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DEVELOPMENT AND VALIDATION OF A DENSITOMETRIC HPTLC METHOD FOR QUANTIFICATION OF OPUNTIOL IN *OPUNTIA ELATIOR* MILL.

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ABSTRACT: Opuntiol is a biologically active flavonol present in *Opuntia elatior* Mill. (Cactaceae) that exhibits significant pharmacological activities such as antioxidant, anti-inflammatory, and anticancer effects. Reliable analytical methods are therefore required for the quantitative estimation in plant extracts and pharmaceutical preparations. The present study aimed to develop and validate a rapid, sensitive, and reproducible densitometric HPTLC method for the quantification of Opuntiol. Opuntiol was isolated from the fruits of *Opuntia elatior* using column chromatography, and its purity was confirmed by TLC and UV spectroscopy. Chromatographic separation was achieved on silica gel 60 F254 plates using an optimized mobile phase consisting of ethyl acetate: water: formic acid: glacial acetic acid: n-hexane (7:1:1.1:1.1:5 v/v). Densitometric scanning was performed at 280 nm, yielding a well-resolved band with an R_f value of 0.42 ± 0.002. The method showed excellent linearity in the range of 10–120 ng/spot with a correlation coefficient of 0.9956. The limits of detection and quantification were 10.14 ± 3.04 ng/spot and 30.74 ± 9.24 ng/spot, respectively. Precision studies demonstrated %RSD values below 2%, confirming excellent repeatability and intermediate precision. The robustness of the developed method was found within acceptable limits, with %RSD values ≤2%, indicating that the method is robust and can tolerate minute deliberate changes. The accuracy study was assessed at three different concentrations: 80, 120, and 150 ng/spot. The percentage mean recoveries were accurate, with <5% deviation and 3–8% RSD, indicating method excellence. The validated HPTLC method is simple, rapid, precise, and suitable for routine quality control and standardization of *Opuntia* in *Opuntia* species and related herbal formulations.

INTRODUCTION: ‘Opuntiol’ is an important phytoactive present in the fruits and cladodes of the cactus *Opuntia elatior* Mill. (*Nagaphani*) as well as in other species of the genus *Opuntia*, which belong to the family “Cactaceae”¹.

It is a potential bioactive with diverse biological activities, such as anti-arthritis², anti-inflammatory³, used to treat glioblastoma⁴, a powerful antioxidant⁵⁻⁶, and an anticancer agent⁷.

It belongs to the class of flavonoids, particularly the subclass ‘flavonol,’ structurally characterized as a glycosylated flavonoid derivative^{6, 8}. Opuntiol has demonstrated a crucial role in modulating the inflammatory pathways by inhibiting pro-inflammatory cytokines and oxidative stress markers.

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This makes *Opuntia* a promising candidate for the development of novel antioxidant, anti-inflammatory, and anticancer agents in the field of phytomedicine and novel drug development⁹. Phytoconstituents in plant matrices are routinely analyzed using chromatographic techniques like TLC, HPTLC, HPLC, GC, LC-MS, and GC-MS¹⁰⁻¹³. Among these, HPTLC and HPLC are the most widely used analytical techniques for both qualitative and quantitative analysis. The HPTLC analytical method development and validation play a pivotal role in new drug discovery, drug development, phytochemical estimation, and pharmaceutical product manufacturing¹⁴.

Some investigations use HPTLC for the identification of various phytoconstituents in the cactus *Opuntia*. The HPTLC method was developed for the crude ethanolic extract of *Opuntia dillenii* cladodes, employing a chloroform:methanol (9:1) mixture as the solvent system, and detected 12 and 13 bands at 254 and 366 nm, respectively¹⁵. The betacyanins were separated and estimated by HPTLC from the fruits of *Opuntia boldinghii* Br¹⁶. The HPTLC analysis of the alcoholic extract of the fruits of *Opuntia elatior* Mill. was performed using toluene:ethyl acetate:acetic acid (7:3:1) as the mobile phase, observing 4 spots in short UV and 2 spots in long UV, with R_f values of 0.04 and 0.97 being common¹⁷.

Accurate analytical methods are essential for the standardization and quality control of herbal medicines. Although several chromatographic techniques have been reported for the analysis of phytoconstituents in *Opuntia* species, to date, no information is available regarding the quantitative estimation of Opuntiol using a validated densitometric HPTLC method. Development of such methods is important for routine quality control, phytochemical profiling, and pharmaceutical formulation analysis.

Thus, the present investigation attempts to develop and validate a novel, sensitive, rapid, precise, and robust densitometric HPTLC method, by International Council for Harmonisation (ICH), guidelines Q2 (R1) for Opuntiol estimation in various *Opuntia* species using an in-house isolated and spectrally characterized (FT-IR, ¹H-NMR, ¹³C-

NMR, GC-MS, and ideally single-crystal XRD; information provided in supplementary files) reference standard, because Opuntiol is not commercially available as a certified reference standard from any major supplier. This method enables the standardization of Opuntiol in various extracts of *Opuntia* species.

MATERIALS AND METHODS:

Materials: The fully matured, dark red colored fruits of *Opuntia elatior* Mill. (Cactaceae), commonly known as “nagphani,” were collected from the local areas of Mahakal, Wardha, Maharashtra, India, in the months of June-July and November-December. Fruits were identified and authenticated by an expert botanist, with a specimen voucher number 08/Botany/2021-22. Opuntiol was separated from the fruits of *Opuntia elatior* Mill. Fruits were collected from the local areas of Mahakal, Wardha, India.

Chemicals: The solvents used for extraction, separation, and analysis were of analytical grade (Loba Chemie, India). HPLC-grade water, ethyl acetate, glacial acetic acid, formic acid, and n-hexane were used for HPTLC and purchased from Loba Chemie, India. HPTLC precoated silica gel 60F254 plates (60F254, Merck, Germany) were used for the experiment.

Extraction and Separation of Opuntiol: The fresh fruits of *Opuntia elatior* Mill. were collected, thoroughly washed, and extracted with hydro-alcoholic solvent (1:1 ratio) for up to 8 days. The extract was filtered, concentrated, and then fractionated with solvents of increasing polarity, such as petroleum ether, n-hexane, toluene, and ethyl acetate. Each fraction was collected and dried in an oven at 40°C. Amongst these fractions, the ethyl acetate fraction was taken into consideration on the basis of TLC analysis for further isolation of phytoconstituents by column chromatography. Pure crystals of Opuntiol were separated by eluting the column with hexane and ethyl acetate at a ratio of 1:1 (50:50). TLC of crystals was carried out, and the purity of Opuntiol was further analyzed by HPTLC.

