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Concurrent analysis of bioactive triterpenes oleanolic acid and β-amyrin in antioxidant active fractions of *Hibiscus calyphyllus*, *Hibiscus deflersii* and *Hibiscus micranthus* grown in Saudi Arabia by applying validated HPTLC method

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ABSTRACT

In this study, we developed a validated HPTLC method for concurrent analysis of two natural antioxidant triterpenes, oleanolic acid (OA) and β-amyrin (BA) in the biologically active fractions (petroleum ether, toluene, chloroform, ethyl acetate and *n*-butanol) of aerial parts of three *Hibiscus* species (*H. calyphyllus*, *H. deflersii* and *H. micranthus*). The chromatography was conducted on normal HPTLC (ready to use glass-plate coated with silica gel 60 F254) plate with chloroform and methanol (97:3, V/V) used as mobile phase. The derivatization of the developed plate was done with p-anisaldehyde and scanned at λ_{max} = 575 nm. Well resolved and intense peaks of OA and BA were obtained at Rf = 0.36 and 0.57, respectively. The linear regression equation/correlation coefficient (r^2) for OA and BA were Y = 6.65x + 553.35/0.994 and Y = 9.177x + 637.23/0.998, respectively in the linearity range of 100–1200 ng/spot indicated good linear relationship. The low values of %RSD for intra-day/inter-day precision of OA (1.45–1.61/1.38–1.59) and BA (1.52–1.57/1.50–1.53) suggested that the method was precise. The recovery/RSD (%) values for OA and BA were found to be 99.21–99.62/1.39–1.95 and 98.75–99.70/1.56–1.80, respectively assures the reasonably good accuracy of the proposed method. Fifteen samples were analyzed to check the content of OA and BA by using the developed HPTLC methods. The content of OA in different samples were found to be 3.87 (HmP) > 1.212 (HcP) > 0.673 (HdC) > 0.493 (HdP) > 0.168 (HdE) > 0.059 (HcC) > 0.015 (HcE) > 0.008 (HmT) μg/mg of the dried weight of extract. However the content of BA was found as: 2.293 (HmP) > 1.852 (HdT) > 0.345 (HdC) > 0.172 (HmT) > 0.041 (HdE) > 0.008 (HcC) μg/mg of the dried weight of extract. Some *Hibiscus* species fractions exhibited good antioxidant potential like: HcE (IC50 = 17.6 ± 1.8) > HdB (IC50 = 32.16 ± 0.9) > HmP (IC50 = 80.4 ± 4.5) > HmT (IC50 = 99.7 ± 8.2) when compared with ascorbic acid (IC50 = 14.2 ± 0.5), while other fractions exhibited only mild antioxidant capability. The developed HPTLC method can be further exploited for analysis of these markers in the quality assessment of raw material as well as herbal formulations available in the market.

1. Introduction

The genus *Hibiscus* (Malvaceae) represents around 275 species distributed in tropical and sub-tropical regions and many of which possess medicinal properties. Most *Hibiscus* species posses a distinct color pattern with the base of corolla (Lowry, 1976) and they are fairly widespread at medium altitudes in the western part of Saudi Arabia. *Hibiscus calyphyllus* (Hc) is a leafy shrub of 1 M height with wide simple serrate leaves and yellow flowers with dark red centre and found mainly in south-west part of Saudi Arabia, particularly NE of Jizan (Collenette, 1999).

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H. deflersii (Hd) (Malvaceae) is an annual or perennial erect leafy straggly shrublet of 1 m height with narrow bright green dentate leaves having around 3 cm wide bright crimson-red flowers. It is grown as an ornamental plant and native to Ethiopia. The flowers are used as emollient and its infusion as a demulcent. Bronchial catarrh in India can be treated by the decoction of flowers of *H. deflersii*. Literature work revealed that *H. deflersii* possesses antidiarrhetic, antiphologistic and anticomplimentary activities. The leaves of *H. deflersii* were found very effective in heart disorders as well as in diabetes (Fryxell, 1980; Nadkarni, 1954, Lakshman et al., 2014).

Hibiscus micranthus (Hm) is a bushy leafy shrub of around 45 cm height having white flowers on short pedicels with very distinctive pea-size fruit capsules and distributed widely in Saudi Arabia, Ceylon, India and tropical Africa. In Saudi Arabia, *H. micranthus* is prevalently found from south to western part of Saudi Arabia (Kirtikar and Basu, 1984). The fruits and flowers of *H. micranthus* are used as antidiabetic (Kakrani et al., 2005) and anti-dandruff agent when applied topically and possesses laxative activity when taken orally (Tamilselvi et al., 2016). The plant has also been approved for its hematological, antipyretic, anti-inflammatory (Al-Yahya et al., 1987), antimicrobial, antiviral, antitumor (Jain et al., 1997), female antifertility, viralizing (Telefo et al., 1998) and anabolizing (Moundipa et al., 1999) effects. The antifungal and anti-tumor activity has been observed from roots of *H. micranthus* and also reported to possess good antiviral activity (Rekha, 2017). Literature reveals that *H. micranthus* possesses a wide range of phytochemicals such as phenolic acids, flavonoids, β-sitosterol, alkanes, fatty alcohols and acids (Jain et al., 1997). Chemical profiling of ethanol extract of *H. micranthus* roots by GC–MS revealed the presence of seventy nine compounds (Kumar et al., 2010). Available literature revealed that a fingerprint profile has been developed by HPTLC and rutin was analyzed by HPLC in *H. micranthus* (stem) hydro alcoholic extracts but there is no evidence yet available on the quantitative analysis of biomarkers using validated HPTLC method in *H. micranthus* extracts.

