RESEARCH PAPER | Biochemistry | Volume : 5 | Issue : 2 | Feb 2015 | ISSN - 2249-555X

TLC Densitometric Method for Caliberation of Eugenol, Ursolic Acid, Oleanolic acid and Beta Sitosterol in Ocimum Tenuiflorum Linn.

KEYWORDS: Thin layer chromatography Eugenol, ursolic acid, oleanolic acid and beta sitosterol Medicinal plants Tulsi Ocimum Tenuiflorum.

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ABSTRACT *OcimumTenuiflorum also addressed as Ocimum sanctum (family Lamiaceae) is a reputed drug of Ayurveda, commonly known as Tulasi. In the present work, we quantified 4 marker compounds viz. eugenol, ursolic acid, oleanolic acid and beta sitosterol, from green varieties of O. Tenuiflorum using high-performance thin-layer chromatography (HPTLC) with densitometry. The mobile phase is cyclohexane: chloroform: ethyl acetate 20:5:8. The method was found to be precise, with relative standard deviation (RSD) values for intraday analyses is 0.59, 0.13, 0.42, and 0.19 and for interday analysis 0.78, 0.14, 0.39 and 0.20 for eugenol, ursolic acid, oleanolic acid, and Beta sitosterol respectively. Instrumental RSD values were 0.18, 0.19, 0.18 and 0.21% for eugenol, ursolic acid, oleanolic acid and beta sitisterol respectively. Accuracy of the methods was checked by conducting a recovery study at 3 different levels for the 4 compounds, and the average recoveries were found to be 98.72, 98.21, 100.5 & 97.31 for eugenol, ursolic acid, oleanolic acid and Beta sitosterol respectively. In present sample we get 0.014% eugenol, 0.012% ursolic acid, 0.06% oleanolic acid and 0.006% Beta sitosterol. We derivatised plate with anisaldehyde–sulfuric acid reagent for quantification of markers. The HPTLC-densitometry methods for the quantification of the 4 markers in O. Tenuiflorum plant will have the applicability in quality control.*

O. Tenuiflorum Linn, commonly known as tulsi or holy basil is widely used in Indian system of medicine. The variety of *Ocimum tenuiflorum* used in *Thai cuisine* is referred to as Thai holy basil (30). Many varieties of *O. Tenuiflorum* are known, *Sri Tulasi/Safed Tulasi* bearing green leaves (OTG) and *Krishna Tulasi/Kali Tulasi* bearing dark purple leaves, of which the latter is claimed to be more potent than the former as per Chunekar (2). In traditional medicine, the plant is used in cardiopathy, blood disorders, leucoderma, asthma, bronchitis, genitourinary disorders, skin diseases, etc. (2).

The major chemical constituents reported from *O. Tenuiflorum* are eugenol (3), luteolin and luteolin-7-*O*- -D-glucuronide (4), apigenin (4), ursolic acid (4, 5), oleanolic acid (6), beta sitoterol (6), galuteolin (7), orientin (4), vicenin-1 (8), vicenin-2 (7), and gallic acid (9). *O. Tenuiflorum* was reported to have anti-inflammatory (10), analgesic (10), antipyretic (10), antioxidant (11), antiulcer (12), antitumor (13), antimutagenic (13), anticarcinogenic (14), and antifertility (15) activities. Leaf powder was shown to reduce blood sugar level by potentiating the action of exogenous insulin (16). Essential oil of *O. Tenuiflorum* was shown to have antibacterial and antifungal activity (17).

In the present paper, we report our work on quantification of eugenol, ursolic acid, oleanolic acid and beta sitosterol (Figure 1) in *O. Tenuiflorum* by high-performance thin-layer chromatography (HPTLC)-densitometry collected from two different places. The 4 marker compounds chosen for the present work have been shown to have important pharmacological activities. Eugenol is known to possess potent anticancer (18) and anti-inflammatory (19) activity and induces dose-dependent hypotension and bradycardia (20); Beta Sitosterol is reported to use in heart disease and high cholesterol (21) and anticancer activity (22); and oleanolic and ursolic acids showed hepatoprotective (23), anti-inflammatory (23), and antihyperlipidemic (23) activity and are recommended in skin cancer therapy (24). For the quantification of eugenol, ursolic acid, oleonolic acid, and β-Sitosterol we developed a simple HPTLC-densitometry method.

