ORIGINAL ARTICLE

In vitro Antioxidant Activity and Stability Indicating High-performance Thin-layer Chromatographic Method for Ximenynic Acid in *Santalum album* Seed Extract

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Abstract

Introduction: Due to emergent concern about the unhealthy consequences of chemicals in the health industry, the interest toward natural and herbal substances have been growing every day; though, regrettably they possess several quality control issues. In this study, the antioxidant effect of *Santalum album* seed extract was evaluated. Furthermore, discover effortless, accurate, responsive, and stability-indicating high-performance thin-layer chromatographic (HPTLC) assay method for the detection and quantification of ximenynic acid in *S. album* seed extract. **Materials and Methods:** Antioxidant activity was evaluated by 2, 2-diphenyl-1, 1-picrylhydrazyl (DPPH) radical scavenging method. The HPTLC method contains aluminum plates precoated with silica gel 60 F254 as a stationary phase. The mobile phase was a combination of toluene: chloroform:methanol: formic acid (2:5:0.3:0.3 v/v/v/v). Densitometric analysis of ximenynic acid was carried out in the absorbance mode at 550 nm using Camag thin-layer chromatography scanner-3. **Results:** Antioxidant potential was observed in DPPH scavenging assay (EC = 4.0 ± 0.02 mg/mL) and by *S. album* seed extract. The HPTLC method was validated as per the ICH guidelines for specificity, precision, linearity, robustness, and accuracy. The method was established to give dense and symmetrical band for ximenynic acid at retention factor 0.45 ± 0.02. The repeatability of the method was found to be 1.25 relative standard deviations and recovery values from 99.94 to 100.10% for ximenynic acid. **Conclusion:** These findings indicate that *S. album* seed extract may have antioxidant potential. Statistical analysis confirmed that the projected method is repeatable, selective, and accurate for estimating the content of ximenynic acid. Since the projected mobile phase successfully resolves the ximenynic acid, this HPTLC method can be useful for identification and quantitation of these phytochemicals in herbal extracts and pharmaceutical dosage form.

Key words: Antioxidant activity, high-performance thin-layer chromatographic, *Santalum album*, ximenynic acid

INTRODUCTION

Herbal medicine plays an important role in the health care of many urbanized, developing countries. The use of herbal products is increasing worldwide due to the distinct advantages.[1] Nearly 80% of African and Asian population depend on traditional medicines for their primary health care.[2] These medicines are readily available in the market from health food stores without prescriptions.[3] In general, it is believed that the risk associated with herbal drugs is very less, but reports on serious reactions are

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indicating the need for development of effective marker systems for identification of the individual components.[4] Consistency, stability and quality control for herbal drugs is possible, but difficult to achieve. Further, the guideline of these drugs is not standardized across all countries. There is variation in the methods used transverse medicine systems and countries in achieving stability and quality control.[5,6]

Santalum album L. (*Santalaceae*), namely Sandalwood, given great respect as "Green Gold," is a traditional hemiparasitic tree and has an extensive record in Indian religious rituals and traditional Chinese medicine.[7] Their essential oil (sandalwood oil) is broadly used in the cosmetic, perfumery, and aromatherapy industries and has been reported to have diverse biological properties such as antiviral, anticarcinogenesis, and antitumor effects.[8] The herb is stated to have α-sangallo, β-sangallo, α-standalone, β-standalone, α-santalalean, β-santalalean, α-curcumene, and tricycloekasantal.[9,10] The seed oil of *S. album* is shadowy red viscid fixed oil containing santalbic acid (or ximenynic acid) and stearolic acid (9-octadecynoic acid).[7,11,12] Ximenynic acid, octadeca-11-trans-en-9-ynoic acid, is one of the few acetylenic fatty acids taking place at higher levels in plant seed oils.[13] In universal, ximenynic acid exhibits many biological activities and pharmacological effects, including antibacterial, antifungal, and anti-inflammatory activities.[14,15]

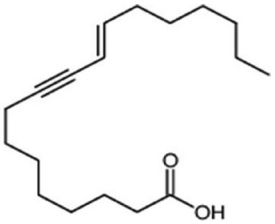
In a present study, the quality assessment *S. album* seed extract was carried out by doing *in vitro* antioxidant activity with their fingerprinting using thin-layer chromatography (TLC). However, in the current literature antioxidant potential and high-performance TLC (HPTLC) method were not stated. Looking at the advantages of HPTLC method, such as, is an inexpensive analytical apparatus because of its less operating price, high sample throughput, and need for minimum sample clean-up. It was thought, needful to develop HPTLC method for analysis of ximenynic acid in formulations.

