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CYTOTOXIC EVALUATION AND CONCURRENT ANALYSIS OF TWO DITERPENES IN THE CHLOROFORM EXTRACT OF *PLECTRANTHUS BARBATUS* USING A VALIDATED HPTLC-UV METHOD

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ABSTRACT. The objective of this study was to develop a validated high-performance thin layer chromatography (HPTLC) method for the concurrent analysis of two diterpenes Sugiol (compound 1) and 11,14-dihydroxy-8,11,13-abietatrien-7-one (compound 2) in *Plectranthus barbatus* chloroform extract (PBCE) and to investigate cytotoxicity of both compounds. The chromatographic estimations were carried out using toluene : ethyl acetate : formic acid (8.2:1.3:0.5 v/v/v) as mobile phase. The compact spots of biomarkers were scanned at λ_{max}= 275 nm. Cytotoxic evaluation of both compounds was performed using follicular thyroid cancer cells (FRO cells). A well resolved, compact and intense peaks of compound 1 (R_f = 0.550 ± 0.001) and compound 2 (R_f = 0.700 ± 0.002) were recorded. The proposed method for both compounds was recorded as simple, linear, precise (% *RSD* = 1.02-1.25), accurate (98-100%), robust and sensitive for the analysis of both compounds. Cytotoxicity evaluation showed significant cell cytotoxicity at 100 µg/mL concentration against the FRO cancer cells after 72 h of incubation. Both compounds were recorded as cytotoxic, however compound **1** showed significant cytotoxic effects. The proposed HPTLC method was found to be suitable for routine analysis of these two biomarkers in chloroform extract of *P. barbatus*. Both compounds were found to be cytotoxic against FRO cancer cells.

KEY WORDS: Diterpenes, Sugiol, *Plectranthus barbatus*, Cytotoxicity, HPTLC

INTRODUCTION

Plectranthus barbatus Andr. belonging to family Lamiaceae, is a perennial herb native to the India and tropical Africa, which was naturalized in the Egypt, Saudi Arabia, Pakistan, Sri Lanka, Brazilian Northeast and Southeast regions [1-3]. This plant has a long history of uses by traditional Hindu and Ayurvedic medicines as well as in the folk medicine of Brazil, tropical Africa and China with potential benefits [4]. In the literature, *P. barbatus* has been ascribed to possess wide range of medicinal uses which includes gastrointestinal disorders, heart diseases such as hypertension and central nervous system (CNS) disorders, respiratory disorders, genito-urinary, skin affections, congestive heart failure, eczema, colic, asthma, angina and psoriasis [5, 6]. A decoction of *P. barbatus* leaves are used in colds, coughs, infections, aetiologies and inflammation [7]. Clinical studies on organic extracts of different parts of *P. barbatus* has demonstrated multiple pharmacological activities such as antimicrobial [8], hypotensive [3, 9], cytotoxic [10], anti-inflammatory [11], antioxidant [12], hepatoprotective [13], antifeedant [14], lipolysis induction in rat adipose tissue [15], total body weight reduction in rats [16], prevention of hair-loss and activation of melanogenesis process [17], antiasthmatic [18], antiaging [19] and antiallergic [20] effects. The aqueous extract of *P. barbatus* showed significant hypotensive, antispasmodic and hypoglycemic effects [21]. Also the aqueous extract of stem and leaves has been reported to enhance the intestinal transit in mice, protected against gastric lesions induced

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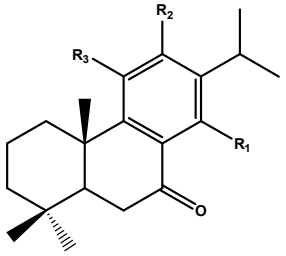
by ethanol or cold-restraint stress [22, 23] and reduced the total pH gastric secretion in pylorus-ligated rats [1]. The ethanolic root extract of *P. barbatus* exhibited potential inhibitory effects against *Escherichia coli* toxin-induced secretory response in Guinea pig and rabbit sileal loops [24] whereas 50 % ethanolic extract showed a variety of toxic effects on different periods of pregnancy in rats [25]. However, the essential oil extracted from leaves has reported to exhibit potential antimicrobial, spasmolytic and relaxant effects [21, 25]. Other significant uses of *P. barbatus* are as a food supplements, in Kenya and Yemen, leaves are being consumed as vegetable, as an ornamental plant or garden herb, in hedges, fences or boundary markers [26]. Phytochemical investigation of different parts of plant material revealed the presence of diterpenoids (abietane and labdane), flavonoids, phenolic compounds, tannins, phlobatannins, sapnonins, cardiac glycosides and essential oils [27-29]. Studies have shown that the leaves of *P. barbatus* growing in Brazil are rich in abietane diterpenes, whereas the roots of the Indian species contain mainly labdane diterpenes such as coleonol and forskolin, main active constituent used in number of patented pharmaceutical preparations as an over-the-counter drug for the treatment of several ailments [30].