Experimental

Materials

(*a*) *Plant material*.—The leaves of *O. Tenuiflorum* were collected from PDKV, district Akola, *Maharashtra*, India, and from Keshav Srushti,, Uttan- Road,, Bhayander (W), Maharashtra India. The samples were authenticated by Dr Rajendra Shinde and Dr Frazer Mascarenhas, and voucher specimens were deposited in Blatter Herbarium ST. Xavier's College (Specimen number 22319 of H. Santapau). The samples were dried in shade, stored at 25 C in air tight containers, and powdered to 40 mesh whenever required.

(*b*) *Standard compounds*.—Eugenol (purity 98%) was procured from Natural Remedies Pvt. Ltd., Bangalore, India; ursolic acid (purity 90%), oleanolic acid (purity 98%) from Sigma-Aldrich, Munich, Germany; and β-sitosterol from Jamia Hamdard New Delhi India.

(*c*) *Chemicals*.—All chemicals used were analytical grade.

Apparatus

(*a*) *Spotting device*.—Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland).

(*b*) *Syringe*.—100 L (Hamilton).

(*c*) *TLC chamber*.—Glass twin trough chamber for 20 10 cm plates (Camag).

(*d*) *Densitometer*.—TLC Scanner 3 linked to WinCATS software (Camag).

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(*e*) *HPTLC plates.*—20 10 cm, precoated with silica gel 60 F_{254}, 0.2 mm layer thickness (Cat. No. 1.05548, Batch No. OB 105659; E. Merck, Darmstadt, Germany).

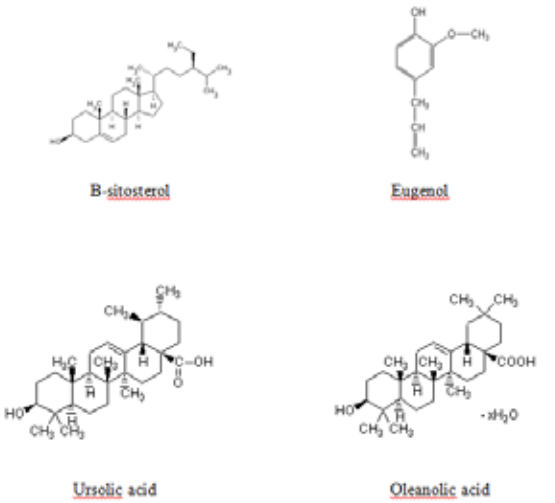

Fig 1. Chemical structures of eugenol, Beta sitosterol, ursolic acid, and oleanolic acid.

Detection Method

(*a*) *Anisaldehyde–sulfuric acid reagent (28).*— Anisaldehyde (0.5 mL) was mixed with 10 mL glacial acetic acid, followed by 85 mL methanol and 5 mL concentrated sulfuric acid, in that order.

(*b*) *Derivatization.*—The plates were dipped in about 20 mL freshly prepared anisaldehyde–sulfuric acid reagent for 1 min and heated at 100 C for 7 min before scanning.

Sample Solutions

(*a*) *Sample Solution 1.*—An accurately weighed 1.0 g quantity of powdered drug was extracted for 15 min with methanol (4×25 mL) under reflux on a water bath at 100 C. The methanolic extract was filtered through Whatman filter paper No. 1, and filtrates were combined, concentrated, and transferred to a 50 mL volumetric flask and the volume was made up to the mark with methanol. This extract was used for the quantification of eugenol, ursolic acid and β-Sitosterol.

(*b*) *Sample Solution 2.*—An accurately weighed 1.0 g quantity of powdered drug was first extracted with *n*-hexane (4×25 mL) for 15 min under reflux on a water bath at 70 C (in order to remove free ursolic acid). The *n*-hexane extract was filtered through Whatman filter paper No. 1, and filtrates were combined and concentrated under vacuum to 25 mL. This *n*-hexane extract was also checked for the presence of oleanolic acid in free form, if any. The marc was dried and then hydrolyzed with 2 M methanolic hydrochloric acid (50 mL) under reflux on a water bath at 100°C for 2 h. The extract was filtered through Whatman filter paper No. 1 and the marc was washed with methanol. The combined filtrates were transferred to a 50 mL volumetric flask, and the volume was adjusted with methanol. This extract was used for the quantification of oleanolic acid.