Sample Preparation: Accurately weighed 10 mg of Opuntiol was solubilized in 10 ml (water: methanol; 20:80% v/v; 1000 µg/ml), and further

serially diluted to obtain a desired concentration of solution of 10 µg/ml. Fresh samples were prepared every day for analysis. It showed maximum UV absorbance (λ_{max}) at 280 nm; hence, 280 nm was used throughout the analysis of *Opuntia*.

Instrumentation: The precoated aluminum plates (10 cm × 10 cm) of silica gel G60 F254 (E. Merck, Darmstadt, Germany), with 250 µm thickness, pre-washed with methanol for up to 10 min and heated for 20 min at 110°C, were used for chromatography.

The standard working solution of Opuntiol was applied using a CAMAG Linomat V semiautomatic applicator (Muttentz, Switzerland) fitted with a 100 µl Hamilton syringe (Switzerland). The application was carried out in band form at an application rate of 10 µL/s, with each band measuring 6 mm in width and a spacing of 6 mm between bands.

The sample was applied from the bottom at 1 cm above and 1.5 cm from the side edges. The plate was developed to approximately 8 cm, and scanning was performed at 280 nm. A 10 µg/ml working stock solution of Opuntiol was prepared, and different volumes of working standard were applied from 1 µl to 12 µl on the HPTLC plate so as to obtain the concentrations of 10 to 120 ng/spot, respectively. A 6 mm × 0.45 mm slit dimension was maintained, and a 20 mm/s scanning speed was set. The optimized solvent system comprised ethyl acetate, water, formic acid, glacial acetic acid, and n-hexane in the ratio of 7:1:1.1:1.1:5 (v/v), and 15.2 ml of the total solvent system was employed for each chromatographic run. The linear ascending development method was implemented in a twin-trough CAMAG glass chamber (100 mm × 100 mm; Muttentz, Switzerland) that had been previously equilibrated with the solvent system. 20 min was set as the optimized chamber equilibration time, at 25°C ± 2 room temperature, and 40% ± 5 relative humidity. After development, the HPTLC plates were dried using an air dryer. The CAMAG TLC scanner III with automated WINCAT software (1.4.2) (Camag, Muttentz, Switzerland) was used to perform the densitometric scanning at 280 nm.

Statistical Analysis: Data were analyzed using WINCAT software (1.4.2). The results were

expressed as mean ± standard deviation (SD). Linear regression analysis was used to generate the calibration curve, and relative standard deviation (%RSD) was calculated to assess precision and robustness. Standard deviation of Response (σ) is calculated using the STEYX Excel function.

Method Development and Optimization: The HPTLC method was developed after screening several mobile-phase combinations. A quercetin-based solvent system (toluene: ethyl acetate: methanol, 50:30:20 v/v) structural similarity of Opuntiol to flavonols;; however, this solvent system failed to achieve adequate chromatographic resolution.

The HPTLC method was developed to quantify the Opuntiol. For that, ethyl acetate and water were used to obtain polarity, and n-hexane was used to achieve the required non-polarity in the solvent system so as to attain an ideal R_f value. To remove the tailing and fronting effects of chromatography, formic acid, and glacial acetic acid were used. Amongst the multiple mobile phases tried, the combination of ethyl acetate, water, glacial acetic acid, formic acid, and n-hexane (7:1:1.1:1.1:5 v/v) was selected as an optimized solvent system. The best resolution of the Opuntiol spot was obtained with the R_f value of 0.42, **Fig. 2**, accomplishing the criteria of an ideal solvent system for the separation of pharmaceutical compounds and/or phytoconstituents. In order to minimize the neckless effect and ensure uniform solvent front development, the TLC chamber was previously equilibrated with the solvent system for 20 minutes prior to plate development. The solvent system was allowed to migrate up to 8 cm, which took approximately 15 minutes for the complete development.

HPTLC Method Validation: The optimized HPTLC method was used to examine several parameters, including linearity, sensitivity (in terms of LOD - Limit of Detection and LOQ - Limit of Quantification), precision, specificity, robustness, and recovery/accuracy, in accordance with ICH guidelines.

Linearity and Range: The linearity and the range of the HPTLC method were determined by plotting the calibration curve for Opuntiol at various

concentrations. 1–12 μ l of samples of 10 μ g/ml concentration were applied to the HPTLC plates so as to achieve the desired concentrations (10–120 ng/spot). The linearity curve was prepared by plotting the different concentrations (10–120 ng/spot) of the working standard solution of Opuntiol against the peak area. On the basis of Opuntiol's linearity curve equation, the limit is selected. A 280 nm wavelength was used for each scan, **Fig. 4**.

Sensitivity of the Method (LOD and LOQ): LOD and LOQ were calculated based on the Where σ is the standard deviation of the response and the slope of the calibration curve. $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$, where σ is the standard deviation of the response, and S is the slope of the calibration curve.

Precision of the Method:

Precision of Instrument: Instrument precision was assessed by plotting a working standard solution of Opuntiol 6 times (100 ng/spot) and calculating %RSD (relative standard deviation). As per ICH guidelines, the %RSD values must be <2%.

Repeatability: The method's intraday precision was determined by analyzing 6 identical aliquots (100 ng/spot) within the day. The % RSD was <2%, indicating excellent repeatability.

Intermediate Precision: The reproducibility of the method was evaluated by analyzing sample aliquots (100 ng/spot) on three consecutive days at varied concentrations. The %RSD value was within permissible limits, suggesting the method's reproducibility.

Robustness of the Method: The robustness of the developed analytical method was confirmed by studying the effect of small, controlled variations in solvent composition (± 1), chamber saturation duration, and solvent volume. The impact of these variations on analytical results was evaluated, and the corresponding %RSD values were calculated. Values <2% suggested the robustness of the method.

Accuracy/Recovery: The accuracy of the developed method was assessed by conducting recovery trials at three levels (80, 120, and 150 ng/spot of the target concentration).