MATERIALS AND METHODS

Chemicals and reagents

Standard ximenynic acid was purchased from Sami Lab India and used without additional purification, due to its high purity, at least 99.12%. The molecular structure of the ximenynic acid is given in Table 1. 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyl toluene (BHT), and Vitamin C were purchased from Merck Co. (Mumbai, India). Purified water (Millipore Equipment, France) was used to prepare the stock solution. Analytical-reagent grade solvents such as toluene, chloroform, formic acid, anisaldehyde reagent, and methanol were obtained from Merck Ltd., India. As stationary phases, the following plate (Merck) was used: HPTLC aluminum plates precoated with silica gel $60F_{254}$ (20 cm × 20 cm, 0.2 mm thickness).

Table 1: Chemical properties of ximenynic acid[16]

Molecule	Ximenynic acid
CAS number	557-58-4
Chemical formula	$C_{18}H_{30}O_2$
IUPAC name	(E)-octadec-11-en-9-ynoic acid
Representation	
Molecular weight	278.43
Appearance	White powder

Plant material

The seed extract of *S. album* (Batch number: C182203) was obtained from Sami Lab India. Which are manufactured in November 2018 and expired in October 2019.

DPPH radical scavenging activity

The free radical scavenging capacity of seed extract of *S. album* was determined using the established DPPH method. To obtain an indication of the antioxidant activity of *S. album* seed extraction, 5 mL of a freshly prepared 0.004% of DPPH in methanol was mixed with 50 µl of different concentration 5, 10, 15, 25, 35, and 50 mg/mL of each sample and the absorbance of each dilution after 30 min was measured at 517 nm. BHT and Vitamin C were the antioxidant used as a positive control.[17]

All tests were performed in triplicate and the methanol was used as a blank solution. The percentage DPPH reduction (or DPPH radical scavenging capacity) was calculated as:

$$\% \text{ Reduction} = (\text{Abs DPPH} - \text{Abs Dil.})/\text{Abs DPPH} \times 100$$

Whereby:
Abs DPPH = Average absorption of the DPPH solution,
Abs Dil. = Average absorption of the three absorption values of each dilution.

Statistical analysis

The statistical analyses were performed using the Prism software. Values were compared to control using analysis of variance (ANOVA) followed by Tukey multiple comparison test.

Simultaneous quantification of ximenynic acid using HPTLC

Preparation of standard solution of ximenynic acid

Standard stock solution was prepared by dissolving 30 mg of ximenynic acid into 100 mL volumetric flask. Added 70 mL of methanol and sonicated to dissolve. Make up the volume to 100 mL with methanol and mixed well, which yields a solution of concentration 300 ppm and was used as working standard for the analysis.

Preparation of test solution for analysis

Weighed and dissolved *S. album* seed extract equivalent to 30 mg of ximenynic acid into 100 mL volumetric flask. Added 70 mL of methanol and sonicated to dissolve. Make up the volume to 100 mL with methanol and mixed well.

Chromatographic conditions and instrumentation

A Camag HPTLC system consisting of Linomat V automatic sample applicator with Camag TLC Scanner 3 and Camag WinCAT software was used for the detection and quantification of ximenynic acid in the formulations. The samples were speckled in the form of band of width 6 mm with CAMAG 100 µL syringe on precoated silica gel $60F_{254}$ aluminum plate (20 cm × 10 cm or 10 cm × 10 cm with 0.2 mm thickness) using Linomat 5 applicator CAMAG (Switzerland) built-in with a CAMAG 100 µL syringe. The volume applied on each track was 5 µL. The ascending development was carried out in the mobile phase toluene: chloroform:methanol:formic acid (2:5:0.3:0.3 v/v/v/v) in a CAMAG twin trough chamber (20 cm × 10 cm). The optimized chamber saturation time for the mobile phase was 15 min at room temperature (25 ± 2°C). The length of the chromatogram run was just about 80 mm. After development, plates were dried by dryer and sprayed with anisaldehyde reagent as derivatization reagent. Again the plates were air-dried after development in a current of air. The densitometric scanning was performed using CAMAG TLC scanner-3 (Switzerland) operated by win CATS software V 1.4.3.6336 at 550 nm after derivatization. The slit dimension was 5 mm × 0.45 mm with a scanning speed of 20 mm/s. The HPTLC chromatographic condition are shown in Table 2. The amount of ximenynic acid present in the samples was evaluated by peak area with linear regression.