TLC Densitometric Quantification of Eugenol

Preparation of standard solutions of eugenol.—A stock solution of eugenol (1 mg/mL) was prepared by dissolving 50 mg of accurately weighed eugenol in methanol and diluting to 50 mL with methanol in a volumetric flask. Aliquots (0.2 to 1.0 mL) of stock solution were transferred to 10 mL volumetric flasks and diluted with methanol to obtain standard solutions containing 20, 40, 60, 80, and 100 g/mL eugenol, respectively.

(*b*) *Preparation of calibration curve of eugenol.*—10 µL each of the standard solutions of eugenol (200 to 1000 ng/spot) were applied (bandwidth, 6 mm; distance between the tracks,

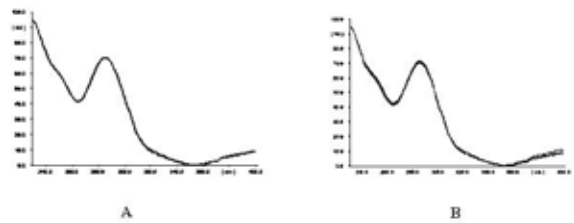

Figure 2. (A) Overlay UV absorption spectra of eugenol and the corresponding band in the sample extract and standard; (B) overlay UV absorption spectra of eugenol in the sample track at the start, middle, and end positions.

12 mm) in triplicate on an HPTLC plate using the Linomat V. The plates were developed in a twin trough chamber with 20 mL of the mobile phase cyclohexane: chloroform: ethyl acetate 20:5:8. v/v/v) for a distance of 6.0 cm at 25 ± 2 C and 40% relative humidity. The plates were dried at room temperature in air and scanned at 280 nm in absorbance mode using the deuterium lamp source of the densitometer. The peak areas were recorded. The calibration curve of eugenol was obtained by plotting peak areas vs applied concentrations of eugenol.

(*c*) *Quantification of eugenol in samples.*—15 µL each of suitably diluted Sample Solution 1 was applied in triplicate on an HPTLC plate. The plate was developed and scanned as described above. The peak areas and absorption spectra were recorded, and the amount of eugenol was calculated using the calibration curve.

TLC Densitometric Quantification of Beta sitosterol

For the quantification of Beta sitosterol

(*a*) *Preparation of standard solutions of* Beta sitosterol.—A stock solution of (40 µg/mL) was prepared by dissolving 4 mg of accurately weighed Beta sitosterol in methanol and diluting to 100 mL with methanol in a volumetric flask. Aliquots (2 to 6 mL) of stock solution were transferred to 10 mL volumetric flasks and diluted with methanol to obtain standard solutions containing 8, 12, 16, 20, and 24 g/mL Beta sitosterol, respectively.

(*b*) *Preparation of calibration curve of* Beta sitosterol.—10 µL each of the standard solutions (80 to 240 ng/spot) were applied and HPTLC was performed as described above for eugenol, except developed and dried plates were derivatized with anisaldehyde–sulfuric acid reagent, heated at 105 C and scanned at 540 nm in the absorbance mode using the White lamp. The peak areas were recorded. Calibration curve of Beta sitosterol was obtained by plotting peak areas vs concentrations of Beta sitosterol applied.

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(*c*) *Quantification of* Beta sitosterol *in the samples.*—15 µL of suitably diluted Sample Solution 1 was applied in triplicate on an HPTLC plate. The plate was developed and scanned as described above. The peak areas and absorption spectra were recorded, and the amount of Beta sitosterol was calculated using the calibration curve.

TLC Densitometric Quantification of Ursolic Acid

(*a*) *Preparation of standard solutions of ursolic acid.*—A stock solution of ursolic acid (90% pure, 72µg/mL) was prepared by dissolving 2 mg of accurately weighed ursolic acid in methanol and diluting to 25 mL with methanol in a volumetric flask. Aliquots (1 to 8 mL) of stock solution were transferred to 10 mL volumetric flasks and diluted with methanol to obtain standard solutions containing 7.2, 14.4, 21.6, 28.8, 36, 43.2, 50.4, and 57.6 µg/mL ursolic acid, respectively.