As evident from earlier reports the aerial parts of selected *Hibiscus* species (*H. calyphyllus*, *H. deflersii* and *H. micranthus*) mainly contain β-ionone, flavonoid and phenolic acids, oleic acid and other organic acids along with their esters (Kumar et al., 2011). As part of our research based on the reported phytochemicals, we selected three species of genus *Hibiscus* (*H. calyphyllus*, *H. deflersii* and *H. micranthus*) for quantitative analysis of biomarkers oleanolic acid (Fig. 1A) and β-amyrin (Fig. 1B) by using validated HPTLC method as well as evaluation of their antioxidant potential.

2. Experimental

2.1. Plant material

Aerial parts of three different species of genus *Hibiscus*; *H. calyphyllus* (Voucher specimen number: HA-234), *H. deflersii* (Voucher specimen number: HA-567) and *H. micranthus* L. (Voucher specimen number: HA-16,240) were collected from Jabal As-Sahla', As-Sarawat mountains (18°42′0″N 42°13′50″E), Asir region of Saudi Arabia in March 2009 and authenticated by Dr. Mohamed Yousef, taxonomist at Pharmacognosy Department, College of Pharmacy, King Saud University. Voucher specimens were deposited in the herbarium, Department of Pharmacognosy. Aerial parts of plant sample were thoroughly washed broken into small pieces and evenly distributed in aluminum trays. The samples were dried in shade at normal temperature, powdered and stored in airtight containers for further use.

2.2. Extraction of plant material

The dried powder of selected plant species were weighed (400 g) and soaked in 95% ethanol (1 L) for 48 h at room temperature with occasional stirring. In each experiment, process of extraction was repeated three times under similar conditions. The ethanolic extracts were then filtered using Whatman filter paper and the filtrates were combined. The obtained filtrates were concentrated under reduced pressure using rotavapour to get dark green solid mass. The obtained yields for *H. calyphyllus*, *H. deflersii* and *H. micranthus* were 12.23 g (3.06%, w/w), 10.21 g (2.6%, w/w) and 12.01 g (3.0%, w/w), respectively. The obtained extracts of *H. calyphyllus*, *H. deflersii* and *H. micranthus* were dissolved in a mixture of methanol and water (7:3) and successively partitioned three times with petroleum ether, toluene, chloroform, ethyl acetate and n-butanol to obtain the respective fractions. The yield of each fraction was calculated as *H. calyphyllus* [petroleum ether fraction (HcP; 2.25 g); toluene fraction (HcT; 3.21 g); chloroform fraction (HcC; 4.30 g); ethyl acetate fraction (HcE; 2.8 g) and n-butanol fraction (HcB; 2.21 g)]; *H. deflersii* [petroleum ether fraction (HdP; 2.19 g); toluene fraction (HdT; 3.10 g); chloroform fraction (HdC; 2.56 g); ethyl acetate fraction (HdE; 3.01 g) and n-butanol fraction (HdB; 1.98 g)] and *H. micranthus* [petroleum ether fraction (HmP; 2.21 g); toluene fraction (HmT; 2.96 g); chloroform fraction (HmC; 2.95 g); ethyl acetate fraction (HmE; 3.86 g) and n-butanol fraction (HmB; 2.01 g)] and stored in refrigerator at 4 °C until the time of use.

2.3. Apparatus and reagents

The two biomarkers, oleanolic acid (OA) and β-amyrin (BA) were purchased from Sigma Aldrich (USA) and the solvents of analytical grade from BDH (UK). The glass-backed silica gel $60F_{254}$ plates for the HPTLC analysis were purchased from Merck (Germany). Furthermore, biomarkers OA and BA along with extract sample were applied band wise to the chromatographic plates using method described by Siddiqui et al. (2016), with slight modification.

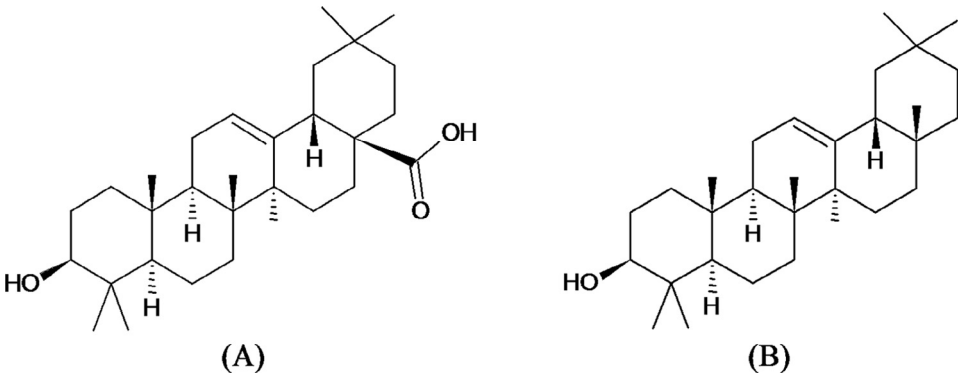

Fig. 1. Chemical structure of biomarkers oleanolic acid (A) and β-amyrin (B).

2.4. HPTLC instrumentation and conditions

The HPTLC analyses of OA and BA in samples (15 fractions) were carried out on pre-coated 20 × 10 cm HPTLC plates and almost similar instrumentation and conditions were used as described by Siddiqui et al. (2015) with slight modifications.

2.5. Preparation of standard stock solutions

The stock solutions of OA and BA (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 to provide a linearity range of 100–1200 ng/band.