Recovery trials were conducted in triplicate. Known amounts of Opuntiol in-house working standard were added to the previously prepared ethyl acetate extract, and the recovery rates were evaluated. % recovery was calculated directly from the peak areas with the formula;

$$\% \text{ Recovery} = \frac{\text{Peak area of the spiked sample}}{\text{Peak area of the standard}} \times 100$$

RESULTS AND DISCUSSION:

Method Development and Optimization: In the present investigation, a novel densitometric HPTLC method was developed and validated for the qualitative and quantitative estimation of Opuntiol isolated from the fruits of *Opuntia elatior* Mill. HPTLC is widely used for phytochemical analysis because of its simplicity, cost-effectiveness, minimal solvent consumption, and ability to analyze multiple samples simultaneously. The development of a reliable analytical method is essential for the standardization and quality control of herbal medicines, especially for bioactive phytoconstituents such as Opuntiol, which possess significant pharmacological activities including antioxidant, anti-inflammatory, and anticancer properties.

Mobile Phase Optimization: Optimizing the mobile phase is critical for achieving proper chromatographic resolution in HPTLC analysis. The migration behavior of an analyte depends primarily on the polarity of the solvent system and the interaction between the analyte, the stationary phase, and the mobile phase. In the present study, several solvent systems consisting of ethyl acetate, water, formic acid, glacial acetic acid, methanol, toluene, and n-hexane were investigated in various proportions to obtain optimal chromatographic separation of Opuntiol¹⁸⁻¹⁹ **Supplementary Fig. 9, 10, 11, 12, 13, 14.**

Initially, solvent systems composed mainly of ethyl acetate, water, formic acid, and glacial acetic acid produced relatively high R_f values (>0.75), indicating excessive polarity of the mobile phase. Such high R_f values may lead to poor separation and unreliable densitometric quantification. To overcome this limitation, n-hexane was introduced to decrease the polarity of the solvent system.

The gradual increase in the proportion of n-hexane resulted in a progressive reduction of the Rf value **Table 1**, Supplementary **Fig. 15, 16, 17, 18, 19**. Ultimately, the solvent system consisting of ethyl acetate: water: formic acid: glacial acetic acid: n-hexane (7:1:1.1:1.1:5 v/v) produced a well-resolved, compact, and symmetrical spot of

Opuntiol with an Rf value of 0.42 ± 0.002 , which lies within the ideal Rf range (0.2–0.8) recommended for reliable chromatographic analysis. This optimized solvent system provided excellent separation and reproducible chromatographic behavior **Fig. 4**.

Supplementary Figures:

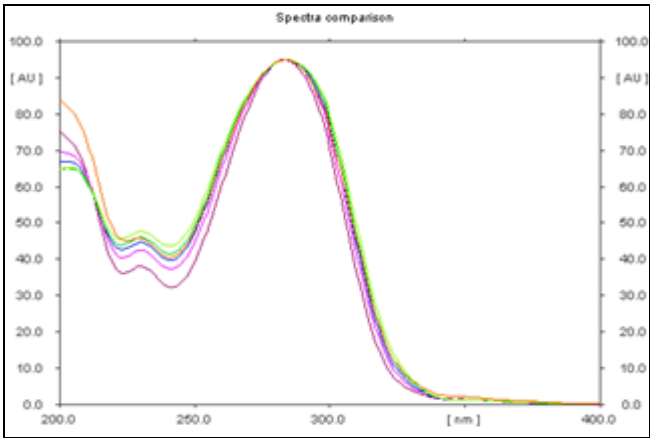


FIG. 1: DENSITOGAM INDICATING PEAK PURITY FOR RF 0.42

TABLE 1: PEAK PURITY TABLE FOR RF 0.42

Track	Rf	Assigned Substance	Max. Signal	Display	r(s,m)	r(m,e)	Purity
1	0.42	OPN	853 AU @ 283 nm		0.999884	0.999831	97%
2	0.42	OPN	925 AU @ 283 nm		0.999618	0.999611	97%
3	0.42	OPN	943 AU @ 284 nm		0.99956	0.999498	97%
4	0.42	OPN	951 AU @ 284 nm		0.999441	0.999384	97%
5	0.42	OPN	954 AU @ 284 nm		0.999545	0.999436	97%
6	0.42	OPN	736 AU @ 283 nm		0.999958	0.999889	97%

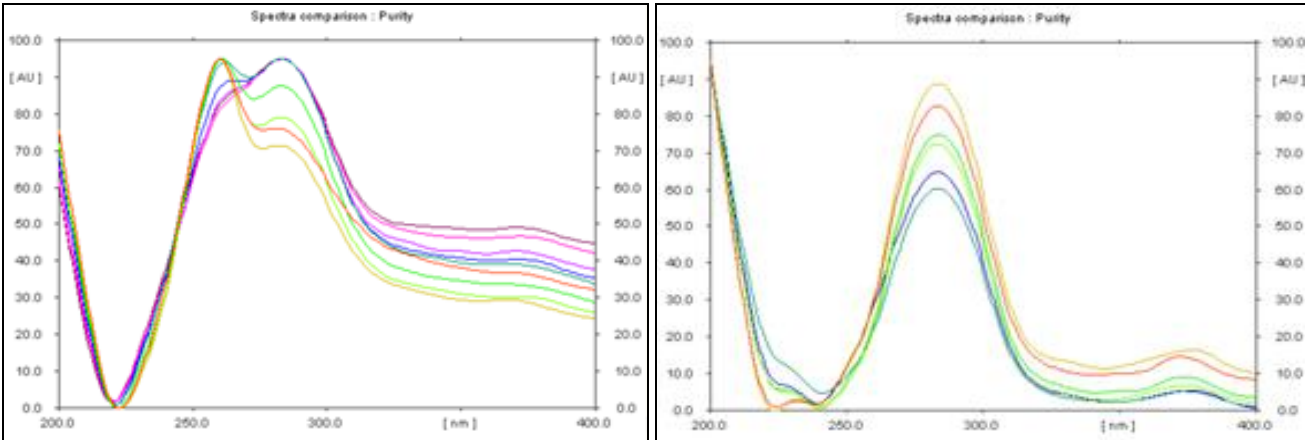


FIG. 2: DENSITOGAM INDICATING PEAK PURITY FOR RF 0.42

TABLE 2: PEAK PURITY TABLE FOR RF 0.42

Track	Rf	Assigned Substance	Max. Signal	Display	r(s,m)	r(m,e)	Purity
1	0.42	OPN	99 AU @ 259 nm		0.999975	0.999898	97%
2	0.42	OPN	71 AU @ 259 nm		0.999924	0.999952	97%
3	0.42	OPN	51 AU @ 260 nm		1	0.999941	97%
4	0.42	OPN	67 AU @ 284 nm		1	0.999936	97%
5	0.42	OPN	84 AU @ 284 nm		0.99997	0.999947	97%
6	0.42	OPN	46 AU @ 283 nm		0.999983	0.999997	97%

