DIETARY SUPPLEMENTS

Quantification of Eugenol, Luteolin, Ursolic Acid, and Oleanolic Acid in Black (*Krishna Tulasi*) and Green (*Sri Tulasi*) Varieties of *Ocimum sanctum* Linn. Using High-Performance Thin-Layer Chromatography

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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, luteolin, ursolic acid, and oleanolic acid, from the leaf of green and black varieties of *O. sanctum* using high-performance thin-layer chromatography (HPTLC) with densitometry. The methods were found to be precise, with relative standard deviation (RSD) values for intraday analyses in the range of 0.52 to 0.91%, 0.77 to 1.29%, 0.11 to 0.16%, and 0.34 to 0.42% and for interday analyses in the range of 0.73 to 0.96%, 1.02 to 2.08%, 0.11 to 0.12%, and 0.39 to 0.64% for different concentrations of eugenol, luteolin, ursolic acid, and oleanolic acid, respectively. Instrumental RSD values were 0.24, 0.39, 0.21, and 0.18% for eugenol, luteolin, ursolic acid, and oleanolic acid, 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 99.73, 99.3, 100.58, and 100.57%, respectively. Eugenol content ranged from 0.175 to 0.362% (w/w) and luteolin from 0.019 to 0.046% (w/w) in the samples analyzed. Green variety was found to contain higher amounts of ursolic acid [0.478 and 0.348% (w/w), from Sources 1 and 2, respectively] than the black variety [0.252 and 0.264% (w/w) from Sources 1 and 2, respectively]. Black variety had 0.174 and 0.218% (w/w) of oleanolic acid from Sources 1 and 2, respectively, while it was not detected in the green variety. Ursolic acid and oleanolic acid ran at the same R_f value and could not be resolved in several solvent systems tried. However, we observed that only ursolic acid gave yellow fluorescence under 366 nm ultraviolet light after derivatization with anisaldehyde–sulfuric acid reagent. The HPTLC-densitometry methods for the quantification of the 4 markers in *O. sanctum* leaf will have the applicability in quality control.**

Received January 3, 2006. Accepted by AP May 17, 2006.
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Ocimum sanctum Linn. (family Lamiaceae), commonly known as *Tulasi* or Holy Basil, is widely used in Indian systems of medicine. At least 2 varieties of *O. sanctum* are known, *Sri Tulasi*/*Safed Tulasi* bearing green leaves (OSG) and *Krishna Tulasi*/*Kali Tulasi* bearing dark purple leaves (OSB; 1, 2), 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. sanctum* are eugenol (3), luteolin and luteolin-7-*O*-β-D-glucuronide (4), apigenin (4), ursolic acid (4, 5), oleanolic acid (6), galuteolin (7), orientin (4), vicenin-1 (8), vicenin-2 (7), and gallic acid (9). *O. sanctum* 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. sanctum* was shown to have antibacterial and antifungal activity (17).

In the present paper, we report our work on quantification of eugenol, luteolin, ursolic acid, and oleanolic acid (Figure 1) in the 2 varieties of *O. sanctum* by high-performance thin-layer chromatography (HPTLC)-densitometry and made efforts to distinguish the varieties. 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); luteolin is reported to have anti-inflammatory (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

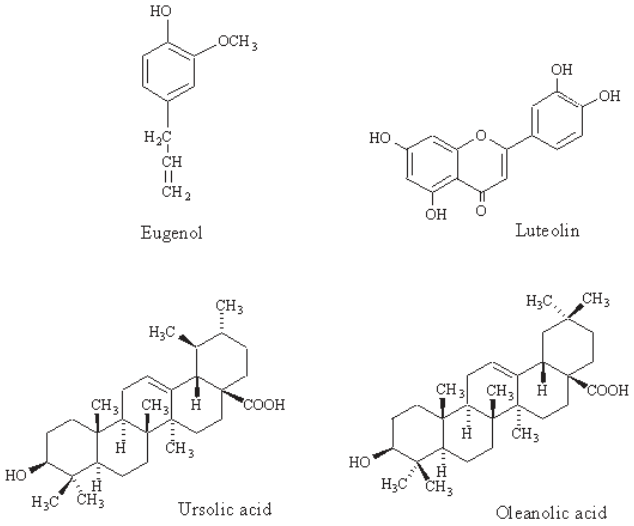

Figure 1. Chemical structures of eugenol, luteolin, ursolic acid, and oleanolic acid.

quantification of eugenol, luteolin, and oleonolic acid, we adopted methods reported from our laboratory earlier (25–27), and for the quantification of ursolic acid we developed a simple HPTLC-densitometry method.

