESI-MS) in addition to X-ray Crystallography. The molecules were packed in the crystal structure by eight intermolecular O—H···O and C—H···O interactions. The structure of compound 1 belongs to monoclinic, $P2_1$, a = 9.1989 (4) Å, b = 4.6651 (2) Å, c = 20.4042 (7) Å, β = 97.862 (3)°, V = 867.31 (6) Å^3, Z = 2, $wR_{ref}(F^2)$ = 0.101, T = 100 K. Thus, the bond angles, bond lengths and absolute structure of compound 1 were confirmed by its X-ray structure. A validated HPTLC method was developed for the quantitative analysis of compound 1 in chloroform and methanol extracts of *A. fruticosa*. It was found to furnish a compact and sharp band of compound 1 at R_f = 0.13 ± 0.005 using chloroform, methanol and glacial acetic acid [17:3:0.5 (v/v/v)] as mobile phase. The LOD and LOQ for compound 1 were found to be 17.86 and 54.13 ng/band, respectively. Compound 1 was found in both chloroform and methanol extracts of the plant (0.03% w/w and 0.31% w/w, respectively). The proposed HPTLC method can be used for the further analysis of schumanniofioside A in different plant extracts, herbal formulations and biological samples as well as in process quality control.

1. Introduction

Chromones (γ-benzopyrones) and their 2- and 3-phenyl analogues (flavones and isoflavones) are naturally occurring compounds ubiquitously found in the plant kingdom, and therefore present in representative amounts in normal human diet. These chromones and their derivatives possess a wide spectrum of biological activities (Ismail et al., 2009; Machado and Marques, 2010) viz. antibacterial (Tchouya et al., 2014), antifungal (Ma et al., 1996), antiviral (Ma et al., 2002; Park et al., 2008), antioxidant (Kuroda et al., 2009), anticancer (Semba et al., 2002; Ren et al., 2003), anti-inflammatory (Hutter et al., 1996) and neuroprotective (Yoon et al., 2006) activities.

In our previous phytochemical study on *Acalypha fruticosa* Forssk. Fl. Aeg growing in Saudi Arabia, we isolated a chromone glucoside 2-methyl-5,7-dihydroxychromone-5-*O*-β-D-glucopyranoside (compound 1) (Fig. 1) from the aerial parts of the plant, and proved its dual PPAR activator effect (Fawzy et al., 2017). Compound 1 was also previously isolated from the root bark of *Schumanniophyton magnificum*, and was named as schumanniofioside A (Tane et al., 1990).

Based on the biological importance of compound 1, it was of interest to fully investigate compound 1 structure by single crystal X-ray diffraction, NMR (^{1}H, ^{13}C and 2D) spectra and electrospray ionization mass spectrum (ESI-MS).

The high-performance thin-layer chromatography (HPTLC) has recently become a conventional analytical tool for the quality-control of herbal drugs because of its low operation-cost, high sample-throughput and need for minimum sample clean-up (Alam et al., 2014). The developed HPTLC chromatograms are

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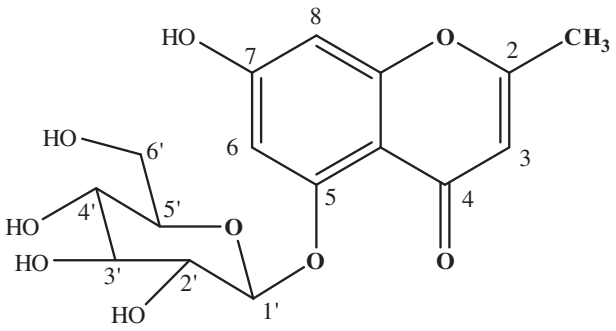

Fig. 1. The chemical structure of compound 1.

useful in identification of biomarkers in various herbal formulations by comparing the fingerprints with standard (Siddiqui et al., 2015; Alam et al., 2015a). It is widely employed for the identification, purity testing, stability, dissolution or content uniformity of crude extracts of plant and animal origin, fermentation mix, drugs and excipients, including pharmaceutical, cosmetic and nutrient formulations (Alajmi et al., 2013; Alam et al., 2015b). Thus, our goal was to develop a validated HPTLC method for the quantitative estimation of the compound 1 in the chloroform and methanol extracts of *A. fruticosa*.