In the recent years, high performance thin layer chromatography (HPTLC) has become one of the most widely applied analytical techniques for the estimation of chemical and biochemical markers in herbal materials. It is due to its numerous advantages which include simplicity, low operating cost and good resolution of active constituents with reasonable accuracy in short time, possibility of multiple sample detection, high sample throughput and rapidly obtained results [31]. The major advantage of HPTLC is that parallel analysis of multiple samples using a small amount of mobile phase, unlike HPLC, thus reducing the analysis time and cost of analysis [32]. HPTLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extract. A little information is available regarding analytical methods for the qualitative and/or quantitative estimation of sugiol (12-hydroxyabieta-8(14), 9(11),12-trien-7-one) and 11,14-dihydroxy-8,11,13-abietatrien-7-one (Figure 1). These two abietane diterpene occur naturally in many plant species and are reported to possess significant biological activities [33, 34]. Studies have shown that sugiol exhibit anti-inflammatory, anti-tumor, antimicrobial and aldose reductase inhibitory activities [33-36].

Consideration the importance of HPTLC and the need for chemical standardization of herbal extracts on the basis of isolated chemical entities, especially bioactive ones for global acceptances, it was decided to carry out the chemical profiling of chloroform extract of *P. barbatus* on the basis of two bioactive diterpenes. In the present study, we herein report on cytotoxic activity of chloroform extract as well as the pure chemical constituents isolated from *P. barbatus*, followed by the development of a sensitive selective hyphenated HPTLC method for the quantization of sugiol and11,14-dihydroxy-8,11,13-abietatrien-7-one in the extract.



Sugiol (R_1= R_3 = H, R_2 = OH) 1
11, 14-dihydroxy-8, 11, 13-abietatrien-7-one
(R_1= R_3 = OH, R_2 = H) 2

Figure 1. Molecular structure of sugiol and 11,14-dihydroxy-8,11,13-abietatrien-7-one.

EXPERIMENTAL

Plant material

The aerial parts of *P. barbatus* Andrews (Lamiaceae) were collected from Makkah roadside, Al-Taif, Al-Hada region of Saudi Arabia in March 2014. The plant was taxonomically identified and authenticated by Dr. Muhammed Yousef, Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. A voucher specimen (15732) has been deposited in the herbarium of the Department.

Chemicals and reagents

High purity grades of methanol, ethanol and n-hexane were procured from E-Merck (Darmstadt, Germany). High purity grades of chloroform and n-butanol were procured from Sigma Aldrich (St. Louis, MO, USA). AR grade toluene, ethyl acetate and formic acid were obtained from BDH Laboratory Supplies (Poole, UK). FRO thyroid cancer cell line was kindly gifted from King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia.

Extraction of plant material

The air-dried powdered aerial parts of *P. barbatus* (2000 g) was soaked in 70% ethanol for seven days at room temperature, extracted three times in the same solvent and filtered. The combined filtrate was concentrated under reduced pressure at 50 ± 5 ^{o}C, residue (220.2 g) obtained was dissolved in a mixture of methanol and water (9:1) and the resulting solution was partitioned successively with n-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and n-butanol (n-BuOH) to obtain n-hexane (25.5), $CHCl_3$ (130.6), EtOAc (30.3 g) and n-BuOH (10.9 g) fractions, respectively. The $CHCl_3$ soluble fraction (120 g) rich in quantity and chemical constituents was subjected to gravity column chromatography. It was dissolved in minimum amount of methanol, uniformly adsorbed over 200 g of silica gel (230-400 mesh) and freed from solvent to obtain free flowing material. A glass column was packed with 500 g of SiO_2 gel (230-400 mesh) in n-hexane–$CHCl_3$ and successively eluted with hexane–$CHCl_3$ (9:1), hexane–$CHCl_3$ (8:2), hexane–$CHCl_3$ (7:3), hexane–$CHCl_3$ (6:4), $CHCl_3$ and then MeOH. The combined 10 and 20% $CHCl_3$ in n-hexane fractions (23.0 g) was re-chromatographed over SiO_2 (230-400 mesh (E-Merck, Darmstadt, Germany) using hexane–$CHCl_3$ mixtures of increasing polarity. A total of 220 fractions (150 mL each) were collected. Fractions 25-45 obtained from hexane–$CHCl_3$ (9:1) were pooled on the basis of similar TLC pattern using $CHCl_3$:MeOH (98:2) as a developing solvent and spots were visualized by spraying plate with *p*-anisaldehyde:H_2SO_4 (0.5 mL *p*-anisaldehyde in 50 mL glacial acetic acid and 1 mL 97% H_2SO_4) followed by heating over hot plate for 5 min at 110 ^{o}C. Sugiol (23.2 mg) was crystallized from MeOH as colorless crystals (m.p. 283-284 ^{o}C; $C_{20}H_{28}O_2$. ESIMS: m/z = 323 $[M+Na]^+$, m/z = 301$[M+H]^+$, ^{1}H and ^{13}C-NMR) [37]. Similarly, fractions eluted in 30 % chloroform in hexane resulted in the isolation of another abietane diterpene, identified as 11,14-dihydroxy-8,11,13-abietatrien-7-one, crystallized in MeOH as yellow needles (m.p. 178-180 ^{o}C; $C_{20}H_{28}O_3$. ESIMS: m/z =339 $[M+Na]^+$, ^{1}H and ^{13}C-NMR) [38]. The structures of both the compounds were confirmed by comparisons of physical constants and spectral data of with previously reported values in literature [37, 38].