Experimental

Materials

(**a**) *Plant material.*—The leaves of both varieties of *O. sanctum* were collected from Dharampur, district Valsad, Gujarat, India, and from Ahmedabad, district Ahmedabad, Gujarat, India. The samples were authenticated, and voucher specimens were deposited in our Pharmacognosy and Phytochemistry Department. The samples were dried in shade, stored at 25°C in airtight containers, and powdered to 40 mesh whenever required.

(**b**) *Standard compounds.*—Eugenol (purity 98%) was procured from Natural Remedies Pvt. Ltd., Bangalore, India; luteolin (purity 99%) and ursolic acid (purity 90%) from Sigma-Aldrich, Munich, Germany; and oleanolic acid (purity 98%) from RRL, Jammu, India.

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

Apparatus

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

(**b**) *Syringe.*—100 µL (Hamilton).

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

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

(**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).

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 10 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 No. 1 filter paper, 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 and ursolic acid.

(**b**) *Sample Solution 2.*—An accurately weighed 1.0 g quantity of powdered drug was 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 No. 1 filter paper, and the residue left after extraction (marc) was washed with methanol. The combined filtrates were 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 luteolin.

(**c**) *Sample Solution 3.*—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 No. 1 filter paper, 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 No. 1 filter paper 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

For the quantification of eugenol, we adopted the method reported by us earlier (25).

(**a**) *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 µg/mL eugenol, respectively.

(**b**) *Preparation of calibration curve of eugenol.*—10 µL each of the standard solutions of eugenol (200 to

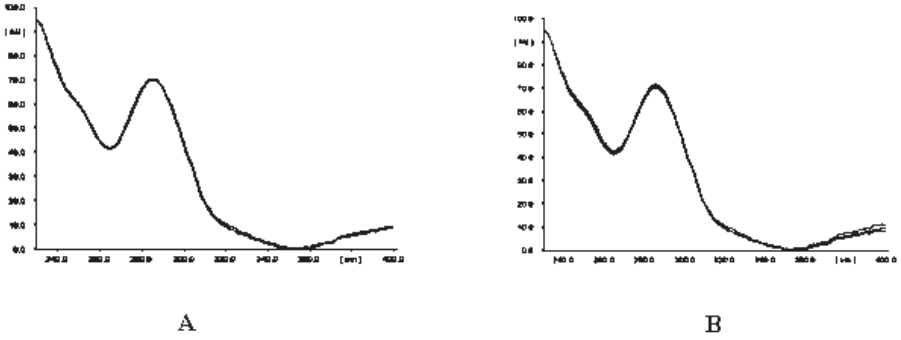

Figure 2. (A) Overlay UV absorption spectra of eugenol and the corresponding band in the sample extract and standard; (B) overlay UV absorption spectra of eugenol in the sample track at the start, middle, and end positions.

1000 ng/spot) were applied (bandwidth, 6 mm; distance between the tracks, 12 mm) in triplicate on an HPTLC plate using the Linomat V. The plates were developed in a twin trough chamber with 10.2 mL of the mobile phase toluene–ethyl acetate–formic acid (7 + 3 + 0.2, 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.*—10 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 Luteolin

For the quantification of luteolin, we adopted the method reported by us earlier (26).

(**a**) *Preparation of standard solutions of luteolin.*—A stock solution of luteolin (40 μg/mL) was prepared by dissolving 4 mg of accurately weighed luteolin 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 μg/mL luteolin, respectively.

(**b**) *Preparation of calibration curve of luteolin.*—10 μL each of the standard solutions (80 to 240 ng/spot) were applied and HPTLC was performed as described above for eugenol, except dried plates were scanned at 350 nm in the absorbance mode using the deuterium lamp. The peak areas were recorded. Calibration curve of luteolin was obtained by plotting peak areas vs concentrations of luteolin applied.