Purity Assessment and Characterization Data:

FTIR:

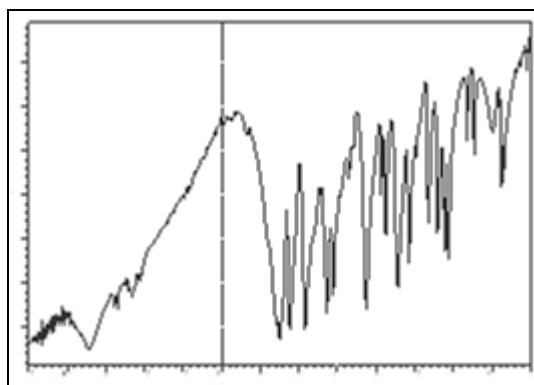


FIG. 3: FT-IR SPECTRA OF OPUNTIOIOL

^1H and ^{13}C NMR:

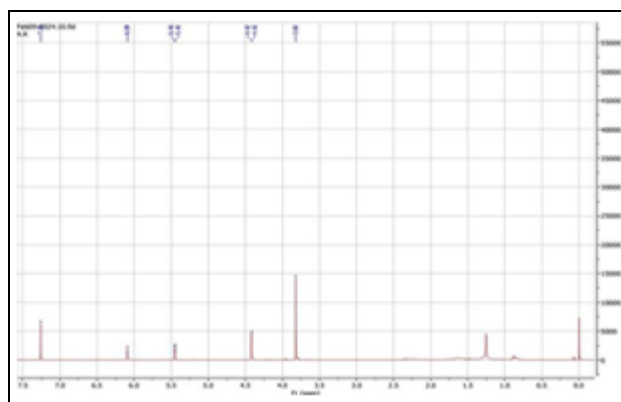


FIG. 4: ^1H -NMR OF OPUNTIOIOL

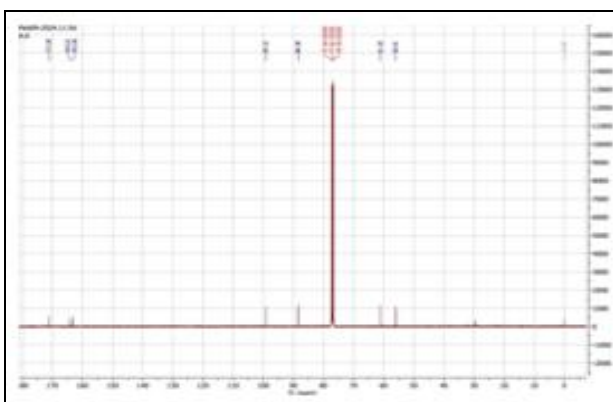


FIG. 5: ^{13}C -NMR OF OPUNTIOIOL

GC-MS:

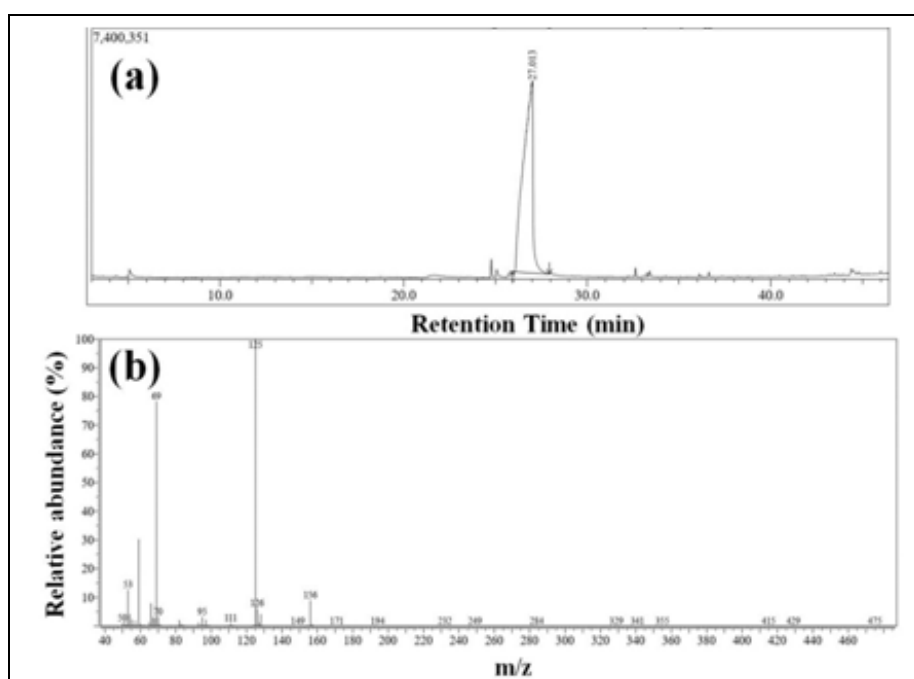


FIG. 6: (A) GC PROFILE AND (B) MASS SPECTRUM OF OPUNTIOIOL

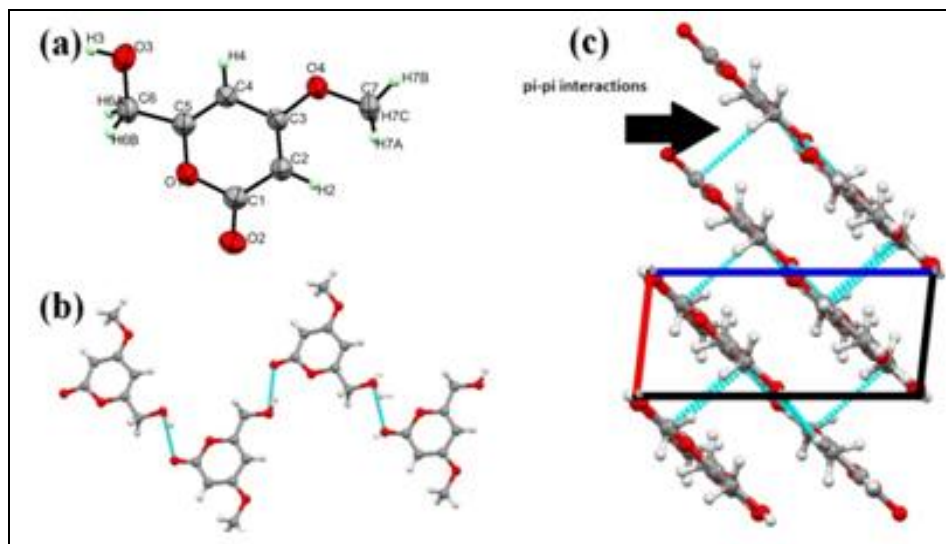
SC-XRD Analysis and DSC:

FIG. 7: (A) SCHEMATIC MOTIF OF OPUNTIOL CRYSTAL STRUCTURE (B) PLANER OPUNTIOL MOLECULES WITH INTERMOLECULAR HYDROGEN BONDING REPRESENTED BY DASHED CYAN LINE (C) π - π INTERACTIONS BETWEEN STACKED MOLECULES

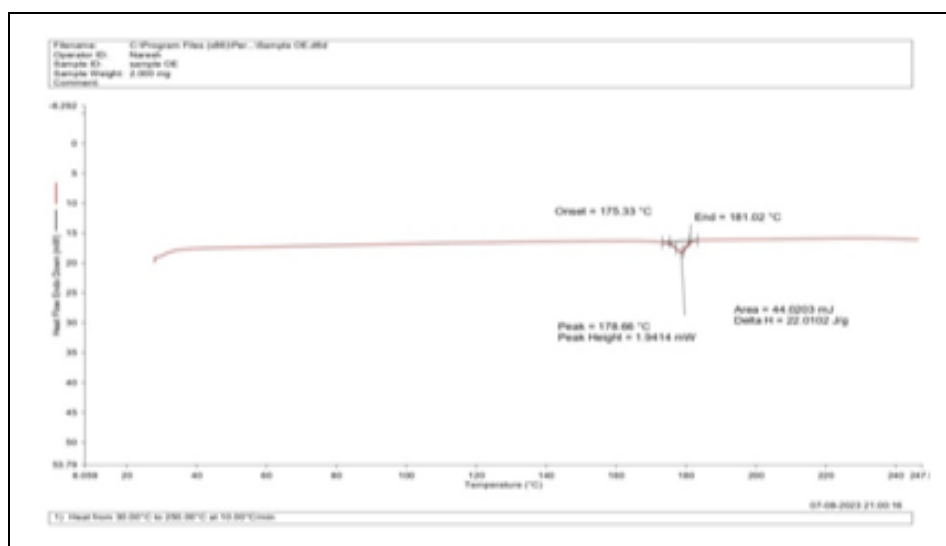


FIG. 8: DIFFERENTIAL SCANNING CALORIMETRY OF OPUNTIOL

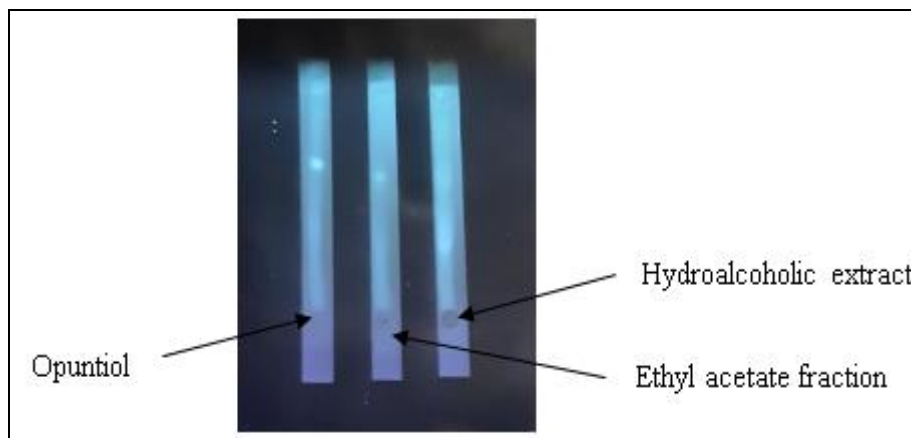
Mobile Phase Optimization by TLC:

FIG. 9: TLC OF HYDROALCOHOLIC EXTRACT, ETHYL ACETATE FRACTION AND OPUNTIOL

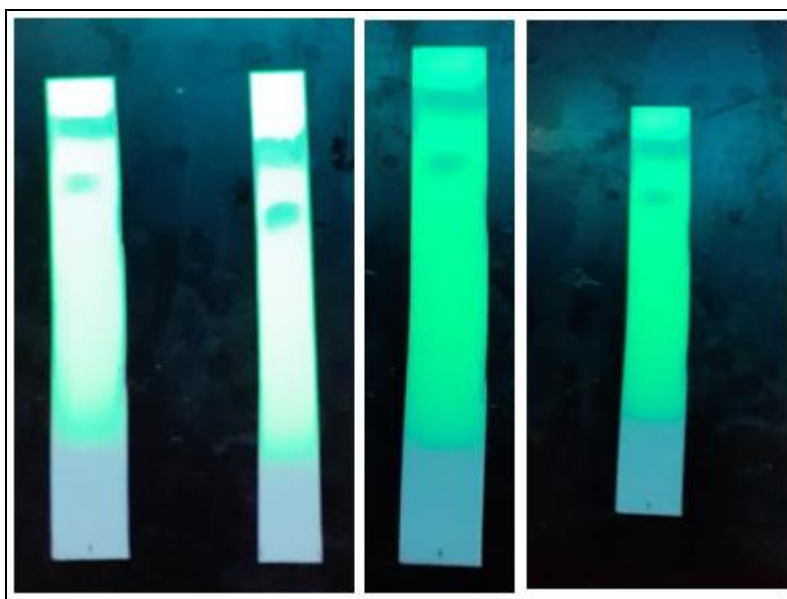


FIG. 10: TLC OF OPUNTIOL

The TLC trials were done for mobile phase optimization. The above mentioned mobile phase was tried at least 4 times for pure Opuntiol but Rf value was 0.78, 0.77, 0.78, and 0.79, Rf value

calculated using the formula. Therefore there was need to optimize the mobile phase for the achievement of ideal Rf value.