Apparatus and reagents

The two cytotoxic diterpene biomarkers sugiol (**1**) and 11,14-dihydroxy-8,11,13-abietatrien-7-one (**2**) were isolated as pure compound from *P. barbatus*. In addition, the AR grade toluene, ethyl acetate and formic acid were purchased from BDH (Poole, UK). The glass-backed silica

gel $60F_{254}$ plates for the HPTLC analysis were purchased from E-Merck (Darmstadt, Germany). Furthermore, biomarkers compound **1** and **2** along with extract (PBCE) were applied band wise to the chromatographic plates using CAMAG Automatic TLC Sampler-4 (Switzerland) and the plates were development in automatic development chamber (ADC2) (Switzerland). The developed HPTLC plates were then scanned and documented by CATS 4 (CAMAG) and TLC Reprostar 3 (CAMAG), respectively.

HPTLC instrumentation and conditions

The HPTLC analysis of compounds **1** and **2** in PBCE was carried out on 10 × 10 cm HPTLC plates, where the band size of each track was 6 mm wide and 7.0 mm apart. Both the markers as well as the extract were applied on HPTLC plate at a rate of 160 nL/s using microlitre syringe fitted with the automatic TLC Sampler-4. The plates were developed in a pre-saturated twin-trough glass chamber (20 × 10 cm) under the chamber saturation condition (at 25 ± 2°C and under 60 ± 5% humidity). Further, the developed HPTLC plates were dried to furnish compact spots of the biomarkers as well as the different phytoconstituents present in the extract. The mixture of toluene: ethyl acetate: formic acid (8.2:1.3:0.5 v/v/v) was used as a mobile phase. The dried plate was quantitatively analyzed at λ_{max} = 275 nm wavelength in absorbance mode.

Preparation of standard stock solutions

The stock solutions of compound **1** and **2** (1 mg/mL) were prepared in chloroform, following further dilution with chloroform to provide seven different concentrations ranging from 10 to 120 µg/mL. 10 µL of each dilution of both biomarkers were applied on the HPTLC plate through the microliter syringe attached with the applicator to provide the linearity range of 100-1200 ng/band.

Validation of method

The proposed HPTLC method was validated for the determination of limit of detection (LOD), limit of quantification (LOQ), linearity range, precision, recovery as accuracy and robustness according to International Conference on Harmonization (ICH) guidelines [39]. ICH guidelines are generally used for validation of such studies [39-42]. The detail procedure about determination of validation parameters is presented in our previous publications [43, 44].

Cytotoxic evaluation

Undifferentiated/anaplastic thyroid cancer cell line (FRO) was kindly gifted from King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia. The cells were cultured in humidified air with 5% CO_2 at 37 ^oC in RPMI-1640 medium with L-glutamine and sodium bicarbonate (R8758, Sigma Aldrich, St. Louis, MO, USA) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin both from (Gibco).