2. Experimental

2.1. General

The ^{1}H, ^{13}C NMR and 2D NMR spectra and the Electrospray ionization mass spectrum (ESI-MS) were recorded as reported in our previous paper on the plant (Fawzy et al., 2017). Compound 1 was obtained as single crystals by slow evaporation of its solution in ethanol at room temperature. Data were collected on a Bracer APEX-II D8 Venture area diffractometer, equipped with graphite monochromatic Cu Kα radiation at 100 K. Cell refinement and data reduction were carried out by Bruker SAINT. SHELXS-97 (Sheldrick, 1997, 2008) was used to solve structure. The final refinement was carried out by full-matrix least-squares techniques with anisotropic thermal data for nonhydrogen atoms. The crystallographic data of compound 1 are available at the Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif (CCDC 1434862).

2.2. Plant material

The aerial parts of *A. fruticosa* Forssk. Fl. Aeg (1.1 kg) were collected in February 2013, from Southwestern region, Saudi Arabia, (Jabal Shada al-A'la). The plant material was identified by Dr. Mohammed Yusuf, Professor of Taxonomy, College of Pharmacy, King Saud University. A voucher specimen (No. 16018) is kept in the herbarium of Department of Pharmacogonsy, King Saud University.

2.3. Extraction and isolation

The aerial parts of *A. fruticosa* (1.1 kg) were shade-dried, ground and successively extracted at room temperature with chloroform (3 × 2.5 l) and methanol (4 × 2.5 l). Isolation of compound 1 has been described in our recently published article on the plant (Fawzy et al., 2017).

2.4. HPTLC instrumentation and conditions

A validated high-performance thin layer chromatography (HPTLC) method was developed for the quantitative analysis of compound 1 in *A. fruticosa* chloroform extract (AFCE) and *A. fruticosa* methanol extract (AFME), which were prepared according to the procedure described in our previous paper on *A. fruticosa* (Fawzy et al., 2017). The chromatograms were performed on 10 × 10 cm precoated silica gel F_{254} HPTLC plate. Numerous combinations of mobile phases were tried to get good separation and resolution of various components present in *A. fruticosa*. Based on our observations chloroform, methanol and glacial acetic acid in the ratio of 17: 3: 0.5, (v/v/v) were selected as mobile phase to carry out the analysis. Different concentrations of the compound 1 along with different samples were applied by CAMAG automatic TLC sampler-4 on the HPTLC plate while CAMAG Automated Developing Chamber-2 (ADC-2) was used to develop the HPTLC plate under controlled condition. The developed plate was dried and scanned by CAMAG TLC Scanner-3 (λ = 297 nm).

2.5. Preparation of standard stock solutions and sample solution

Standard stock solution of compound 1 (1 mg/mL) was prepared in methanol, following further dilution with methanol to furnish different concentrations ranging from 10 to 100 μg/mL. All the dilutions (10, 20, 40, 60, 80 and 100 μg/mL) were applied (10 μL, each) through microliter syringe attached with the applicator on the HPTLC plate to furnish the linearity range of 100–1000 ng/band. The sample concentrations prepared for AFCE and AFME were 30 mg/mL and 10 mg/mL, respectively and applied in equal volume on the HPTLC plate by the applicator.

2.6. Validation of method

Validation of the proposed HPTLC method was performed according to the ICH guidelines (ICH, 2005), for the determination of linearity range, limit of detection (LOD), limit of quantification (LOQ), precision, recovery as accuracy and robustness.

2.6.1. Linearity range

All the dilutions (10, 20, 40, 60, 80 and 100 μg/mL) prepared from the standard stock solution were applied (10 μL, each) through microliter syringe attached with the applicator on the HPTLC plate to furnish the linearity range of 100–1000 ng/band.

2.6.2. Accuracy

Accuracy was determined by standard addition method. The preanalyzed sample of compound 1 (200 ng/spot) was spiked with the extra 0, 50, 100 and 150% of compound 1 and the solutions were reanalyzed in six replicates. The % recovery and percent relative standard deviation (%RSD) were calculated.

2.6.3. Precision

Precision (inter and intraday) of the proposed method was evaluated by performing replicate analyses (n = 6) at three different concentration levels 400, 600 and 800 ng/spot of compound 1. Inter-day precision was determined by repeating the intra-day assay on three different days.

2.6.4. Robustness

Robustness was studied in triplicate at 300 ng band^{-1} by making deliberately small change to mobile phase composition, mobile phase volume and duration of saturation. The results were studied in terms of SD and %RSD of peak areas. Mobile phases prepared from *Chloroform, methanol and glacial acetic acid (v/v/v),* in different proportions were used for chromatography. Mobile phase volume and duration of saturation investigated were 20 ± 2 mL (18, 20, and 22 mL) and 20 ± 10 min (10, 20 and 30 min), respectively. The plates were activated at 110 °C for 30 min before chromatography.