2.6. Method validation

The latest ICH guidelines (2005) were being followed for the validation of developed HPTLC method. The parameters observed for validation were the determination of limit of detection (LOD), limit of quantification (LOQ), linearity range, precision, recovery as accuracy and robustness.

2.7. Antioxidant assay

The antioxidant activity of extract was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University by the DPPH free radical scavenging assay in triplicate and average values were considered.

2.7.1. DPPH free radical scavenging assay

The evaluation of all the fifteen fractions of *H. calyphyllus*, *H. deflersii* and *H. micranthus* was done for antioxidant activities in terms of quantity by free-radical scavenging ability against DPPH according to the method described by Lee et al. (2013), with minor modification to suite 96-well microtitre plate format. In brief, 40 μL of DPPH (0.2 mM in methanol) was mixed with 100 μL of different concentrations (31.25, 62.5, 125, 250 and 500 μg/mL) of each fraction in wells of a 96-well microtitre plate. Pure solvent along with same amount of DPPH was used as control to rule out the effect of solvent. After 30 min incubation in dark at 25 °C, the decrease in absorbance (Abs) was measured immediately with a UV–visible spectrophotometer (Milton Roy, Spectronic 1201) at λ = 515 nm using microtitre plate reader. The absorbance of the DPPH radical without antioxidant (control) and the reference com-

Table 1
Rf, Linear regression data for the calibration curve of oleanolic acid and β-amyrin (n = 6).

Parameters	Oleanolic acid (OA)	β-Amyrin (BA)
Linearity range (ng/spot)	100–1200	100–1200
Regression equation	Y = 6.651X + 553.35	Y = 9.177X + 637.23
Correlation (r^2) coefficient	0.994	0.998
Slope ± SD	6.651 ± 0.023	9.177 ± 0.042
Intercept ± SD	553.35 ± 11.93	637.23 ± 12.85
Standard error of slope	0.009	0.017
Standard error of intercept	4.87	5.24
Rf	0.36 ± 0.001	0.57 ± 0.001
LOD (ng)	11.47	15.29
LOQ (ng)	34.78	46.35

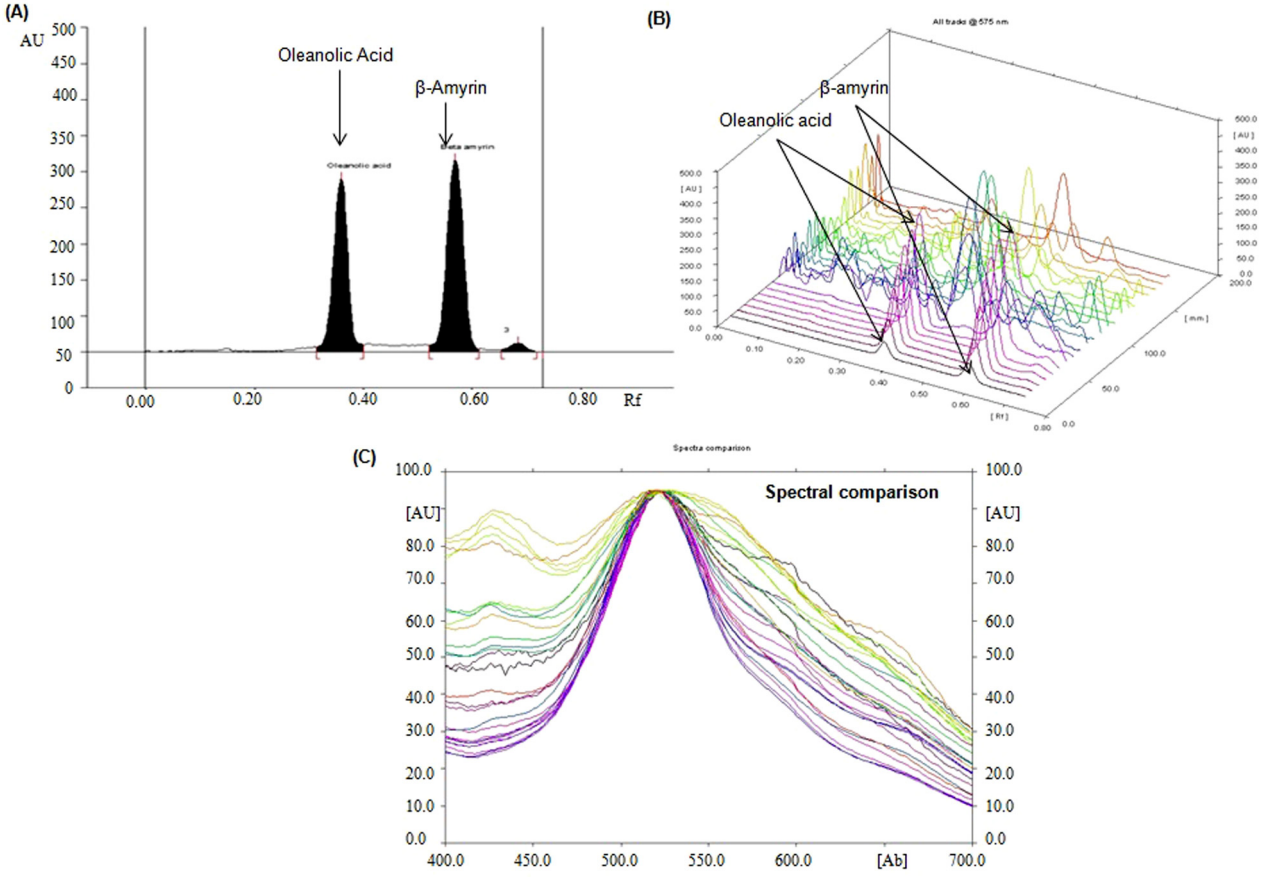

Fig. 2. Quantification of oleanolic acid and β-amyrin in different fractions of *H. calyphyllus*, *H. deflersii* and *H. micranthus* by HPTLC. (A) Chromatogram of standards oleanolic acid (Rf = 0.36; 800 ng/spot) and β-amyrin (Rf = 0.57; 800 ng/spot) at 575 nm. (B) 3-D display of all tracks at 575 nm. (C) Spectral comparison of all tracks at 575 nm.