Calibration curve and linearity

The acceptability of linearity data is often evaluated by examining the correlation coefficient and intercept of the linear regression line for the response versus concentration plot. The stock solution of ximenynic acid was diluted to five different concentrations between 50% and 150% of working concentration. The plate was developed and analyzed to engender the calibration equation for quantification of ximenynic acid in samples. The curves demonstrated coefficient of correlation (r^2) ≥0.9996.

Table 2: Chromatographic condition

Chromatographic condition	Details
Saturation time	15 min
Plate activation	At 105°C for 5 min
Mobile phase chamber	Twin through chamber (10×10 and 20×10)
Wavelength detection	550 nm after derivatization
Derivatization reagent	Anisaldehyde reagent
Photo documentation	At white R light
Application volume	5 µL
Retention factor	0.45
Type of application	Band type
Room temperature	22°C at the time of experimentation
Storage conditions of sample and STD	At 2–8°C and in dark chamber
Diluent	Methanol

Method validations[18-21]

Validation of the analytical method was done according to the International Conference on Harmonization guideline. The method was validated for specificity, solution stability, recovery, robustness, and precision.

Specificity

Specificity was ascertained by applying 5 µL band of standard, blank, and sample solutions on the HPTLC plates. The bands for ximenynic acid from sample solutions were authenticated by comparing the retention factor (Rf) and spectra of the bands to those of the standards. The peak purity of ximenynic acid was analyzed by comparing the spectra at three different levels, i.e., at peak start, peak apex, and peak end positions of the spot. The sensitivity of measurement was anticipated in terms of the limit of quantification (LOQ) and the limit of detection (LOD). The LOQ and LOD were calculated by the use of equations LOD = 3 × N/B and LOQ = 10 × N/B where N is the standard deviation of the peak area of the drug (n = 3), taken as a measure of noise and B is the slope of the corresponding calibration plot.

Precision

The precision of the developed method was studied by performing system, method, and intermediate precision studies. The sample application and measurement of peak area were resolute by performing six replicate measurements of the same band using a sample solution containing 300 ppm of ximenynic acid.

Solution stability

The sample solution and standard solution were prepared as per the proposed method and subjected to stability study at room temperature for 6 h. The change in response of ximenynic acid in sample solution with respect to time is calculated as an absolute percent difference against initial response.

Robustness

The volume of the mobile phase, polar solvent volume, and saturation time was involved in this study. The effect of these changes on both the Rf values and peak areas was evaluated by calculating the relative standard deviations (RSD) for each parameter.

Table 3: DPPH scavenging activity of extract

Samples	% EC50 (mg/mL)		
	Seed extract (50 mg/mL)	Vitamin C	BHT
1	4.1*	2.2**	2.0**
2	3.8*	2.1**	1.9***
3	3.9*	1.9***	1.9***
Mean	4.0*	2.0***	2.0***

Results are expressed as mean standard error of mean (n=6), one-way analysis of variance followed by Tukey multiple comparison test. *P<0.05, **P<0.01, ***P<0.001, When compared with control groups. DPPH: 2,2-diphenyl-1-picrylhydrazyl, BHT: Butylated hydroxyl toluene

Table 4: Result of linearity of xymenynic acid

Conc. of xymenynic acid (ppm)	Average peak area of xymenynic acid
150	3254
200	4282
300	6245
350	7365
450	9456

Accuracy (recovery)