2.6.5. LOD and LOQ

The LOD is the lowest amount of an analyte that may be differentiated from the assay background at a distinct level of confidence and the LOQ is the minimum amount that can be quantified at a distinct level of precision or accuracy. The LOD and the LOQ were calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formulae: [LOD = 3.3 (SD/S) and LOQ = 10 (SD/S)]. The standard deviation of the response was determined based on the standard deviation of y-intercepts of regression lines.

2.6.6. Assay of compound 1

Compound 1 and test samples (AFCE and AFME) were spotted in duplicate on HPTLC plates. The percentage of compound 1 present in test samples was determined by measuring area for the standard and test samples.

2.7. Statistical analysis

Data were expressed as means ± SD. The unpaired Student's *t*-test was conducted using the Graph Pad InStat (ISI Software) computer program. Differences were considered significant at P values of less than 0.05.

3. Results and discussion

3.1. X-ray crystallographic analysis of compound 1

A series of column chromatographic separations of the methanol extract of the aerial parts of *Acalypha fruticosa* resulted in the isolation of a chromone glucoside,2-methyl-5,7-dihydroxychromone 5-*O*-β-D-glucopyranoside (compound 1), whose spectral data (^{1}H, ^{13}C NMR and ESI-MS) supported the structure compound 1 (Fawzy et al., 2017). The structure of compound 1 was further supported by single-crystal X-ray crystallographic analysis of its isolated dehydrate derivative.

The asymmetric unit of the crystal contains one independent molecule with two molecules of water (Fig. 2) and the pyran ring (C11-C15/O6) shows a chair conformation. Some substituents are equatorially attached to this ring and others are axially attached.

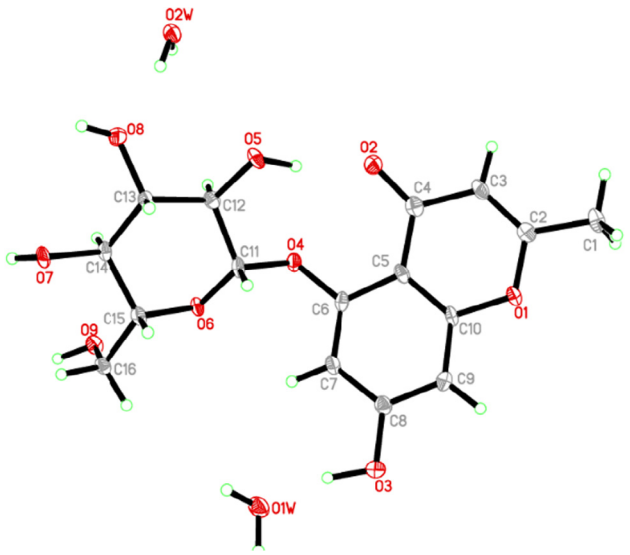

Fig. 2. The ORTEP diagram of compound 1. Displacement ellipsoids are plotted at the 40% probability level for non-H atoms.

The structure of the five chiral centers were found to be C11(*S*), C12(*R*), C13(*S*), C14(*S*) and C15(*R*).The crystallographic data and refinement information of compound 1 are summarized in Table 1. All the bond lengths and bond angles are in normal ranges (Parson et al., 2013).

3.2. HPTLC method development and validation

The mobile phase used in HPTLC analysis was selected by analyzing various compositions of different solvents. Of these, a mixture of *Chloroform, methanol and glacial acetic acid* in the ratio of 17:3:0.5 (*V/V/V*) was found to be the best mobile phase for development and analysis of compound 1 in AFCE and AFME. The developed HPTLC method gave an intense, compact and sharp peak of compound 1 at $R_f = 0.13 \pm 0.005$ (Fig. 3). This method was found to be very efficient in clearly separating the compound 1 and various constituents of AFCE and AFME. During the development of the HPTLC plate the optimized mobile phase volume for saturation was found to be 20 ml and saturation time was found to be 20 min. The developed HPTLC method was found to be selective with high resolution baseline. The regression equation and square of correlation coefficient (r^2) for compound 1 were found to be Y = 6.99x + 1021.67 and 0.9975 ± 0.0002 in the linearity range 100–1000 ng/spot. The Limit of detection (LOD) and limit of quantification (LOQ) for compound 1 were found to be 17.86 and 54.13 ng/band, respectively (Table 2). The recoveries as accuracy study for the proposed method was recorded (Table 3). The recovery (%) and RSD (%) for compound 1 were found to be 98.79–99.79 and 1.72–2.13, respectively. The intra-day and inter-day precision for the proposed method was recorded (Table 4). The %RSD for intra-day and inter-day precisions (n = 6) were found to be 1.64–1.51% and 1.47–1.63%, respectively, which showed the good precision of the proposed method. The robustness study was performed by

Table 1
X-ray crystal and experimental data collection of compound 1.