Table 1. Method validation parameters for the quantification of eugenol, Beta sitosterol, ursolic acid, and oleanolic acid by the proposed method

Serial No.	Parameter	Eugenol	Beta sitosterol	Ursolic acid	Oleanolic acid
1	Instrumental precision [RSD (%), $n = 7$]	0.18	0.21	0.19	0.18
2	Repeatability [RSD (%), $n = 5$]	0.42	0.57	0.16	1.10
4	Accuracy (average recovery, %)	98.72	97.31	98.21	100.57
5	Limit of detection, ng	67	40	28	28
6	Limit of quantification, ng	200	80	85	85
7	Specificity	Specific	Specific	Specific	Specific
8	Linearity (r)	0.998	0.997	0.991	0.993
9	Range, ng/spot	200–700	85–590	85–590	100–500

(*b*) *Preparation of calibration curve of ursolic acid.*—10 µL each of the standard solutions of ursolic acid (72 to 576 ng/spot) were applied and HPTLC was performed as described above for eugenol. After development, the plates were dried at room temperature in air, derivatized with anisaldehyde–sulfuric acid reagent, heated at 105°C until colored bands appeared, and scanned densitometrically at 530 nm in absorbance mode using the tungsten lamp. The peak areas were recorded. Calibration curve of ursolic acid was obtained by plotting peak areas vs applied concentrations of ursolic acid.

(*c*) *Quantification of ursolic acid in the samples.*—15µL of suitably diluted Sample Solution 1 was applied in triplicate on an HPTLC plate. The plate was developed and scanned as described above. The peak areas and absorption spectra were recorded, and the amount of ursolic acid was calculated using its calibration curve.

TLC Densitometric Quantification of Oleanolic Acid

For the quantification of oleanolic acid

(*a*) *Preparation of standard solutions of oleanolic acid.*—A stock solution of oleanolic acid (100 µg/mL) was prepared by dissolving 2 mg of accurately weighed oleanolic acid in methanol and diluting to 20 mL with methanol in a volumetric flask. Aliquots (1 to 5 mL) of stock solution were transferred to 10 mL volumetric flasks and diluted with methanol to obtain standard solutions containing 10, 20, 30, 40, and 50 µg/mL oleanolic acid.

(*b*) *Preparation of calibration curve of oleanolic acid.*—10µL each of the standard solutions of oleanolic acid (100 to 500 ng/spot) were applied and HPTLC, detection, and scanning were performed as described above for ursolic acid. The peak areas were recorded. Calibration curve of oleanolic acid was obtained by plotting peak areas vs applied concentrations of oleanolic acid.

(*c*) *Quantification of oleanolic acid in the samples.*—15µL of suitably diluted Sample Solution 2 was applied in triplicate on an HPTLC plate. The plate was developed and scanned as described above. The peak areas and absorption spectra were recorded, and the amount of oleanolic acid was calculated using its calibration curve.

Validation of the Methods

International Conference on Harmonization (ICH) guidelines were followed for the validation of the analytical procedures (CPMP/ICH/281/95 and CPMP/ICH/381/95). The methods were validated for precision, repeatability, and accuracy. Instrumental precision was checked by repeated scanning (n = 7) of the same spot of eugenol (300 ng/spot), Beta sitosterol (160 ng/spot), ursolic acid (216 ng/spot), and oleanolic acid (200 ng/spot) and expressed as relative standard deviation (RSD). The repeatability of the method was affirmed

Table 2. Intraday and interday precision for eugenol, , ursolic acid, Beta sitosterol and oleanolic acid determination

Marker	Concn, ng/spot	Intraday precision[a]	Interday precision[a]
Eugenol	200	0.61	0.96
	300	0.55	0.84
Beta sitosterol	80	1.29	2.07
	160	1.13	1.02
Ursolic acid	216	0.11	0.12
	288	0.15	0.15
Oleanolic acid	100	0.42	0.39
	200	0.42	0.61

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Table 3. Recovery study of eugenol, ursolic acid, Beta sitosterol and oleanolic acid by the proposed HPTLC-densitometric method