pound (ascorbic acid) were also measured. All the observations were made in triplicate and average was considered for each observation. The percentage radical scavenging activity was calculated according to the formula:

$$\%\text{Radical scavenging activity} = [1 - (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}})] \times 100$$

The IC_{50} value of each fraction was calculated and reported in Table 6.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnet's test for the estimation of total variation was used for statistical analysis. Results were expressed as mean ± SD. $P < .05$ was considered as significant.

3. Results and discussion

3.1. HPTLC method development and validation

The mobile phase was selected by the rigorous exercise of permutation-combination of many solvents in different proportions. The combination of chloroform and methanol in the proportion of 97:3 (v/v) was found to be the optimized mobile phase for the development and quantitative analysis of OA and BA. An intense, sharp and compact peak of OA and BA were found at R_f = 0.36 ± 0.001 and 0.57 ± 0.002, respectively (Fig. 2A). The proposed method separated the biomarkers OA and BA as well as different constituents of selected *Hibiscus* species fractions (Fig. 2B). The saturation time for the saturation of developing chamber and volume of mobile phase was found to be 20 min and 20 mL, respectively. The recognition of the bands of the extracts were established by overlaying their spectra along with the spectra of standard OA and BA (Fig. 2C). The regression equation/correlation coefficient (r^2) for biomarkers OA and BA were found as Y = 6.651x + 553.35/0.994 and Y = 9.177x + 637.23/0.998, respectively in the linearity range 100–1200 ng/spot while the limit of detection (LOD)/limit of quantification (LOQ) for OA and BA were found as 11.47/34.78 ng and 15.29/46.35 ng, respectively (Table 1). The recoveries as accuracy study for the proposed method was recorded (Table 2). The recovery/RSD (%) for biomarkers OA and BA were found as 99.21–99.62/1.39–1.95 and 98.75–99.70/1.56–1.80, respectively. The intra-day and inter-day precision for the proposed method was recorded in Table 3 and the % RSD for intra-day/inter-day precisions (n = 6) of biomarkers OA and BA were recorded as 1.45–1.61/1.38–1.59 and 1.52–1.57/1.50–1.53, respectively, which exhibits the good precision of the proposed method. Some small intentional changes were made in for mobile phase volume, composition, saturation time etc. to check the robustness of the proposed method. The data reported in Table 4 show low values of SD and % RSD which indicate that the proposed method was robust and not significantly affected by slight changes in the experimental environment.

Table 2
Recovery as accuracy studies of the proposed HPTLC Method (n = 6).

Percent (%) of oleanolic acid and β-amyrin added to analyte	Theoretical concentration of oleanolic acid and β-amyrin (ng/μL)	Concentration found (ng/μL) ± SD		%RSD		% Recovery	
		Oleanolic acid	β-amyrin	Oleanolic acid	β-amyrin	Oleanolic acid	β-amyrin
0	200	199.25 ± 2.77	198.84 ± 3.11	1.39	1.56	99.62	99.41
50	300	297.64 ± 4.39	296.25 ± 4.69	1.47	1.58	99.21	98.75
100	400	398.16 ± 6.37	398.84 ± 6.71	1.59	1.68	99.54	99.70
150	500	497.45 ± 9.74	497.47 ± 8.97	1.95	1.80	99.49	99.49

Table 3
Precision of the proposed HPTLC Method (n = 6).

Concentration of standards added (ng/spot)	Oleanolic acid				β-Amyrin			
	Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
	Average Conc. found ± SD	% RSD	Average Conc. found ± SD	% RSD	Average Conc. found ± SD	% RSD	Average Conc. found ± SD	% RSD
200	199.25 ± 2.89	1.45	196.69 ± 2.73	1.38	197.78 ± 3.02	1.52	195.60 ± 2.94	1.50
400	398.16 ± 6.27	1.57	398.62 ± 6.12	1.53	398.96 ± 6.23	1.56	393.51 ± 6.01	1.52
600	597.21 ± 9.67	1.61	597.21 ± 9.54	1.59	597.35 ± 9.43	1.57	596.26 ± 9.13	1.53

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

Optimization condition	Oleanolic acid (300 ng/band)		β-Amyrin (300 ng/band)	
	SD	%RSD	SD	%RSD
Mobile phase composition (Chloroform: methanol)				
(97:3)	4.59	1.54	4.81	1.65
(96.5:3.5)	4.62	1.56	4.89	1.66
(97.5:2.5)	4.55	1.53	4.92	1.68
Mobile phase volume (for saturation)				
(18 mL)	4.72	1.59	4.71	1.61
(20 mL)	4.79	1.61	4.79	1.63
(22 mL)	4.82	1.62	4.85	1.66
Duration of saturation				
(10 min)	4.51	1.51	4.83	1.65
(20 min)	4.58	1.54	4.89	1.66
(30 min)	4.68	1.57	4.94	1.68