Crystal data	
Chemical formula	$C_{16}H_{18}O_9.2H_2O$
Molecular weight	390.33
Crystal system, space group	Monoclinic, $P2_1$
Temperature (K)	100
a, b, c (Å)	9.1989 (4), 4.6651 (2), 20.4042 (7)
β (°)	97.862 (3)
V (Å^3)	867.31 (6)
Z	2
Radiation type	Cu Kα
μ (mm^{-1})	1.11
Crystal size (mm)	0.80 × 0.07 × 0.06
Data Collection	
Diffractometer	Bruker APEX-II D8 Venture diffractometer
Absorption correction	Multi-scan, SADABS Bruker 2014
T_{min}, T_{max}	0.964, 0.987
No. of measured, independent and observed [I > 2σ(I)]σ(I)] reflections	8965, 2975, 2663
R_{int}	0.055
Refinement	
R[F^2 > 2σ(F^2)], wR(F^2), S	0.040, 0.101, 1.14
No. of reflections	2975
No. of parameters	280
No. of restraints	1
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.39, −0.41
Absolute structure	Flack x determination using 1011 quotients [(I+)-(I−)]/[(I+)-(I−)] [25]
Flack parameter	0.35 (16)

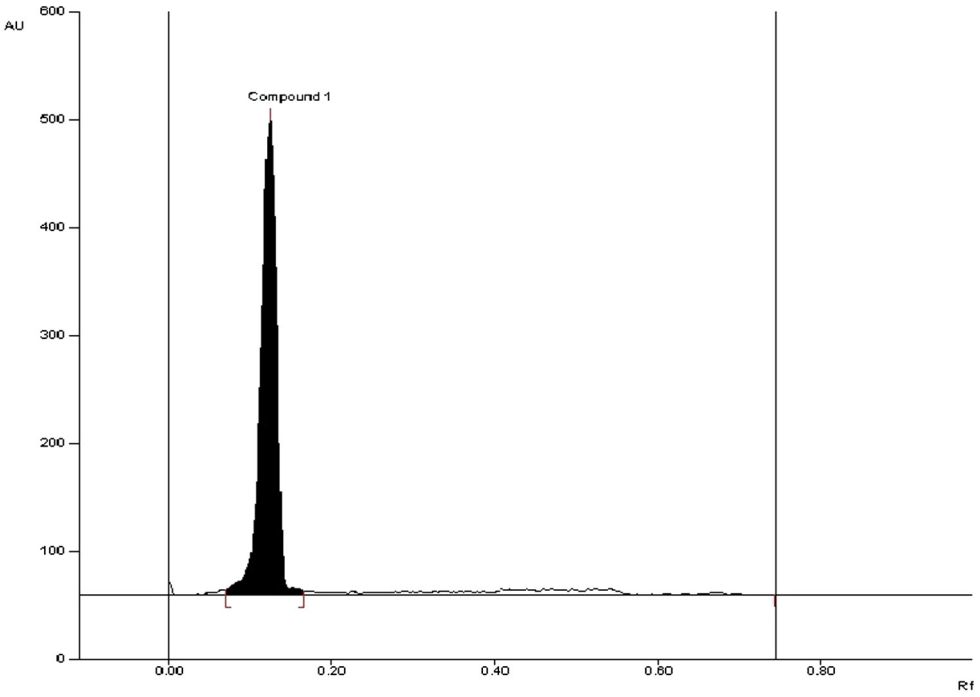

Fig. 3. Chromatogram of compound 1 (R_f = 0.13; 600 ng/spot) at λ = 297 nm; mobile phase: [chloroform: methanol: glacial acetic acid (17:3:0.5, v/v/v)].

Table 2
R_f, Linear regression data for the calibration curve of compound 1 (n = 6).