Accuracy of the method was ascertained by spiking the preanalyzed samples with a known amount of ximenynic acid (80, 100, and 120%). The average percentage recovery was estimated by applying values of peak area to the regression equations of the calibration graph.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. In the DPPH assay, the antioxidants are able to reduce the stable radical DPPH to non-radical form, DPPH-H. The purple-colored alcoholic solution of DPPH radical changes to yellow in the presence of hydrogen-donating antioxidant which could be measured at 517 nm, the activity is expressed as effective concentration EC50, which is the concentration of the sample leading to 50% reduction of the initial DPPH concentration.[17] Table 3 shows the DPPH scavenging activity of *S. album* seed extract, BHT and Vitamin C at different concentrations, as assayed by DPPH (EC50 mg/mL). The effectiveness of antioxidant properties is inversely correlated with EC50 values. If the EC50 value of an extract <10 mg/mL, that is, mean the extract is an effective antioxidant.[22] In this study, the EC50 value of *S. album* seed extract was 5 mg/mL <50 mg/mL this indicates that the samples have effective antioxidant activity.

Method optimization for the HPTLC-densitometric measurements

Like so many other unusual fatty acids, ximenynic acid is a distinguishing constituent of the seed oils of only a few closely related plant families. It comes about in the order *Santalales*, i.e., in the traditional "Santalalean" plant families *Santalaceae*, *Olacaceae,* and *Opiliaceae* and has never been found outside the *Santalales*.[23-25]

Table 5: Result of method precision and intermediate precision study

S. No.	Peak area of xymenynic acid	Assay (% W/W, method precision)	Assay (% W/W, intermediate precision)
1.	6155	100.91	101.10
2.	6258	98.29	100.17
3.	6139	101.09	98.96
4.	6287	98.53	100.23
5.	6123	98.71	100.50
6.	NA	99.09	101.75
Average	6192.4	99.44	100.45
% RSD	1.21	1.25	0.94
Overall % RSD	NA	1.17	