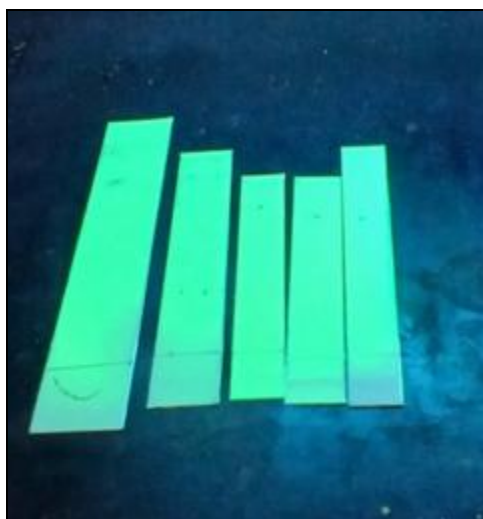


FIG. 11: TLC OF OPUNTIOL

The mobile phase used was Ethyl acetate: water: formic acid: acetic acid (100:26:11:11) which is generally used for flavonoid detection.

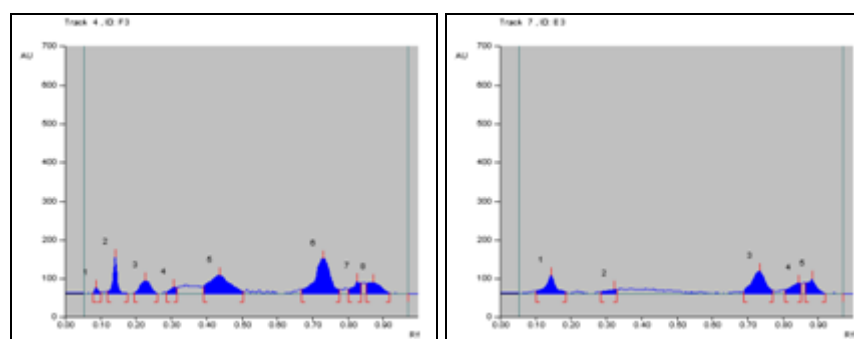


FIG. 12: HPTC CHROMATOGRAM OF ETHYL ACETATE FRACTION

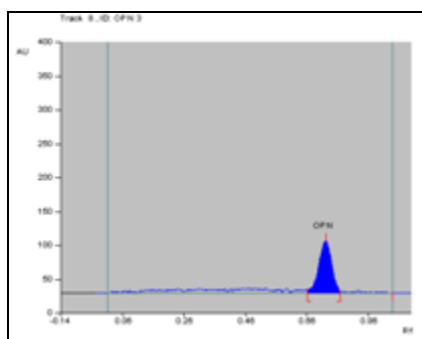


FIG. 13: HPTLC CHROMATOGRAM OF PURIFIED OPUNTIOL

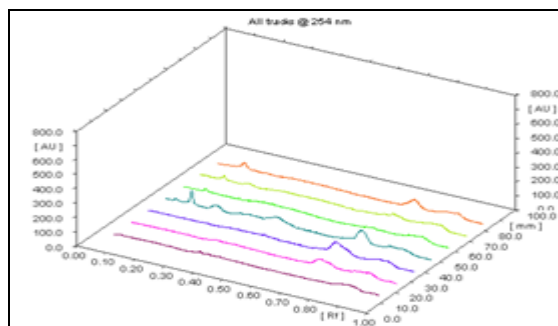


FIG. 14: DENSITOGAM OF ALL SAMPLES ALONG WITH BLANK (1-TRACK)

From the results of TLC, HPTLC was performed for optimization of mobile phase, further the details of the HPTLC chromatogram and densitogram is provided of 120ng/spot concentration.

HPTLC Trials: Total 6 trials were performed for optimization of ideal mobile phase by using HPTLC.

Trial 1: Ethyl acetate: water: formic acid: acetic acid (10:2.6:1.1:1.1); Rf value = 0.83.

By using same mobile phase concentration single HPTLC run also carried, the photograph is given below:

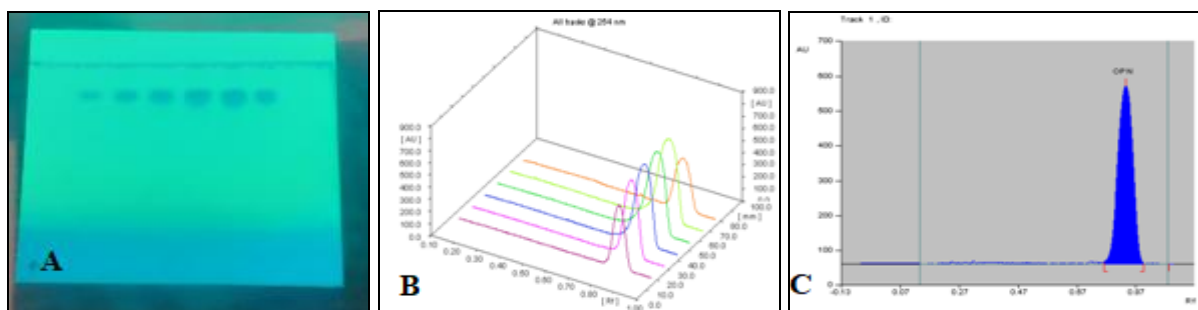


FIG. 15: A-HPTLC CHROMATOGRAM OF OPUNTIOL. (Concentration 1000 ppm) Sample application volume per track: 2.0 µl, 4.0 µl, 6.0 µl, 8.0 µl, 10.0 µl, 5.0 µl) B: HPTLC densitogram of Opuntiol, C: HPTLC chromatogram of Opuntiol

Trial 4: Ethyl acetate: water: formic acid: acetic acid: n-hexane (8:1:1.1:1.1:2); Rf value = 0.72.