Parameters	Compound 1
Linearity range (ng/spot)	100–1000
Regression equation	Y = 6.99X + 1021.67
Correlation (r^2) coefficient	0.9975 ± 0.0002
Slope ± SD	6.99 ± 0.04
Intercept ± SD	1021.67 ± 26.41
Standard error of slope	0.02
Standard error of intercept	15.25
R_f	0.13 ± 0.005
LOD	17.86 ng/band
LOQ	54.13 ng/band

introducing small deliberate changes in the mobile phase composition, duration of saturation and mobile phase volume used in the saturation at the 300 ng/band concentration of compound 1. The obtained data of this study in the form of SD and %RSD was reported in Table 5. The SD and %RSD values were found to be very low which indicated that the method was robust.

Table 5
Robustness of the proposed HPTLC method (n = 6).

Optimization condition	Compound 1 (300 ng/band)	
	SD	%RSD
Mobile phase composition; (Chloroform: methanol: glacial acetic acid, v/v/v)(mL)		
17:3:0.5	8.03	2.71
16.8:3.2:0.5	8.45	2.83
17.2:2.8:0.5	8.59	2.92
Mobile phase volume (for saturation)		
18 mL	6.98	2.35
20 mL	7.11	2.39
22 mL	7.22	2.44
Duration of saturation		
10 min	7.03	2.37
20 min	7.14	2.40
30 min	7.19	2.43

Table 3
Recovery as accuracy studies of the proposed HPTLC Method (n = 6).

Percent (%) of compound 1 added to analyte	Theoretical concentration of compound 1 (ng/ml)	Concentration of compound 1found (ng/mL) ± SD	%RSD	% Recovery
0	200	197.59 ± 3.39	1.72	98.79
50	300	297.93 ± 5.69	1.91	99.31
100	400	399.17 ± 8.18	2.05	99.79
150	500	495.45 ± 10.55	2.13	99.09

Table 4
Precision of the proposed HPTLC Method (n = 6).

Conc. of compound 1 (ng/band)	Intra-day precision		Inter-day precision	
	Average Conc. found ± SD	%RSD	Average Conc. found ± SD	%RSD
400	398.16 ± 6.01	1.51	395.30 ± 5.81	1.47
600	596.24 ± 9.48	1.59	592.09 ± 9.05	1.53
800	795.96 ± 13.05	1.64	791.81 ± 12.87	1.63

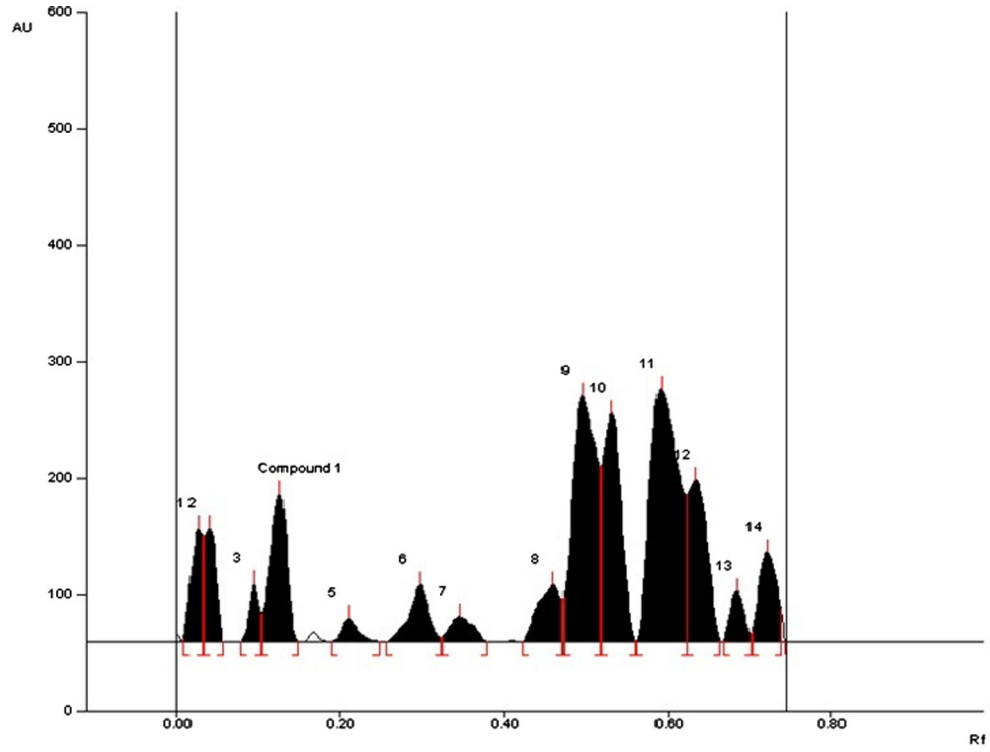

Fig. 4. Chromatogram of AFCE scanned at λ = 297 nm (compound 1; R_f = 0.13, peak 4); mobile phase: [chloroform: methanol: glacial acetic acid (17:3:0.5, v/v/v)].