(**c**) *Quantification of luteolin in the samples.*—15 μL 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 luteolin 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, 72 μg/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,

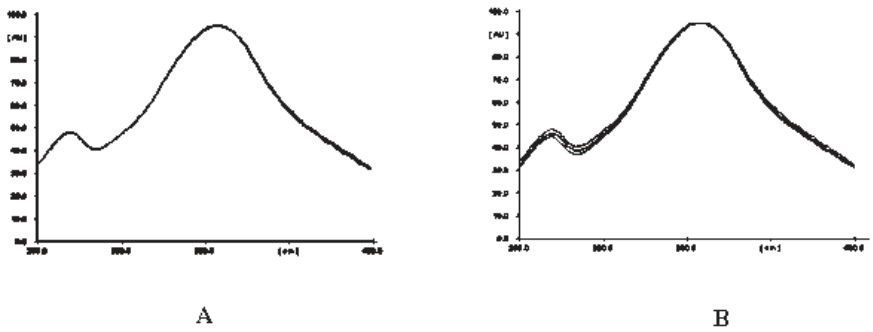

Figure 3. (A) Overlay UV absorption spectra of luteolin and the corresponding band in the sample extract and standard; (B) overlay UV absorption spectra of luteolin in the sample track at the start, middle, and end positions.

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

Serial No.	Parameter	Eugenol	Luteolin	Ursolic acid	Oleanolic acid
1	Instrumental precision [RSD (%), *n* = 7]	0.24	0.39	0.21	0.18
2	Repeatability [RSD (%), *n* = 5]	0.42	0.57	0.16	1.10
4	Accuracy (average recovery, %)	99.73	99.30	100.58	100.57
5	Limit of detection, ng	60	40	18	20
6	Limit of quantification, ng	200	80	72	100
7	Specificity	Specific	Specific	Specific	Specific
8	Linearity (r)	0.998	0.997	0.991	0.993
9	Range, ng/spot	200–1000	80–240	72–576	100–500

21.6, 28.8, 36, 43.2, 50.4, and 57.6 μg/mL ursolic acid, respectively.

(**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.*—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 ursolic acid was calculated using its calibration curve.

TLC Densitometric Quantification of Oleanolic Acid

For the quantification of oleanolic acid, we adopted the method reported by us earlier (27).

(**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.*—10 μL 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.*—15 μL of suitably diluted Sample Solution 3 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 (600 ng/spot), luteolin (160 ng/spot), ursolic acid (216 ng/spot), and oleanolic acid (300 ng/spot) and expressed as relative standard deviation (RSD). The repeatability of the method

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

Marker	Concn, ng/spot	Intraday precision[a]	Interday precision[a]
Eugenol	200	0.61	0.96
	300	0.52	0.86
	400	0.91	0.73
Luteolin	80	1.29	2.08
	160	1.12	1.02
	240	0.77	1.24
Ursolic acid	216	0.11	0.12
	288	0.16	0.11
	360	0.12	0.12
Oleanolic acid	100	0.42	0.39
	200	0.40	0.64
	300	0.34	0.46

[a] RSD (%), n = 3.

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

Marker	Amount of marker present, μg	Amount of marker added, μg	Amount of marker found[a], μg	Recovery[a], %	Avg. recovery, %
Eugenol	530	250	757.22 ± 0.25	97.08 ± 0.35	99.73
	530	500	1043.18 ± 1.05	101.28 ± 1.2	
	530	650	1189.91 ± 0.89	100.84 ± 0.91	
Luteolin	320	160	478.6 ± 0.32	99.7 ± 0.56	99.3
	320	320	632.4 ± 0.25	98.8 ± 0.38	
	320	400	716.7 ± 0.96	99.5 ± 1.3	
Ursolic acid	252	126	382.73 ± 0.25	101.25 ± 0.38	100.58
	252	252	500.52 ± 0.63	99.31 ± 0.53	
	252	324	582.85 ± 1.16	101.19 ± 1.02	
Oleanolic acid	442	240	680.72 ± 0.32	99.81 ± 0.29	100.57
	442	400	850.47 ± 0.41	101.01 ± 0.38	
	442	560	1010.99 ± 0.77	100.89 ± 0.69	

[a] Mean ± SD (n = 3).