	Amount of marker	Amount of marker	Amount of marker			
Marker	present, mg	added, mg	found[a], mg		Recovery[a], %	Avg. recovery, %
Eugenol 1	1.44	1.93	3.22 ± 0.25		97.08 ± 0.35	99.73
2	1.2	2.86	3.18 ± 1.05		99.28 ± 1.2	
Beta sitosterol 1	1.47	1.6	2.11± 0.11		99.7 ± 0.56	99.3
2	1.21	1.18	3.24	± 0.25	98.8 ± 0.38	
Ursolic acid 1	1.16	2.6	2.73	± 0.25	101.25 ± 0.38	100.58
2	1.45	4.2	5.52	± 0.63	99.31 ± 0.53	
Oleanolic acid 1	1.9	1.05	6.72	± 0.32	99.81 ± 0.29	100.57
2	1.01	2.01	5.47	± 0.41	101.01 ± 0.38	

[a] Mean ± SD (*n* = 3).

by analyzing 300 ng/spot eugenol, 160 ng/spot of beta sitosterol, 216 ng/spot ursolic acid, and 200 ng/spot oleanolic acid individually on the HPTLC plate (*n* = 5) and was expressed as RSD. Variability of the method was studied by analyzing aliquots of standard solution containing 200, 400, and 600 ng/spot eugenol; 80, 160, and 240 ng/spot beta sitosterol; 216, 288, and 360 ng/spot ursolic acid; and 100, 200, and 300 ng/spot oleanolic acid on the same day (intraday precision) and on different days (interday precision), and the results were expressed as RSD. Limit of detection (LOD) and limit of quantification (LOQ) were evaluated by applying different dilutions of the standard solutions of eugenol, beta sitosterol, ursolic acid, and oleanolic acid along with the blank (methanol).

The accuracy of the method was assessed by performing recovery studies at 3 different levels (approximately 50, 100, and 125% addition of eugenol, beta sitosterol, oleanolic acid, and ursolic acid). The recoveries and average recoveries were calculated.

Results and Discussion

In the present study, we quantified 4 marker compounds, eugenol, beta sitosterol, ursolic acid, and oleanolic acid, in 2 samples of *O.Tenuiflorum* by HPTLC-densitometry. We developed a new method for quantification of ursolic acid, eugenol, beta sitosterol, and oleanolic acid to resolve all the compounds in 1 solvent system. Preliminary experiments showed that, of the 4 compounds, eugenol, beta sitosterol, and ursolic acid were in free form, whereas oleanolic acid were detected only after hydrolysis. Consequently, eugenol, beta sitosterol, oleanolic acid, and ursolic acid were quantified from the methanolic extract, and the samples were hydrolyzed to obtain the aglycones of oleanolic acid. The optimized mobile phase resolved all of the marker compounds with the following R_f values: eugenol, 0.77, beta sitosterol, 0.49; ursolic acid, 0.56; and oleanolic acid, 0.56. Other compounds in the sample extracts did not interfere.

Table 4. Eugenol, betasitosterol, ursolic acid, and oleanolic acid content estimated in 2 samples of O. Tenuiflorum by the proposed HPTLC-densitometric method

		Eugenol	Beta sitosterol	Ursolic acid	Oleanolic acid
Source	Sample	(%, w/w)[a]	(%, w/w)[a]	(%, w/w)[a]	(%, w/w)[a]
Akola	1	0.029 ± 0.011	0.051 ± 0.001	0.052 ± 0.002	0.016 ± 0.009
Mumbai	2	0.014 ± 0.010	0.006 ± 0.001	0.012 ± 0.023	0.018 ± 0.002

Mean ± SD (*n* = 3).

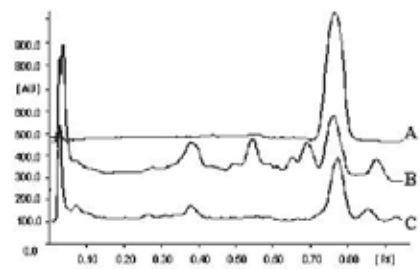

Figure 4. HPTLC-densitogram at 280 nm of a methanolic extract of O. Tenuiflorum, with eugenol standard: (A) eugenol standard;
(B) Sample Solution 1 (Akola); (C) Sample Solution 2 (Mumbai)