RSD: Relative standard deviations

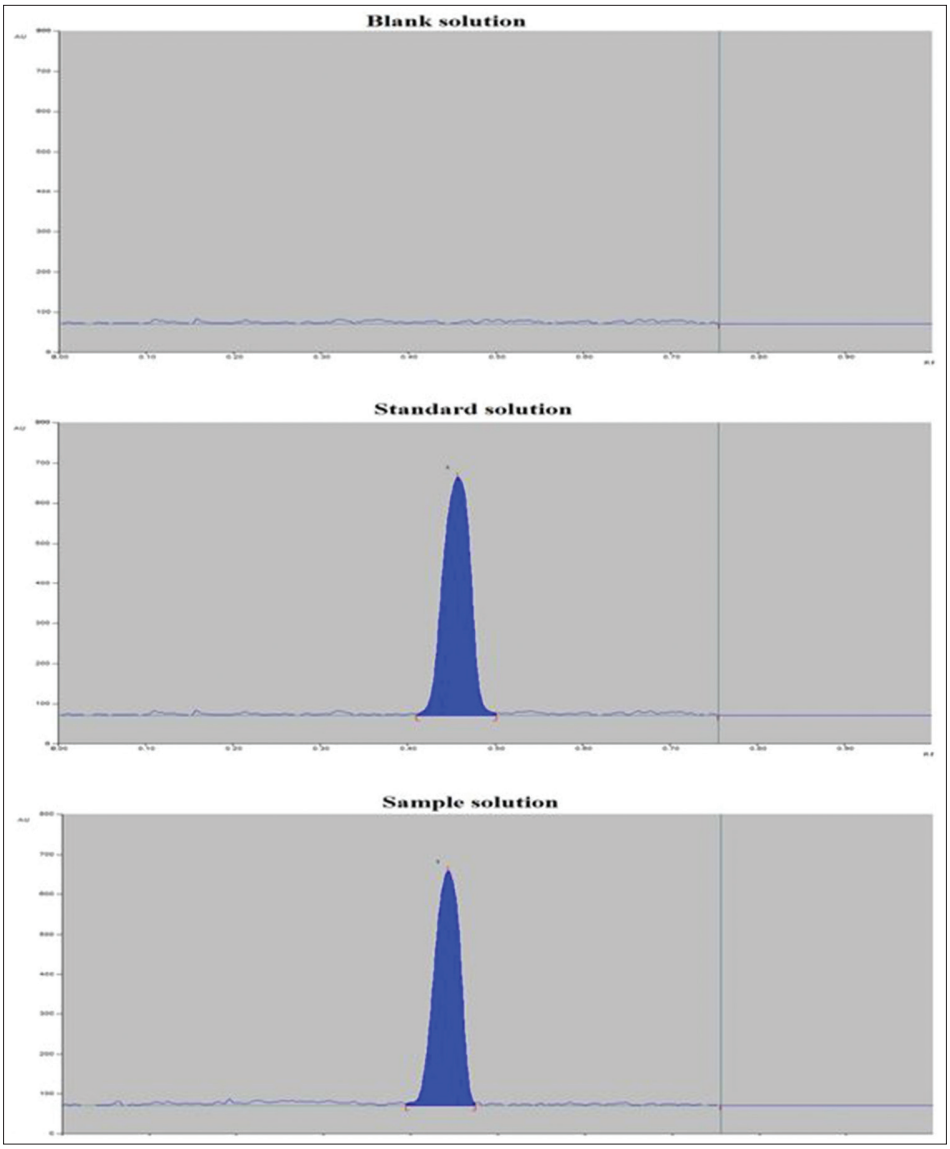


Figure 1: High-performance thin-layer chromatography of blank solution, standard solution, and sample solution