was affirmed by analyzing 600 ng/spot eugenol, 160 ng/spot of luteolin, 216 ng/spot ursolic acid, and 300 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 luteolin; 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, luteolin, 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, luteolin, 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, luteolin, ursolic acid, and oleanolic acid, in 2 varieties of *O. sanctum* by HPTLC-densitometry. We developed a new method for quantification of ursolic acid, whereas for quantification of eugenol, luteolin, and oleanolic acid, we adopted the methods proposed by us earlier (25–27), with some modifications, to resolve all the compounds in 1 solvent system. Preliminary experiments showed that, of the 4 compounds, eugenol and ursolic acid were in free form, whereas luteolin and oleanolic acid were detected only after hydrolysis. Consequently, eugenol and ursolic acid were quantified from the methanolic extract, and the samples were hydrolyzed to obtain the aglycones of luteolin and oleanolic acid. The optimized mobile phase resolved all of the marker compounds with the following R_f values: eugenol, 0.77; luteolin, 0.27; ursolic acid, 0.56; and oleanolic acid, 0.56. Other compounds in the sample extracts did not interfere.

Table 4. Eugenol, luteolin, ursolic acid, and oleanolic acid content estimated in 2 varieties of *O. sanctum* by the proposed HPTLC-densitometric method

Source	Sample	Eugenol (%, w/w)[a]	Luteolin (%, w/w)[a]	Ursolic acid (%, w/w)[a]	Oleanolic acid (%, w/w)[a]
Dharampur	OSB	0.219 ± 0.011	0.031 ± 0.001	0.252 ± 0.002	0.173 ± 0.009
	OSG	0.175 ± 0.003	0.032 ± 0.001	0.478 ± 0.004	ND[b]
Ahmedabad	OSB	0.208 ± 0.010	0.046 ± 0.001	0.264 ± 0.023	0.218 ± 0.002
	OSG	0.362 ± 0.012	0.019 ± 0.001	0.348 ± 0.005	ND

[a] Mean ± SD (n = 3).
[b] ND = Not detected.

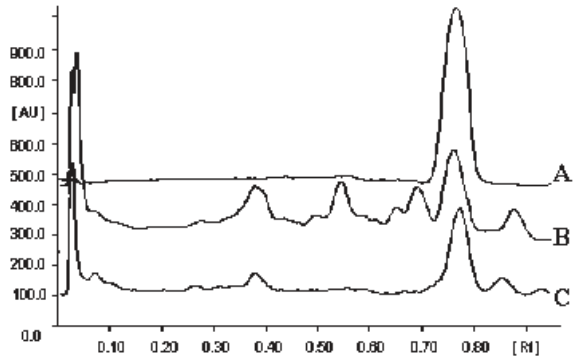

Figure 4. HPTLC-densitogram at 280 nm of a methanolic extract of *O. sanctum*, black and green varieties, with eugenol standard: (A) eugenol standard; (B) Sample Solution 1 of green variety; (C) Sample Solution 1 of black variety.

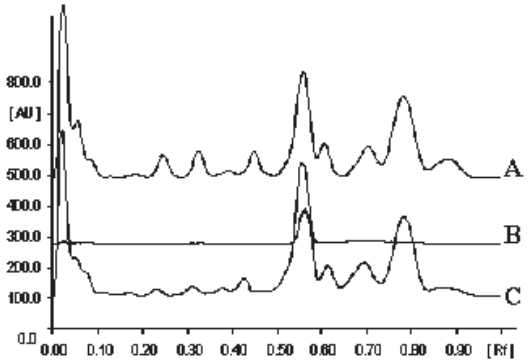

Figure 6. HPTLC-densitogram at 530 nm of *O. sanctum*, black and green varieties with ursolic acid standard after derivatization with anisaldehyde-sulfuric acid reagent: (A) Sample Solution 1 of green variety; (B) ursolic acid standard; (C) Sample Solution 1 of black variety.

The identity of the bands of eugenol and luteolin 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 and 3A). 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 (Figures 2B and 3B).

The methods were validated in terms of precision, repeatability, and accuracy (Table 1). The linearity ranges for eugenol, luteolin, ursolic acid, and oleanolic acid were found to be 200–1000, 80–240, 72–576, and 100–500 ng/spot, respectively, with correlation coefficients (r values) of 0.998, 0.997, 0.991, and 0.993, respectively. The intraday and interday precision expressed as RSD (Table 2) indicate that the proposed method is precise and reproducible. The LOD values for eugenol, luteolin, ursolic acid, and oleanolic acid were found to be 60, 40, 18, and 20 ng, respectively, and LOQ values were 200, 80, 72, and 100 ng, respectively. The average recoveries at 3 different levels of eugenol, luteolin, ursolic acid, and oleanolic acid were found to be 99.73, 99.3, 100.58, and 100.57%, respectively (Table 3).