3.2. HPTLC analysis of biomarkers OA and BA in different fractions of selected Hibiscus species

The developed HPTLC method was used for the concurrent analysis of biomarkers OA and BA in the different fractions of selected *Hibiscus* species (Table 5; Figs. 3–5). By applying the above developed method the quantity of biomarker OA and BA in HmP was found as 3.87 and 2.29 μg/mg, respectively of the dried weight of extracts (Fig. 3A). The quantity of OA in HdP and HcP and was found as 0.49 μg/mg and 1.21 μg/mg, respectively (Fig. 3B and C) with respect to the dried weight of the extract, but BA was altogether absent in HdP as well as HcP. As evident from the Fig. 4 HdC, HcC, HdE and HcE fractions showing very little or insignificant amount of both OA as well as BA. HmT also exhibited low amount of OA and BA (Fig. 5A) while HdT showed good amount of BA but no OA (Fig. 5B). The previous literature indicates that the authors reporting the quantification of oleanolic acid and β-amyrin by HPTLC method for the first time in the petroleum ether, toluene, chloroform, ethyl acetate and n-butanol fractions of *H. calyphyllus*, *H. deflersii* and *H. micranthus*.

High-performance thin-layer chromatography (HPTLC) is a more precise, calibrated and automated TLC which has many

Table 5
HPTLC analysis of Oleanolic acid and β-amyrin in different fractions of *H. calyphyllus*, *H. deflersii* and *H. micranthus*.

S. No.	Samples	Oleanolic acid content (μg/mg of dried weight of extract)	β-amyrin content (μg/mg of dried weight of extract)
1	*H. micranthus* petroleum ether fraction (HmP)	3.87	2.29
2	*H. deflersii* petroleum ether fraction (HdP)	0.49	Not detected
3	*H. calyphyllus* petroleum ether fraction (HcP)	1.21	Not detected
4	*H. micranthus* toluene fraction (HmT)	0.008	0.17
5	*H. deflersii* toluene fraction (HdT)	Not detected	1.85
6	*H. calyphyllus* toluene fraction (HcT)	Not detected	Not detected
7	*H. micranthus* chloroform fraction (HmC)	Not detected	Not detected
8	*H. deflersii* chloroform fraction (HdC)	0.673	0.345
9	*H. calyphyllus* chloroform fraction (HcC)	0.059	0.008
10	*H. micranthus* ethyl acetate fraction (HmE)	Not detected	Not detected
11	*H. deflersii* ethyl acetate fraction (HdE)	0.168	0.041
12	*H. calyphyllus* ethyl acetate fraction (HcE)	0.015	Not detected
13	*H. micranthus* n-butanol fraction (HmB)	Not detected	Not detected
14	*H. deflersii* n-butanol fraction (HdB)	Not detected	Not detected
15	*H. calyphyllus* n-butanol fraction (HcB)	Not detected	Not detected