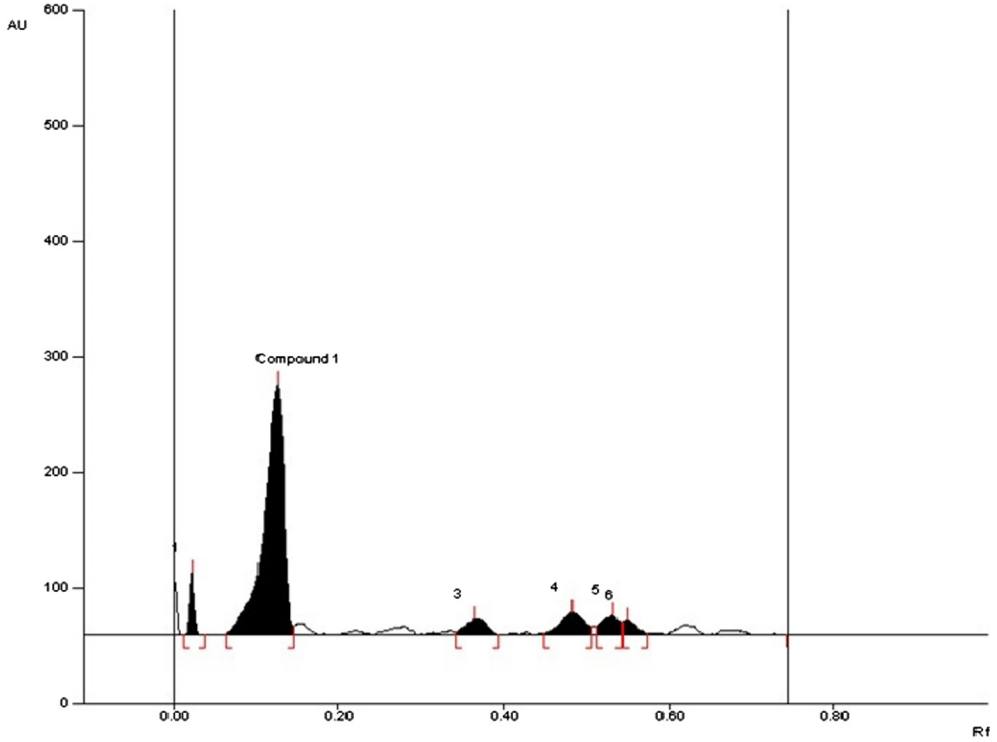

Fig. 5. Chromatogram of AFME scanned at λ = 297 nm (compound 1; R_f = 0.13, peak 2); mobile phase: [chloroform: methanol: glacial acetic acid (17:3:0.5, v/v/v)].

3.3. Estimation of compound 1 in AFCE and AFME by HPTLC

The proposed validated HPTLC method was employed for quantitative analysis of compound 1 in *A. fruticosa* chloroform extract (AFCE) and *A. fruticosa* methanol extract (AFME). This biologically active marker was found to be present in the both extracts, AFCE (Fig. 4) and AFME (Fig. 5). The dried *A. fruticosa* chloroform extract (AFCE) and *A. fruticosa* methanol extract (AFME) were found to contain 0.03%, w/w and 0.31%, w/w, respectively of compound 1.

4. Conclusion

2-Methyl-5,7-dihydroxychromone-5-*O*-β-D-glucopyranoside (compound 1), a chromone glucoside was isolated from the aerial parts of *Acalypha fruticosa* growing in Saudi Arabia. The structure of the compound was fully characterized based on nuclear magnetic resonance (^{1}H, ^{13}C and 2D NMR spectra), ESI-MS and single-crystal X-ray analysis. This is a maiden report, which demonstrated the development of an economical, precise, accurate and simple HPTLC method for quantitative analysis of the biomarker compound in different extracts of *A. fruticosa.* The proposed HPTLC method can be further used for the further analysis of compound 1 in different plant extracts, herbal formulations and biological samples as well as in process quality control.

5. Conflicts of interest

The authors declare no conflict of interest.

Supplementary materials

The crystallographic data of compound 1 are available at the Cambridge Crystallographic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif (CCDC 1434862).

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