The cancer cell lines were maintained in RPMI-1640 that included L-glutamine (GIBCO) with 10% FBS (GIBCO) and 1% penicillin–streptomycin (GIBCO). Cells were cultured at 37 ^oC in a 5% CO_2 incubator [45, 46]. The effect of chloroform extract of plant (PBCE) along with pure isolated compounds **1** and **2** were evaluated on cellular viability using Alamar Blue assay (BUF012B; AbDSerotec, Langford Ln, Kidlington OX5 1GE, UK). The Alamar Blue assay is used to assess cell viability based on the reduction potential of metabolically active cells. Viable cells were seeded in the growth medium into 96-well microtiter plates (1 x 10^4 cells/well) and were incubated at 37 ^oC in a 5% CO_2 incubator for 24 h. The extract (PBCE), compounds 1 and 2 were adjusted to final 30, 50 and 100 µg/mL concentrations by diluting with the growth medium. After standing for 24 h, media were removed and the test sample was added to each

well. Control wells consisted of cells alone. After 24, 48 and 72 h of addition of test sample, 10 µL of Alamar Blue reagent was added to the each well (final concentration, 10 µg/mL) and the plates were incubated at 37 ^{o}C for 4 h. After incubation, plates were read using a spectrophotometric microplate reader (Biotek Synergy 2; Biotek Instruments, Highland Park, Winooski, Vermont, NE, USA) and the relative fluorescence unit was recorded. Cell viability was calculated using Eq. (1):

$$\% \text{ Cell viability } = \frac{\text{Flourescence of treated cells}}{\text{Flourescence of untreated (control) cells}} \times 100 \quad (1)$$

Results were expressed as percentage cell viability versus the control.

Statistical analysis

The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnet's test for the estimation of total variation in a set of data. Results were expressed as mean ± SD. $p < 0.01$ was considered significant.

RESULTS AND DISCUSSION

HPTLC method development and validation

The mobile phase selection for the HPTLC analysis was carried out by testing various compositions of different solvents. Of these, the combination of toluene: ethyl acetate: formic acid (8.2:1.3:0.5 v/v/v) was found to be the best mobile phase for the development and quantitative analysis of compound **1** and 2. An intense, sharp and compact peak of compounds **1** and **2** were found at R_f = 0.550 ± 0.001 and 0.700 ± 0.002, respectively (Figure 2).

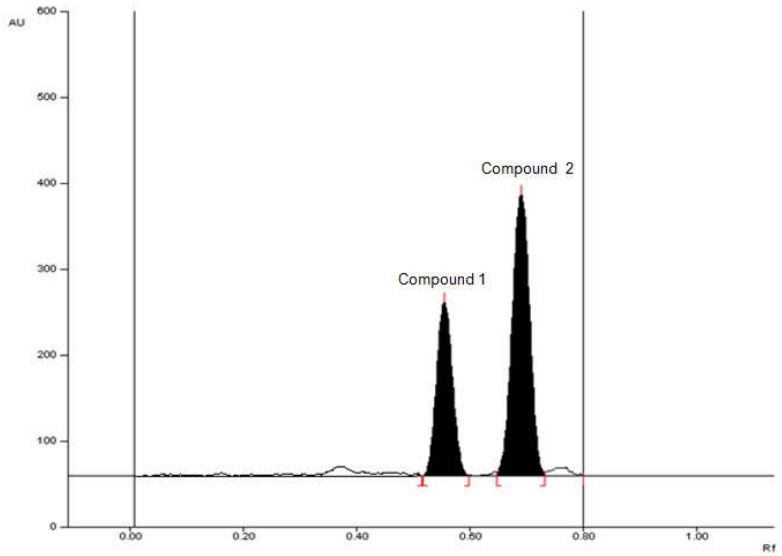


Figure 2. Chromatogram of compound 1 (R_f = 0.550 ± 0.001) and compound 2 (R_f = 0.700 ± 0.002) at λ_{max} =275 nm; mobile phase: toluene: ethyl acetate: formic acid (8.2:1.3:0.5 v/v/v).

This method clearly separated the biomarkers compound **1** and **2** as well as different constituents of PBCE (Figure 3A and B).