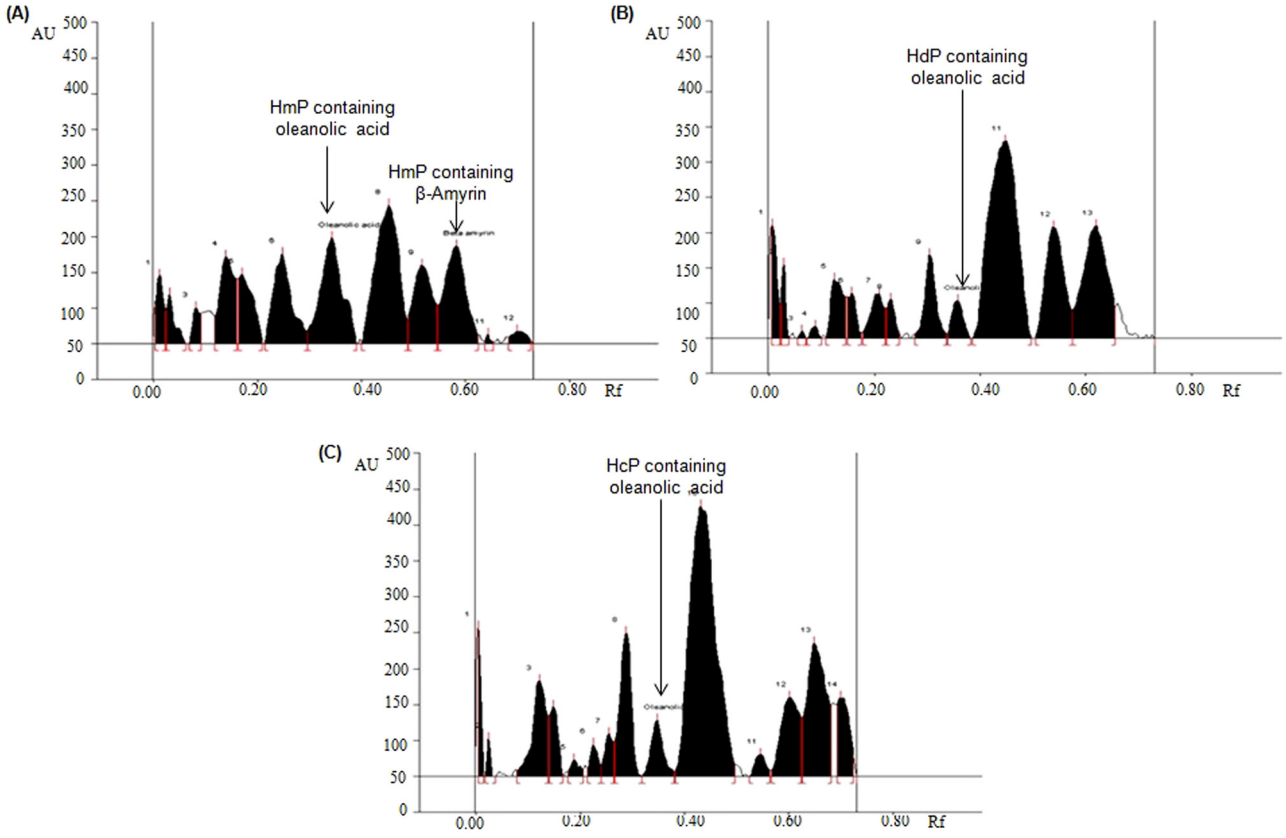

Fig. 3. Chromatogram of oleanolic acid and β-amyrin estimation in petroleum ether fractions of *H. calyphyllus*, *H. deflersii* and *H. micranthus* at 575 nm [mobile phase: chloroform: methanol (97:3)]. (A) *H. micranthus* Petroleum ether fraction [HmP (oleanolic acid, spot 7, Rf = 0.36; β-amyrin, spot 10, Rf = 0.57)]; (B) *H. deflersii* Petroleum ether fraction [HdP (oleanolic acid, spot 10, Rf = 0.36)]; (C) *H. calyphyllus* Petroleum ether fraction [HcP (oleanolic acid, spot 9, Rf = 0.36)].

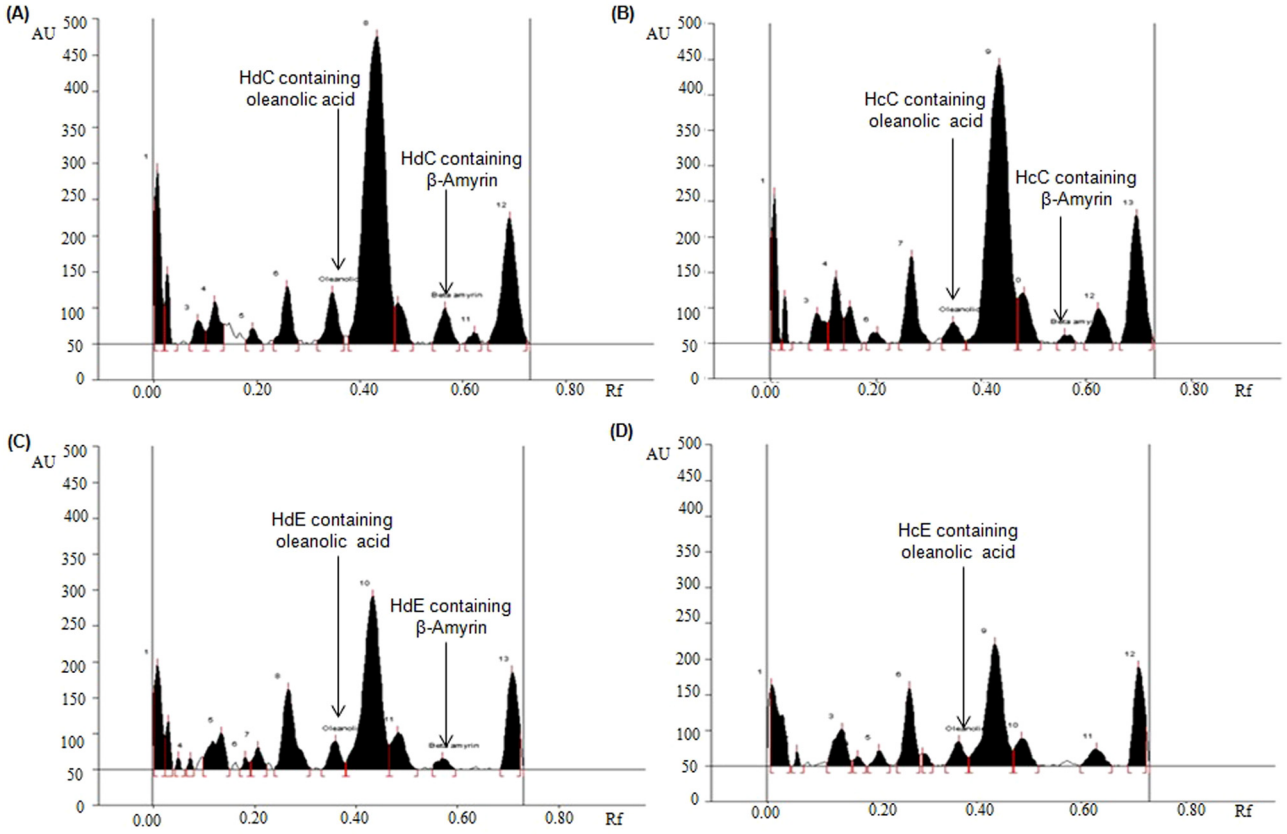

Fig. 4. Chromatogram of oleanolic acid and β-amyrin estimation in the dichloromethane and ethyl acetate fractions of *H. calyphyllus* and *H. deflersii* at 575 nm [mobile phase: chloroform: methanol (97:3)]. (A) *H. deflersii* dichloromethane fraction [HdC (oleanolic acid, spot 7, Rf = 0.36; β-amyrin, spot 10, Rf = 0.57)]; (B) *H. calyphyllus* dichloromethane fraction [HcC (oleanolic acid, spot 8, Rf = 0.36; β-amyrin, spot 11, Rf = 0.57)]; (C) *H. deflersii* ethylacetate fraction [HdE (oleanolic acid, spot 9, Rf = 0.36; β-amyrin, spot 12, Rf = 0.57)]; (D) *H. calyphyllus* ethylacetate fraction [HcE (oleanolic acid, spot 8, Rf = 0.36)].