The content of eugenol, luteolin, ursolic acid, and oleanolic acid in 2 samples of both of the varieties of *O. scantum* was quantified by the proposed methods (Table 4; Figures 4–7). Green variety contains a high amount of ursolic acid when compared to the black variety, and oleanolic acid was not detected in the green variety. The quantification of eugenol and luteolin does not help in distinguishing the 2 varieties, although they will serve as markers in standardization and quality control.

As mentioned above, ursolic acid and oleanolic acid are present in OSB. 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

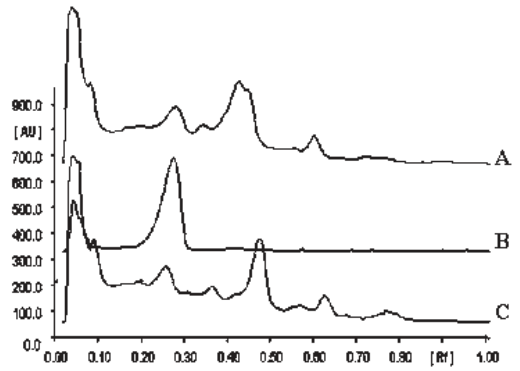

Figure 5. HPTLC-densitogram at 350 nm of *O. sanctum*, black and green varieties with luteolin standard: (A) Sample Solution 2 of green variety; (B) luteolin standard; (C) Sample Solution 2 of black variety.

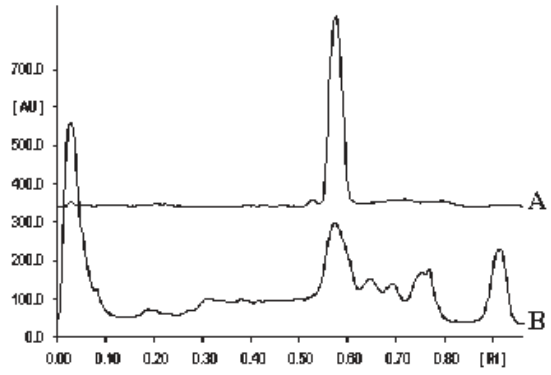

Figure 7. HPTLC-densitogram at 530 nm of *O. sanctum* black variety with oleanolic acid standard after derivatization with anisaldehyde-sulfuric acid reagent: (A) oleanolic acid standard; (B) Sample Solution 3 of black variety.

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 the band at R_f 0.56 was oleanolic acid in the Sample Solution 2 of the black variety and ursolic acid in the green variety of *O. sanctum*.

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.

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 OSB sample (Dharampur), where the same sample was extracted ($n = 3$) as described in Sample Solution 3 and each sample solution was applied in triplicate on a TLC plate. It was found to contain 0.1727% (w/w) of oleanolic acid, with a standard deviation (SD) of 0.009 ($n = 9$).

(*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 OSB and OSG samples studied, ursolic acid was present in free form, whereas oleanolic acid was present in bound form (only in OSB), 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 the sample of OSB 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 in OSG.

Simultaneous quantification of all 4 markers, eugenol, luteolin, 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 and ursolic acid are present in free form, but eugenol is detected under UV light (λ_{max} 280 nm) without derivatization, whereas ursolic acid can be detected only after derivatization with anisaldehyde–sulfuric acid reagent (λ_{max} 530 nm).

(*2*) Luteolin and oleanolic acid are present in bound form, but luteolin is detected under UV light (λ_{max} 350 nm) without derivatization, whereas oleanolic acid can be detected 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, luteolin, ursolic acid, and oleanolic acid in black and green varieties of *O. sanctum*. The methods prove to be helpful in distinguishing the 2 varieties of *O. sanctum*, black and green. The developed methods are simple, precise, specific, sensitive, and accurate, and they can be used for multiple marker-based evaluation of the 2 varieties of *O. sanctum* and formulations containing either of the 2 varieties for standardization and quality control purposes.

Acknowledgments

We are thankful to Harish Padh, B.V. Patel PERD Centre, for providing the facilities, and Industries Commissionerate, Government of Gujarat, for the financial aid towards the instrumentation facility.

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