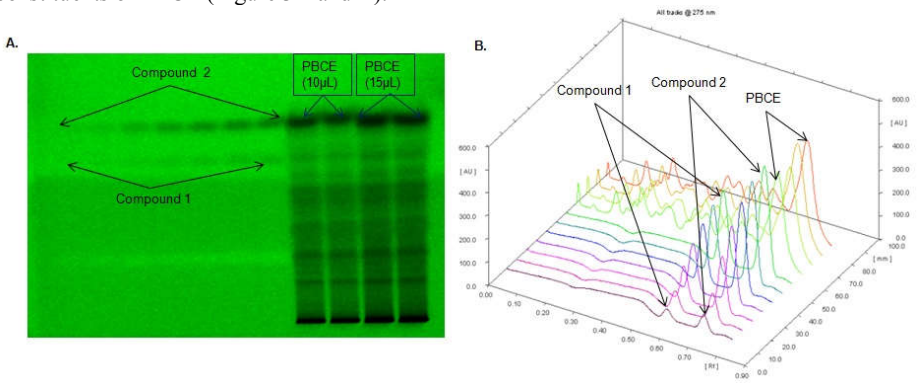


Figure 3. Quantification of compound **1** and compound **2** in the chloroform extract of *P. barbatus* (PBCE) by HPTLC; (A) pictogram of HPTLC plate at short UV (λ = 254 nm) and (B) 3-D display of all tracks at λ = 275 nm.

The mobile phase volume and optimized saturation time for the saturation of developing chamber were found to be 20 mL and 20 min, respectively. The identities of the bands of the extracts were confirmed by overlaying their spectra along with the spectra of compound **1** and **2** (Figure 4).

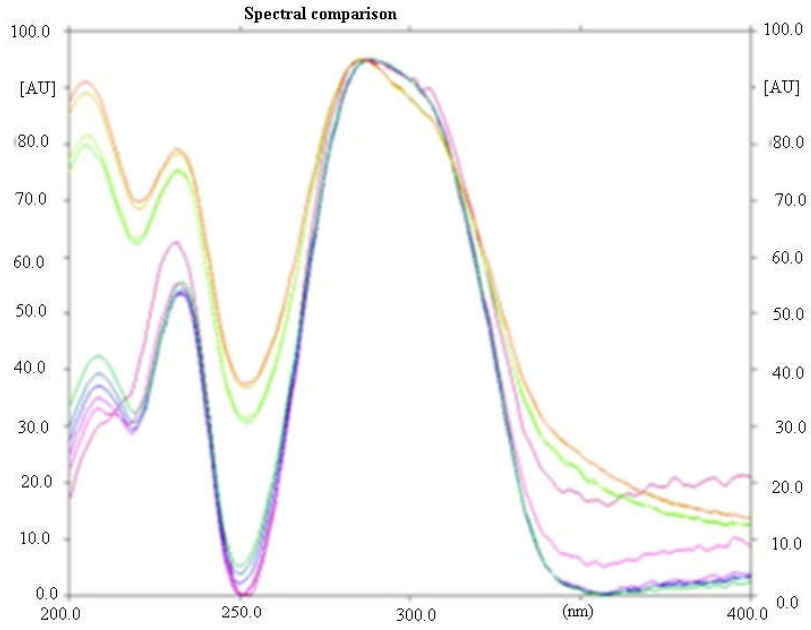


Figure 4. Spectral comparisons of different tracks at 275 nm (different color codes represent different concentrations of compounds).

The developed method was found to be quite selective with high baseline resolution. The regression equation/correlation co-efficient (r^2) for compounds **1** and **2** were found as $y = 6.32\ x + 552/0.996 \pm 0.0004$ and $y = 11.1\ x + 760/0.998 \pm 0.0002$, respectively, in the linearity range of 100-1200 ng/spot while the LOD/LOQ for compounds **1** and **2** were found as 9.96/30.1 ng/band and 13.1/39.7 ng/band, respectively. The recoveries as accuracy study for the proposed method was recorded (Table 1). The % recovery/RSD (%) for compounds **1** and **2** were found to be 98.4-100/1.18-1.63 and 98.0-99.5/1.51-2.15, respectively. % RSD for intra-day/inter-day precisions (n = 6) of compounds **1** and **2** were recorded as 1.07-1.19/1.02-1.15 and 1.06-1.25/1.04-1.22, respectively, which exhibits the good precision of the proposed method. The robustness of proposed method was checked by making a small deliberate change in the mobile phase composition, saturation time and mobile phase volume and the obtained data are reported in the Table 3. The low values of % RSD indicated that the proposed method was robust.

Table 1. Recovery as accuracy studies of the proposed HPTLC method (n = 6). Comp. **1**: Compound **1**; Comp. **2**: Compound **2**

Percent (%) of Comp. **1** and Comp. **2** added to analyte	Theoretical concentration of Comp. **1** and Comp. **2** (ng/mL)	Concentration found (ng/mL) ± *SD*		% Recovery	
		Comp. **1**	Comp. **2**	Comp. **1**	Comp. **2**
0	200	197 ± 3.21	196 ± 4.21	98.4	98.0
50	300	301 ± 4.26	299 ± 5.51	100	99.5
100	400	395 ± 4.86	395 ± 6.71	98.7	98.8
150	500	498 ± 5.91	496 ± 7.49	99.6	99.1

Table 2. Precision of the proposed HPTLC method (n = 6).