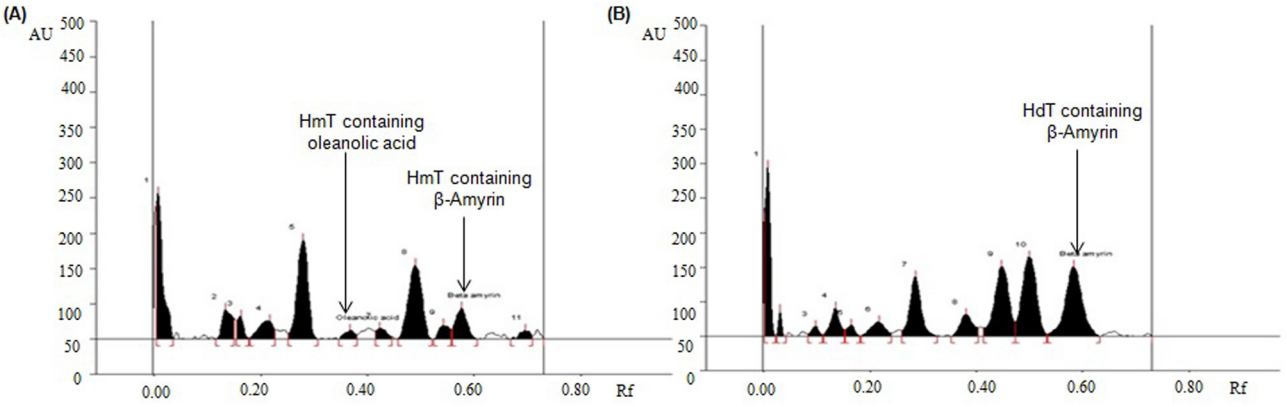

Fig. 5. Chromatogram of oleanolic acid and β-amyrin estimation in the toluene extract of *H. micranthus* and *H. deflersii* at 575 nm [mobile phase: chloroform: methanol (97:3)]. (A) *H. micranthus* toluene extract [HmT (oleanolic acid, spot 6, Rf = 0.36; β-amyrin, spot 10, Rf = 0.57)]; (B) *H. deflersii* toluene extract [HdT (β-amyrin, spot 10, Rf = 0.57)].

advantages in comparison to other techniques like high performance liquid chromatography (HPLC) and other chromatographic methods in the analysis of different markers. HPTLC can be used for simultaneous identification and quantification for multiple markers whether UV active or not. A wide range of stationary phases has broadened the application of HPTLC for a variety of samples contrary with the separation on bare silica gel.

3.3. Antioxidant potential of different fractions of H. calyphyllus, H. Deflersii and H. Micranthus

As far as the antioxidant potential is concerned ethyl acetate fraction of *H. calyphyllus* was emerged as most significant [HcE (IC_{50} = 17.6 ± 1.8); Fig. 6A] among all the fractions of *H. calyphyllus*, *H. deflersii* and *H. micranthus* compared to the standard ascorbic