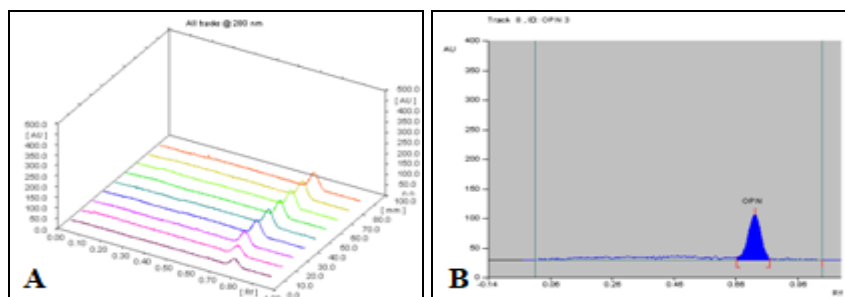


FIG. 16: A- HPTLC DENSITOGAM OF OPUNTIOL AT 10-120 ng/ SPOTCONCENTRATION. B-HPTLC CHROMATOGRAM OF OPUNTIOL AT 120NG/SPOT CONCENTRATION

Trial 5: Ethyl acetate: water: formic acid: acetic acid: n-hexane (8:1:1.1:1.1:3); R_f value = 0.61.

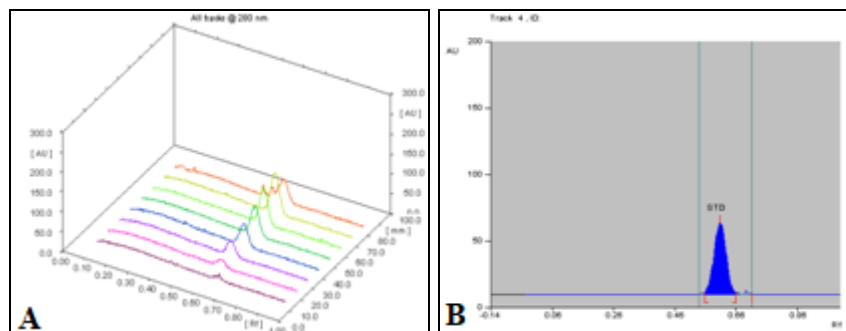


FIG. 17: A- HPTLC DENSITOGAM OF OPUNTIOI AT 10-120 ng/SPOT CONCENTRATION. B- HPTLC CHROMATOGRAM OF OPUNTIOI AT 120ng/SPOT CONCENTRATION

Trial 6: Ethyl acetate: water: formic acid: acetic acid: n-hexane (8:1:1.1:1.1:4); R_f value = 0.56.

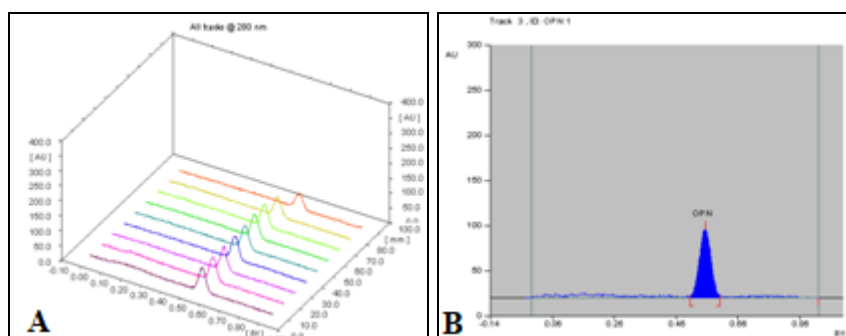


FIG. 18: A- HPTLC DENSITOGAM OF OPUNTIOI AT CONCENTRATION 10-120 ng/SPOT. B-HPTLC CHROMATOGRAM OF OPUNTIOI AT 120 ng/SPOT CONCENTRATION

Trial 7: Ethyl acetate: water: formic acid: acetic acid: n-hexane (7:1:1.1:1.1:5); R_f value = 0.42.

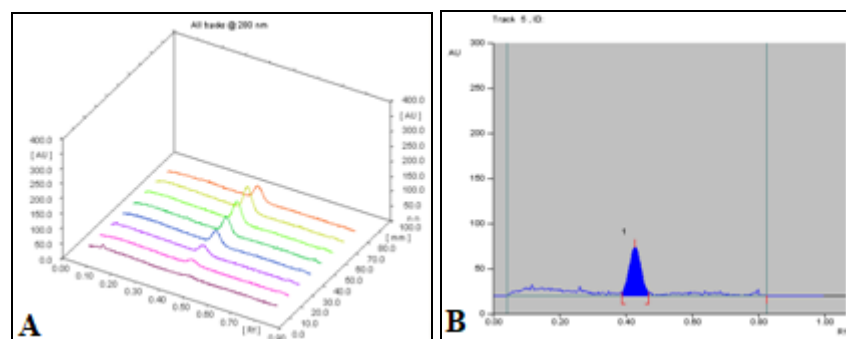


FIG. 19: A- HPTLC DENSITOGAM OF OPUNTIOI AT CONCENTRATION 10-120 ng/SPOT. B-HPTLC CHROMATOGRAM OF OPUNTIOI AT 120 ng/SPOT CONCENTRATION

Linearity and Range: The linearity of the developed HPTLC method was evaluated over the concentration range of 10–120 ng/spot, which demonstrated a directly proportional relationship between the peak area and concentration of Opuntiol. The calibration curve exhibited a high correlation coefficient ($r = 0.9956$), indicating excellent linearity within the studied range. Such strong linear correlation confirms that the detector response is directly proportional to the analyte concentration, enabling accurate quantification of

Opuntiol in plant extracts and pharmaceutical formulations.

The linearity range selected in the present study is suitable for the routine analysis of phytoconstituents at low concentrations in plant matrices. The results therefore demonstrate that the developed method is capable of providing reliable quantitative measurements over a wide concentration range **Table 3**.

TABLE 3: LINEAR REGRESSION ANALYSIS OF CALIBRATION CURVES FOR OPUNTIAL (n=3)

Sample (In-house standard)	Run	Rf (mean $\pm$ SD)	Linearity range (ng/spot)	Regression equation	Slop (mean $\pm$ SD)	Intercept (mean $\pm$ SD)	Correlation coefficient (r ²)
Opuntial	1	0.425 $\pm$ 0.002	10-120	y = 21.15x + 78.794	21.120 $\pm$ 0.116796	98.037 $\pm$ 18.54303	R ² = 0.997
Opuntial	2	0.425 $\pm$ 0.002	10-120	y = 20.992x + 115.79	21.120 $\pm$ 0.116796	98.037 $\pm$ 18.54303	R ² = 0.9966
Opuntial	3	0.425 $\pm$ 0.002	10-120	y = 21.22x + 99.529	21.120 $\pm$ 0.116796	98.037 $\pm$ 18.54303	R ² = 0.9916

Sensitivity of the Method (LOD and LOQ): The sensitivity of the analytical method was assessed by calculating the limit of detection (LOD) and limit of quantification (LOQ) using the standard deviation of the response and slope of the calibration curve. The LOD and LOQ values were

found to be 10.14 $\pm$ 3.04 ng/spot and 30.74 $\pm$ 9.24 ng/spot, respectively, (**Table 4**). These low detection limits indicate that the developed HPTLC method possesses adequate sensitivity for detecting trace quantities of Opuntial²⁰⁻²².