Conc. (ng/spot)	Compound **1**		Compound **2**	
	Intra-day precision	Inter-day precision	Intra-day precision	Inter-day precision
	Mean conc. found ± SD	Mean conc. found ± SD	Mean conc. found ± SD	Mean conc. found ± SD
400	395 ± 4.24	392 ± 4.01	397 ± 4.25	395 ± 4.14
600	601 ± 6.87	596 ± 6.59	597 ± 6.82	595 ± 6.69
800	797 ± 9.51	792 ± 9.13	797 ± 9.98	795 ± 9.73

Table 3. Robustness of the proposed HPTLC method (n = 6).

Optimization condition	Compound **1** (400 ng/band)	Compound **2** (400 ng/band)
	SD	SD
Mobile phase composition; (toluene: ethyl acetate: formic acid)		
(8.2:1.3:0.5)	4.59	4.62
(7:2.3:0.7)	4.63	4.52
(6.4:3.2:0.4)	4.69	4.58
Mobile phase volume (for saturation)		
(18 mL)	4.38	4.52
(20 mL)	4.35	4.55
(22 mL)	4.31	4.57
Duration of saturation		
(10 min)	4.55	4.51
(20 min)	4.51	4.53
(30 min)	4.59	4.56

The intra-day and inter-day precision for the proposed method is recorded in Table 2 and the HPTLC is an advanced form of instrumental TLC which has various advantages in comparison to other techniques like HPLC and other chromatographic methods in the analysis of different markers. By using HPTLC method, many samples can be separated on the same HPTLC plate simultaneously which resulted in high through-put and lowered operating cost. The compounds having different light absorption capacity or colors can also be identified using different mode of evaluation by HPTLC. The plates used in the HPTLC analysis are disposable hence it does not require regeneration or essential clean-up. The availability of many stationary phases has expanded the HPTLC application to various kinds of samples contrary with the separation on bare silica gel. The HPTLC technique is applied in qualitative and quantitative separations of compounds in mixtures, where the quantitative mode operates in a more optimized way (standardized with a given procedure) and hence applicable in the assay of compounds in samples [47-49].

*HPTLC analysis of compound **1** and compound **2***

The developed HPTLC method was used for the concurrent analysis of compounds **1** and **2** in the extracts PBCE (Figure 5). By applying the above developed method, the quantity of compound **1** and **2** in PBCE were found as 2.04 and 15.9 µg/mg, respectively, of the dried weight of extracts. This is a maiden report which demonstrated the development of an economical, precise, accurate and simple HPTLC method for the concurrent analysis of cytotoxic biomarkers sugiol (**1**) and 11,14-dihydroxy-8,11,13-abietatrien-7-one (**2**) in the chloroform extract of *P. barbatus* (PBCE).

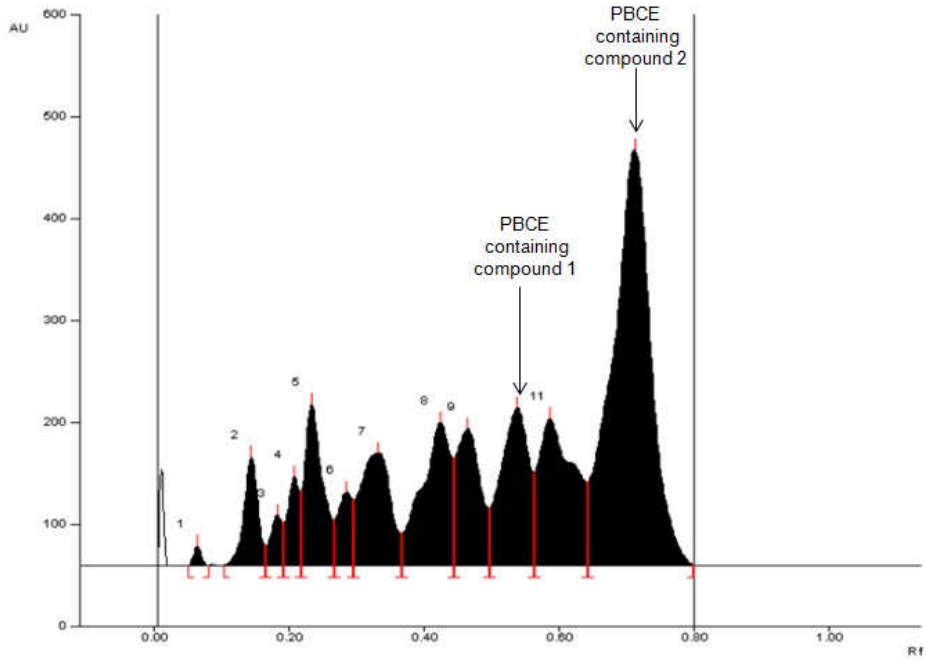


Figure 5. Chromatogram of chloroform extract of P. barbatus (PBCE) at λ = 275 nm; [compound **1**, spot 10, R_f = 0.55; compound **2**, spot 12, R_f = 0.70)].