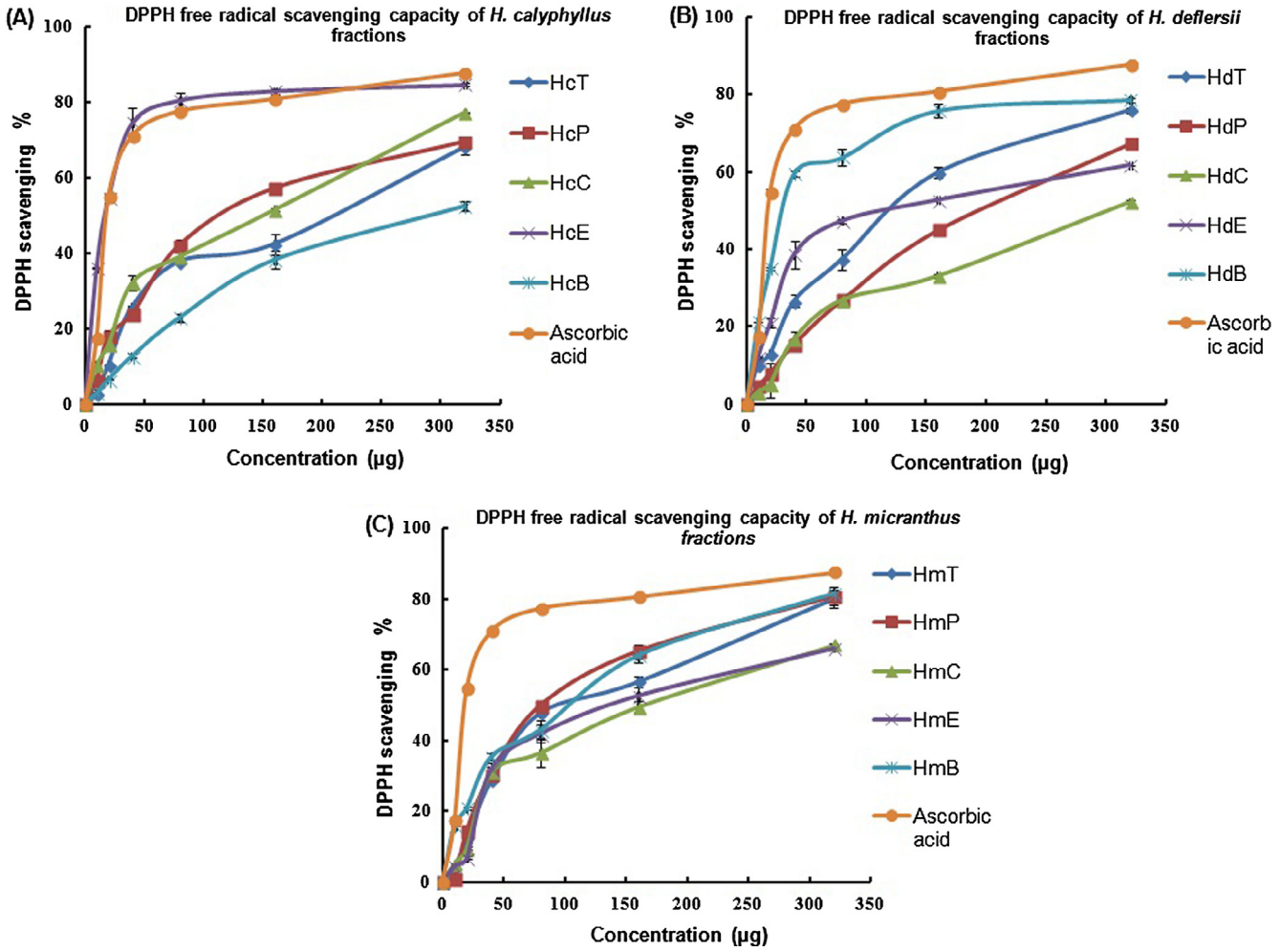

Fig. 6. DPPH free radical scavenging activity of different concentrations (10–320 µg/mL) of different fractions of *H. calyphyllus*, *H. deflersii* and *H. micranthus*. Values are means of three experiments. (A) DPPH free radical scavenging capacity of *H. calyphyllus* fractions. (B) DPPH free radical scavenging capacity of *H. deflersii* fractions. (C) DPPH free radical scavenging capacity of *H. micranthus* fractions.

Table 6
The estimated IC_{50} (µg/mL) values of different fractions of *H. calyphyllus*, *H. deflersii*, *and H. micranthus for antioxidant potential.*

IC_{50} (µg/mL) values of different fractions of *H. calyphyllus*		IC_{50} (µg/mL) values of different fractions of *H. deflersii*		IC_{50} (µg/mL) values of different fractions of *H. micranthus*	
Fractions	IC_{50} (µg/ml) ± SD	Fractions	IC_{50} (µg/ml) ± SD	Fractions	IC_{50} (µg/ml) ± SD
HcT	207.5 ± 1.3	HdT	125 ± 2.5	HmT	99.7 ± 8.2
HcP	122 ± 0.8	HdP	196.5 ± 0.8	HmP	80.4 ± 4.5
HcC	150.9 ± 1.2	HdC	301.5 ± 0.6	HmC	164 ± 1.2
HcE	17.6 ± 1.8	HdE	120 ± 9.2	HmE	139.6 ± 1.8
HcB	292.9 ± 3.6	HdB	32.16 ± 0.9	HmB	106.6 ± 4.5
Ascorbic acid	14.2 ± 0.5	Ascorbic acid	14.2 ± 0.5	Ascorbic acid	14.2 ± 0.5

acid (IC_{50} = 14.2 ± 0.5). These findings also supports the previous literature about antioxidant potential of oleanolic acid with mechanism of significant inhibition in the production of nonenzymatic glycative products, pentosidine and carboxymethyllysine (CML) and it is reported that oleanolic acid exhibited greater antioxidant activity than alpha-tocopherol at different pH. Oleanolic acid also reported to possesses a dose-dependent effect on superoxide anion scavenging activity, chelating effect, xanthine oxidase inhibition activity, and reducing power (Yin and Chan, 2007). The experimental findings also indicate that OA and BA are not the only factors regulating anti-oxidant capabilities otherwise *H. micranthus* would have been the most potent antioxidant being highest contents of OA and BA present in it. *H. deflersii* also exhibited moderate antioxidant capabilities [HdB (IC_{50} = 32.16 ± 0.9); Fig. 6B] but *H. micranthus* [HmP (IC_{50} = 80.4 ± 4.5), HmT (IC_{50} = 99.7 ± 8.2); Fig. 6C] showed least usefulness as anti-oxidant. Some previous findings also depicted the antioxidant capabilities of β-amyrin acetate (Fabiyi et al., 2012). The data obtained from Table 6 exhibited that the butanol fractions free from OA and BA (HcB and HmB) also possess antioxidant potential, that too HdB showed significant IC_{50} value as antioxidant. On the other side HmP which possess highest amount of OA and BA also showed moderate antioxidant capabilities with IC_{50} value 80.4. These findings suggest that OA and BA are active antioxidant biochemicals but the emergence of HcE as most

potent antioxidant among the selected *Hibiscus* species indicates the presence of many other phytochemicals as antioxidants.

4. Conclusion

The authors developed HPTLC method for simultaneous analysis of oleanolic acid and β-amyrin in *H. calyphyllus*, *H. deflersii* and *H. micranthus* for the first time. The proposed method may be further applied for quality analysis of the raw material as well as herbal formulation claiming the presence of oleanolic acid and β-amyrin. Stability studies and degradation kinetics of herbal formulation having oleanolic acid and β-amyrin can also be performed by the method developed by the authors. Exploration of new genera or species possessing oleanolic acid and can be possible by the proposed method. The *Hibiscus* species can also be considered for antioxidant activity because the experimental findings suggest that *H. calyphyllus* possesses significant antioxidant potential.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

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