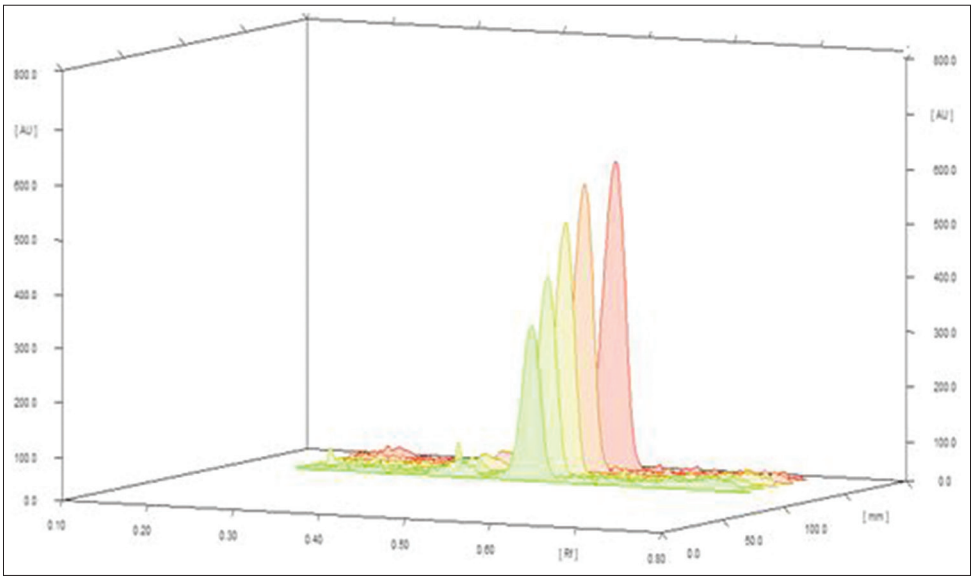


Figure 2: Three-dimensional linearity graph for xymenynic acid

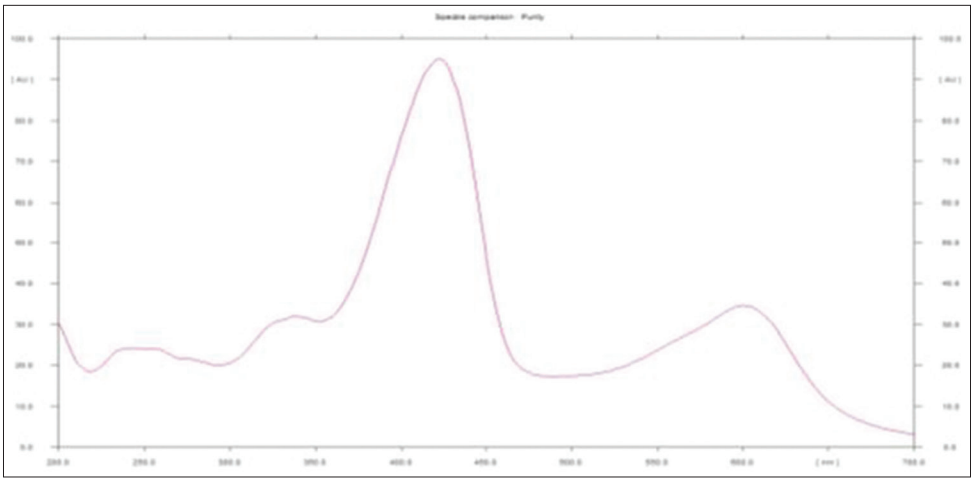


Figure 3: Spectra of xymenynic acid from STD

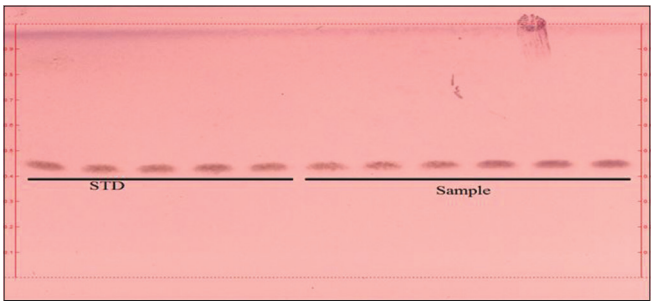


Figure 4: Three-dimensional chromatographs of method precision and intermediate precision study

The HPTLC method was optimized with a sight to develop a stability-indicating assay method. Different composition of the mobile phase for reversed-phase-HPTLC analysis was experimented with a goal to obtain high resolution and reproducible peaks. The required objective was achieved using toluene: chloroform:methanol:formic acid (2:5:0.3:0.3 v/v/v/v) mobile phase. It gave impenetrable, compact, and well-alienated spots of the drug. This mobile phase showed good resolution of ximenynic acid peak from the extract of *S. album*. The wavelength of 550 nm after derivatization was found to be optimal for the highest sensitivity. Sharp and well-defined peaks for the ximenynic acid were obtained at Rf 0.45 ± 0.02. The present method is faster as the time needed for development of plate is reduced considerably to <½ h for chamber saturation. At this wavelength, the ximenynic acid showed optimum response [Figure 1].

Method validation

Calibration curve and linearity

A calibration curve was constructed by plotting peak area against the concentration of ximenynic acid (ppm). The results of linearity are shown in Table 4. They confirm the linearity of the standard curves over the range studied (150–450 ppm). Linear regression of concentration versus peak area plots resulted in an average coefficient of determination (r^2) >0.999. The average equation for calibration curves was y = 20.63x + 135.3. The 3-D chromatographs of all calibration concentrations are shown in Figure 2, respectively.

Table 6: Result of robustness study for xymenynic acid

Robustness parameter	% RSD	Rt	Peak purity
Saturation time (minute)			
14	1.25	0.45	Pass
15	1.36	0.45	Pass
16	1.75	0.45	Pass
Polar solvent volume (Formic acid)			
0.4	1.96	0.44	Pass
0.5	1.25	0.45	Pass
0.6	1.25	0.46	Pass
Mobile phase volume			
9	1.63		Pass
10	1.27		Pass
11	1.96		Pass

RSD: Relative standard deviations

Specificity and sensitivity

The outcome of spectral comparison for ximenynic acid was found to be specific at peak start (S), peak apex (M), and peak-end (E), respectively. The ultraviolet spectra of the standards are presented in Figure 3. The intimacy of peak purity values to one indicates that the spots were only attributed to a single compound. By comparing the photos and chromatograms of the blank solution, standard solution, and sample solution, it was observed that no peak was coeluted with the analyte band from bank solution. LOD and LOQ were found to be 8 ppm and 25 ppm, respectively. Hence, the method was found to be highly sensitive for determination of MIL.