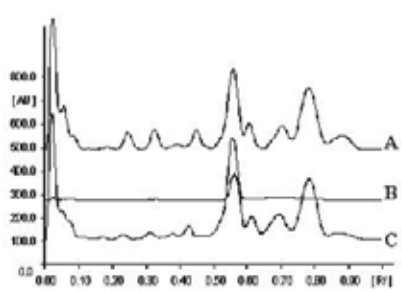

Figure 5. HPTLC-densitogram at 530 nm of O. Tenuiflorum with ursolic acid standard after derivatization with anisaldehyde-sulfuric acid reagent: (A) Sample Solution 1 (Akola); (B) ursolic acid standard; (C) Sample Solution 2 (Mumbai)

The identity of the bands of eugenol, beta sitosterol and ursolic acid in the sample extract was confirmed by overlaying their ultraviolet (UV) absorption spectra with those of the respective reference standards using the Camag TLC Scanner 3 with WinCATS software (Figures 2A). The purity of each of these bands in the sample extract was confirmed by comparing the absorption spectra recorded at start, middle, and end positions of the band.

The methods were validated in terms of precision, repeatability, and accuracy (Table 1). The linearity ranges for eugenol, beta sitosterol, ursolic acid, and oleanolic acid were found to be 200–1000, 85–590, 85–590, and 100–500 ng/spot, respectively, with correlation coefficients (r values) of 0.998, 0.997, 0.991, and 0.993, respectively. The method were found to be precise, with relative standard deviation

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(RSD) values for intraday analyses is 0.59, 0.13, 0.42, and 0.19 and for interday analysis 0.78, 0.14, 0.39 and 0.20 for eugenol, ursolic acid, oleanolic acid, and Beta sitosterol respectively. Instrumental RSD values were 0.18, 0.19, 0.18 and 0.21% for eugenol, ursolic acid, oleanolic acid and beta sitisterol respectively. Accuracy of the methods was checked by conducting a recovery study at 3 different levels for the 4 compounds, and the average recoveries were found to be 98.72, 98.21, 100.5 & 97.31 for eugenol, ursolic acid, oleanolic acid and Beta sitosterol respectively. In present sample we get 0.014% eugenol, 0.012% ursolic acid, 0.06% oleanolic acid and 0.006% Beta sitosterol. We derivatised plate with anisaldehyde–sulfuric acid reagent for quantification of markers. The HPTLC-densitometry methods for the quantification of the 4 markers in *O. Tenuiflorum* plant will have the applicability in quality control.

The intraday and interday precision expressed as RSD (Table 2) indicate that the proposed method is precise and reproducible.

The content of eugenol, betasitosterol, ursolic acid, and oleanolic acid in 2 samples of both of *O. scantum* was quantified by the proposed methods (Table 4; Figures 4–7). The quantification of eugenol and betasitosterol does not help in distinguishing the 2 samples, although they will serve as markers in standardization and quality control.

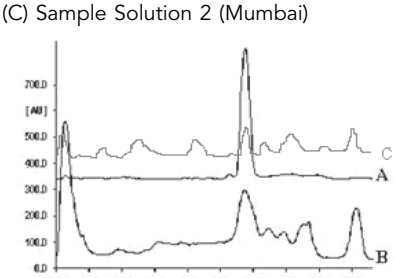
As mentioned above, ursolic acid and oleanolic acid are present in OT. Ursolic acid and oleanolic acid are isomers (29), and both ran at the same R_f value. They do not have a chromophor and, hence, it is not possible to detect them under either 254 or 366 nm UV light. They both turn purple upon derivatization with anisaldehyde–sulfuric acid reagent and, hence, cannot be distinguished. In all the solvent systems tried, it was not possible to resolve them on either TLC or HPTLC plates. However, we observed that, after derivatization with anisaldehyde–sulfuric acid reagent and heating at 100 C for 5 min, ursolic acid gave a yellowish-orange fluorescence when observed under UV 366 nm, while oleanolic acid did not give any fluorescence. From this we could confirm that both markers are present in OT and can be easily distinguished

Although ursolic acid gives yellow fluorescence after derivatization with anisaldehyde–sulfuric acid reagent, it was not possible to quantify in fluorescence mode because its sensitivity was found to be low and reproducibility was poor. However, this feature of ursolic acid showing fluorescence after derivatization can be used to identify ursolic acid, as described above.