TABLE 4: VALIDATION PARAMETERS: PRECISION, LOD AND LOQ (N=6)

Parameters	Opuntial
Instrument Precision (% RSD, n=6)	0.88
Repeatability/intraday (% RSD, n=6)	1.08
Intermediate precision/interday (% RSD, n=6)	1.45
LOD (ng/spot)	10.14 $\pm$ 3.04
LOQ (ng/spot)	30.74 $\pm$ 9.24

The ability to detect such low concentrations is particularly important in phytochemical analysis, where marker compound concentrations may vary significantly across plant species, geographical origin, harvesting season, and extraction conditions. Therefore, the developed method can serve as a useful analytical tool for phytochemical screening and quality control studies.

Precision of the Method: Precision is an important parameter in analytical method validation because it reflects the reproducibility and reliability of the analytical procedure. In the present study, precision was evaluated in terms of instrument precision, repeatability (intra-day precision), and intermediate precision (inter-day precision). Instrument precision was determined by repeatedly applying the same concentration of Opuntial standard solution and scanning the developed spot six times. The obtained %RSD value of 0.88% indicates excellent instrumental repeatability.

Similarly, intra-day and inter-day precision studies produced %RSD values of 1.08% and 1.45%, respectively, (**Table 4**). These values are well below the acceptable limit of 2% recommended by ICH guidelines, confirming the high precision and reproducibility of the developed HPTLC method. The low variability observed in precision studies indicates that the method produces consistent, reliable analytical results under normal laboratory conditions²³.

Robustness of the Method: Robustness testing was conducted to assess the analytical method's reliability under small, deliberate variations in experimental conditions. Minor changes were introduced in solvent composition, chamber saturation time, and solvent volume. The resulting %RSD values remained within the acceptable limit of $\leq 2\%$, indicating that the chromatographic performance of the method was not significantly affected by these small variations **Table 5**.

TABLE 5: ROBUSTNESS PARAMETERS OF THE METHOD AT 100 NG/SPOT, (N=3)

Working standard	Parameters	Area, (Mean $\pm$ SD)	% RSD
Opuntial	Composition of mobile phase EA:W:FA:GAA:H (70:10:11.1:11.1:50), EA:W:FA:GAA:H (70.5:10:11.1:11.1:45.5)	2340.017 $\pm$ 21.41, 2348.133 $\pm$ 24.12	0.91, 1.02
Opuntial	Chamber saturation time 20 min, 25 min	2367.85 $\pm$ 16.68, 2291.97 $\pm$ 33.67	0.70, 1.46
Opuntial	Volume of the mobile phase 15.2, 14.9	2351.18 $\pm$ 14.77, 2346.97 $\pm$ 20.11	0.62, 0.85

The robustness of an analytical method is essential for routine laboratory applications, as slight operational variations are inevitable in day-to-day analysis. The results obtained in the present study demonstrate that the developed HPTLC method is sufficiently robust and reliable for routine analysis of Opuntiol^{24, 25}.

Accuracy/Recovery: The accuracy of the proposed method was assessed by evaluating triplicate trials

at three different concentrations: 80, 120, and 150 ng/spot. A known concentration of Opuntiol was added to the previously determined ethyl acetate extract.

The percentage mean recoveries were found to be accurate with <5% deviation, and % RSD values lie between 3-8 %, indicating the method's excellence in all the 3 runs as shown in **Table 6**.

TABLE 6: RECOVERY STUDY OF OPUNTIOIOL

Theoretical Concentration (ng/spot)	Run 1	Run 1		Run 2	Run 2		Run 3	Run 3	
Theoretical Concentration (ng/spot)	% Recovery	Mean % Recovery, SD	% RSD	% Recovery	Mean % Recovery, SD	% RSD	% Recovery	Mean % Recovery, SD	% RSD
80	98.58	102.01± 3.90	3.82	87.91	96.51 ± 7.64	7.91	91.81	97.85 ± 5.27	5.39
120	106.26	102.01± 3.90		102.50	96.51 ± 7.64		101.57	97.85 ± 5.27	
150	101.18	102.01± 3.90		99.13	96.51 ± 7.64		100.16	97.85 ± 5.27	

Previous chromatographic investigations of *Opuntia* species have mainly focused on qualitative phytochemical profiling of crude extracts rather than the quantitative estimation of specific marker compounds. For example, Patel *et al.* reported HPTLC fingerprinting of alcoholic extracts of *Opuntia elatior* fruits using toluene:ethyl acetate:acetic acid as the mobile phase, which resulted in multiple bands corresponding to different phytoconstituents¹⁷.

Similarly, other studies have reported HPTLC analysis for general phytochemical characterization of *Opuntia* extracts. In contrast, the present study focuses specifically on the development of a validated densitometric HPTLC method for the quantitative estimation of Opuntiol, a pharmacologically important flavonol. The optimized solvent system developed in this study provided a well-resolved single band corresponding to Opuntiol, enabling precise densitometric quantification.

This represents a significant improvement over previously reported qualitative fingerprinting methods. Standardization of herbal medicines requires reliable analytical methods capable of identifying and quantifying marker compounds. The validated HPTLC method developed in the

present study provides a simple, cost-effective analytical tool for routine quality control of *Opuntia* extracts. The method can facilitate batch-to-batch consistency of herbal formulations and support the development of Opuntiol-based phytopharmaceutical products products.

CONCLUSION: The present study successfully developed and validated a rapid, sensitive, and reliable densitometric HPTLC method for the quantitative estimation of Opuntiol isolated from *Opuntia elatior* Mill.

The developed method demonstrated excellent linearity, precision, sensitivity, and robustness, and recovery in accordance with ICH guidelines.

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