Cytotoxic evaluation

Cytotoxicity of chloroform extract of *P. barbatus* along with sugiol (**1**) and 11,14-dihydroxy-8, 11,13-abietatrien-7-one (**2**) were evaluated using Alamar Blue assay. The Alamar Blue assay was used to assess cell viability and cell proliferation and is based on the reduction potential of metabolically active cells. The effect of chloroform extract of *P. barbatus* (PBCE), compound **1** and **2** on FRO cells viability at different concentrations (30, 50 and 100 µg/mL) at different time intervals (24, 48 and 72 h) is demonstrated in Figure 6 A, B and C. As shown in Figure 6A-C, there was no significant alteration in cells viability at 30 µg/mL concentration of extract while some reduction in the viability of FRO cells was observed at 50 µg/mL and 100 µg/mL concentration of the extract after incubation of cells for 48 (Figure 6B) and 72 h (Figure 6C) periods, respectively. At 100 µg/mL concentration of extract, cells viability reduction was the most for extract after 24, 48 and 72 h, respectively (Figure 6A-C). However significant cell cytotoxicity was observed with compound 1 at concentration of 30 µg/mL at 24, 48, and 72 h (Figure. 6A-C). However, no signs of cytotoxicity were observed for compound **2** at 30 µg/mL concentration. Meanwhile at 50 µg/mL concentration, compound **2** showed around 40% reduction in cell viability and even more cytotoxicity was observed for compound **2** at higher concentrations (100 µg/mL) as represented in Figure 6A-C. Thus our results clearly show that compound **1** was the most cytotoxic among others.

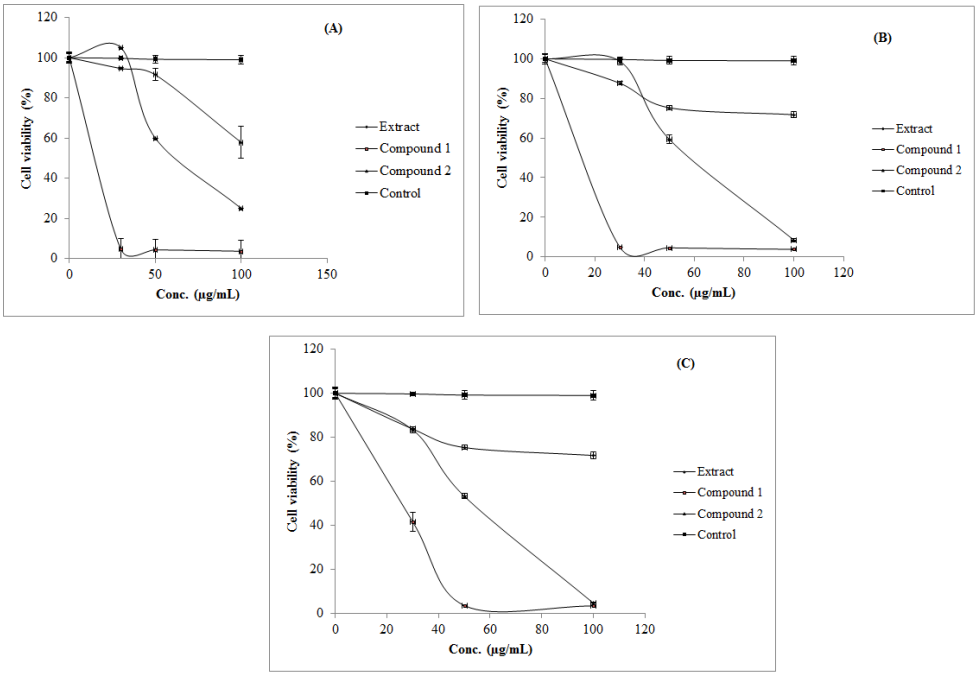


Figure 6. Cell viability (%) of FRO cells after incubation with extract, compound **1** and **2** in a dose dependent manner; cell viability of FRO cells with extract, compound **1**, and compound **2** along with control at 30, 50 and 100 µg/mL concentrations after (A) 24 h, (B) 48 h and (C) 72 h incubation assessed using Alamar Blue assay. Results are expressed as % mitochondrial activity for the Alamar Blue assay. Results are presented as mean ± SD, n = 3.