Figure 6. HPTLC-densitogram at 530 nm of O. Tenuiflorum with oleanolic acid standard after derivatization with anisaldehyde-sulfuric acid reagent:

(A) oleanolic acid standard; (B) Sample Solution 1 (Akola);

(C) Sample Solution 2 (Mumbai)



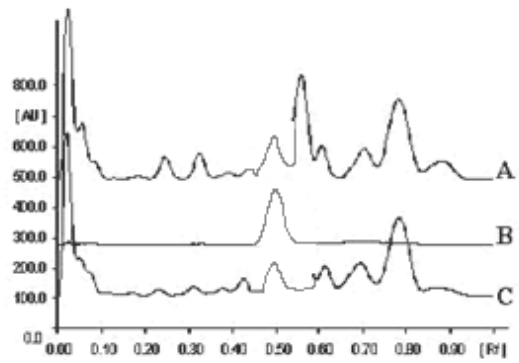


Figure 7. HPTLC-densitogram at 530 nm of O. Tenuiflorum with Beta Sitosterol standard after derivatization with anisaldehyde-sulfuric acid reagent: (A) Sample Solution 1 (Akola), (B) oleanolic acid standard; (C) Sample Solution 2 (Mumbai)

Because both compounds give a purple color upon derivatization with anisaldehyde–sulfuric acid reagent, it is impossible to discern whether a spot at R_f 0.56 is a mixture of the 2 compounds, although the presence of ursolic acid can be ascertained from the yellow fluorescence. However, in the present study, it was possible to quantify both of the compounds in the samples because ursolic acid was present in free form and oleanolic acid in bound form, which was confirmed from the following observations:

(*1*) Oleanolic acid content was quantified in OT samples, where the same sample was extracted (n = 3) as described in Sample Solution 2 and each sample solution was applied in triplicate on a TLC plate. It was found to contain 0.06 % (w/w) of oleanolic acid.

(*2*) Ursolic acid was present in the *n*-hexane extract but was not detected in the defatted plant material after hydrolysis.

From the above observations, it can safely be concluded that, in the OT samples studied, ursolic acid was present in free form, whereas oleanolic acid was present in bound form, which facilitated their quantification separately. If ursolic acid was present in bound form or oleanolic acid in free form, it would have been impossible to quantify these 2 compounds individually in OT samples in the present study, although the presence of ursolic acid would have been ascertained as described above. Still, we cannot rule out the possibility of the presence of oleanolic acid in free form.

Simultaneous quantification of all 4 markers, eugenol, betasitostero, ursolic acid, and oleanolic acid, was not possible even though they were resolved in the same solvent system because of the following reasons:

(*1*) Eugenol, beta sitosterol, oleanolic acid and ursolic acid are present, but eugenol is detected under UV light (λ_{max} 280 nm) without derivatization, whereas ursolic acid and beta sitosterol and oleanolic acid can be detected only after derivatization with anisaldehyde–sulfuric acid reagent (λ_{max} 530 nm).

(*2*) Oleanolic acid are present in bound form(λ_{max} 350 nm) without derivatization, whereas oleanolic acid can be de-

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tected only after derivatization with anisaldehyde–sulfuric acid reagent (λ_{max} 530 nm).

(*3*) The plates were scanned at the respective λ_{max} of the 4 markers for quantification. All 4 markers are visible after derivatization, and this feature can be used for TLC fingerprinting purposes, where the sample extracts can be co-chromatographed with markers and visualized after derivatization with anisaldehyde–sulfuric acid reagent.

Conclusions

HPTLC-densitometry methods were successfully applied for the quantification of eugenol, ursolic acid, beta sitosterol, and oleanolic acid in 2 samples of *O. Tenuiflorum*. The methods prove to be helpful in distinguishing the 2 samples of *O. Tenuiflorum*. The developed methods are simple, precise, specific, sensitive, and accurate, and they can be used for multiple marker-based evaluation of the 2 samples of *O. Tenuiflorum* and formulations containing either of the 2 samples for standardization and quality control purposes.

Acknowledgments

We are thankful to DST, for the financial aid towards this work.

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