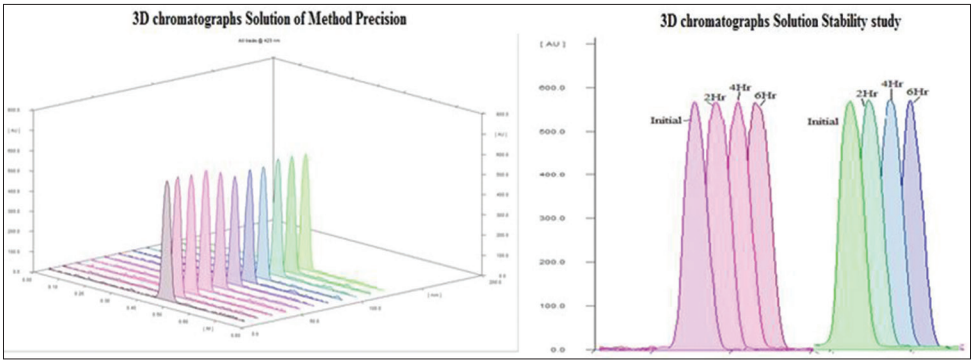


Figure 5: Three-dimensional chromatographs of method precision and solution stability study

Table 7: Result of accuracy study for xymenynic acid

Band	Sample	Recovery levels (%)	STD wt. (spiked)	Amount recover	% recovery	Average % recovery
1.	Sample 1	80	24.13	24.06	99.71	99.94
2	Sample 2	80	24.06	24.05	99.96	
3.	Sample 3	80	24.05	24.09	100.17	
4.	Sample 1	100	30.14	30.16	100.07	100.11
5.	Sample 2	100	30.02	30.18	100.53	
6.	Sample 3	100	30.13	30.05	99.73	
7.	Sample 1	120	36.12	36.13	100.03	100.10
8.	Sample 2	120	36.05	36.17	100.33	
9.	Sample 3	120	36.05	36.03	99.94	

Precision

System, method, and intermediate precision of the developed method were articulated in terms of RSD of the peak area [Figure 4]. The consequences showed that the system, method, and intermediate variations of the results at concentration of 300 ppm for ximenynic acid were within the acceptable range [Figure 5]. The coefficients of variation for system, method, and intermediate precision of the method were found to be <1.21%. The ximenynic acid was also analyzed by two different analysts within the same day, and the results revealed that there is good intermediate precision between analysts [Table 5].

Solution stability

There was no significant divergence in peak area of ximenynic acid (RSD <1.5%) observed on analysis up to 6 h. No decomposition of the drug was observed during chromatogram development. These observations suggest that the drug is stable under the typical processing and storage conditions of the analytical procedure [Figure 5].

Robustness

The results obtained in the new conditions (variation in composition mobile phase, polar solvent volume, and saturation time) were in accordance with the original results, as shown in Table 6, though the Rf varied very slightly (0.45 ± 0.1). The % RSD values for peak area were <1.0 indicating the highly robust nature of the developed method. The low RSD values indicate the robustness of the method [Table 6].

Accuracy (recovery)

The developed method showed high and reliable recoveries at all studied levels. The percentage recovery of ximenynic acid at all three levels was found to be satisfactory [Table 7]. For ximenynic acid, the % recovery was found between 99.94% and 100.10%.

CONCLUSION

The seed extract of *S. album* exhibited antioxidant activity as manifested through DPPH radical scavenging. An extensive literature assessment revealed that the xymenynic acid is currently available for the treatment of skin. In this research HPTLC method for determination of xymenynic acid was developed, as there is no official HPTLC method reported in major pharmacopeias such as USP, EP, JP, BP, and IP. A stability-indicating HPTLC method was developed and validated for the determination of xymenynic acid in seed extract on pre-coated silica gel HPTLC plates using toluene: chloroform:methanol:formic acid (2:5:0.3:0.3 v/v/v/v) as the mobile phase with densitometric detection at 550 nm after derivatization. With virtue of the results obtained, the present method is precise, specific,

accurate stability-indicating assay method. The method can reduce the cost of reagents and time for analysis. It also utilized the advantage of applying numerous sample spots on HPTLC plate, which may be more beneficial for regulatory quality control laboratories particularly to facilitate the post-marketing surveillance program. In addition, the method is economical and not requires certain types of stationary phases.

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