The results of cytotoxicity evaluation in terms of maximum inhibition (MI) and IC_{50} are furnished in Table 4 [46]. The values of IC_{50} and MI for extract, compound **1** and compound **2** were determined by concentration-dependent cell viability curves shown in Figure 6A-C. The MI value for extract was recorded as 42.2 ± 8.00, 28.2 ± 1.50 and 28.2 ± 7.86 after 24, 48 and 72 h, respectively. However, the MI value for compound 1 was recorded as 96.3 ± 5.60, 96.2 ± 1.04 and 96.5 ± 1.88% after 24, 48 and 72 h, respectively, which were significant in comparison with extract ($p < 0.05$). On the other hand, the MI value for compound **2** was recorded as 75.0 ± 1.08, 91.5 ± 1.64 and 95.2 ± 2.02% after 24, 48 and 72 h, respectively, which were also significant in comparison with extract ($p < 0.05$). Overall, compound **1** was found to be more efficacious than extract and compound **2** against FRO cells. Control sample indicated cell growth at negligible extent (Figure 6). The IC_{50} value of extract was obtained as 128, 165 and 166 µg/mL after 24, 48 and 72 h, respectively. However, the IC_{50} value for compound **1** was recorded as 18.4, 18.5 and 31.2 µg/mL after 24, 48 and 72 h, respectively. On the other hand, the IC_{50} value for compound **2** was recorded as 72.1, 61.9 and 55.5 µg/mL after 24, 48 and 72 h, respectively. The IC_{50} value of compound **1** was significantly lower than extract and compound 2.

Table 4. IC_{50} and MI values of extract, compound **1**, compound **2** and control on FRO cells after 24, 48 and 72 h of treatment (Mean ± SD, n = 3).

Sample matrices	IC_{50} (µg/mL) ± SD			MI (%) ± SD		
	24 h	48 h	72 h	24 h	48 h	72 h
Extract	128 ± 6.45	165 ± 4.27	166 ± 5.91	42.2 ± 8.00	28.2 ± 1.50	28.2 ± 7.86
Compound 1	18.4 ± 0.92	18.5 ± 0.83	31.2 ± 1.57	96.3 ± 5.60	96.2 ± 1.04	96.5 ± 1.88
Compound 2	72.1 ± 2.58	61.9 ± 2.22	55.5 ± 2.36	75.0 ± 1.08	91.5 ± 1.64	95.2 ± 2.02
Control	-	-	-	1.00 ± 0.20	1.00 ± 0.20	1.00 ± 0.20

Standard deviation (SD); the percent of maximum inhibition (% MI); the concentration responsible for 50% of cell inhibition (IC_{50}).

The cytotoxicity activity evaluation of the chloroform extract of the plant (100 µg/mL at 72 h) showed enough reduction in the viability of undifferentiated FRO cells. The two abietane-type diterpenes viz., 12-hydroxyabieta-8(14),9(11),12-trien-7-one (**1**) and 11,14-dihydroxy-8,11,13-abietatrien-7-one (**2**) were found to be cytotoxic. However, compound **1** showed significant cytotoxic effects at different time intervals.

Cytotoxicity studies suggested that compound **1** was around four fold more efficacious in comparison with extract after 24 and 48 h of incubation period. Overall, compound 1 was found to be more potent than extract and compound **2**. Therefore, compound **1** could be formulated in different dosage forms for the chemoprevention of cancers in future studies.

CONCLUSION

The maiden HPTLC method developed for the concurrent analysis of cytotoxic biomarkers sugiol and 11,14-dihydroxy-8,11,13-abietatrien-7-one in the *P. barbatus* extract may be further employed in the analysis of these biomarkers in extracts of other species of same genus as well as different genus to establish the chemotaxonomic relationship. The above developed method can also be used in the quantitative analysis of these two cytotoxic biomarker in marketed preparations, quality control of herbal drugs containing these two biomarkers and study of their degradation kinetics. Cytotoxicity evaluation indicated that compound 1 was more efficacious in comparison with extract. Overall, compound **1** was found to be more potent than extract and compound **2**. Hence, compound **1** could be formulated in different dosage forms for the chemoprevention of cancers in future studies.

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