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Development, validation and application of RP-HPLC method for estimation of Xymenynic acid in *Santalum album* seeds extract

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Abstract
A simple, reproducible, feasible and innovative reversed-phase high performance liquid chromatographic method was developed and validated for the quantitative determination of Xymenynic acid present in *Santalum album* L. seeds extract. An extraordinary increase in the demand for skin lightning herbal formulations in the last two decades, and a need has been handled for ensuring quality, safety, and efficacy of herbal skin lightning drugs. High Performance Liquid chromatography with a photo diode array detector at a wavelength of 229 nm using a reversed-phase Zorbax stationary phase has been employed in this study. Isocratic elution is employed using a mixture of methanol- water (60:40v/v). This new method is validated for assay determination of *Santalum album* L. seeds extract, which include accuracy, precision, specificity, linearity, and range. This method expressions enough selectivity, sensitivity, accuracy, precision, and linearity range to satisfy International Conference of Harmonization regulatory requirements. A precise, accurate and reproducible HPLC method has been validated for analysis of xymenynic acid in *S. album* seed extract.

Keywords: *Santalum album* seeds, Xymenynic acid, FTIR, HPLC

Introduction
Indian *Santalum album*, belonging to family Santalaceae, is oldest and important natural source which has medicinal significance. Essential oils which are obtained from *S. album* heart wood are used to treatment and prevention of wide range of diseases and disorders in Ayurveda, Siddha and Unani medicine systems [1]. Every part of the *S. album* has some medicinal and commercial use. *S. album* is a small, medium sized, ever green plant with slim branches 18 m in height and 2.4 m in width. Bark is reddish dark in color; leaves are glabrous, thin, 1.5-8 cm or 1.6-3.2 cm in size [2]. The flowers of *S. album* are small, regular, straw colored, brownish purple, reddish purple or violet in color. Fruit is a drupe, purplish when fully matured and single seeded. Sandal fruits are collected fresh from the tree or as soon as they have fallen on the ground during April-May and September-October [3-6]. In India *S. album* has been cultivated from last 25 centuries, and widely distributed in Karnataka, Andhra Pradesh and Tamil Nadu. *S. album* has ability to grow in different kinds of soils like sand, clay, and laterite. *S. album* is a large source of costliest wood, essential oils obtained from it has been extensively investigated for its chemical constituents. The main constituent of *S. album* oil is santalol [6-11]. Seeds oil of *S. album* contains about 10.07% of oleic acid and 83.95% of Xymenynic acid; the later has large immense use. Xymenynic acid is extracted from fruit kernels of *S. album* [12-14].
Standardization and quality control of herbal products are suffering from big problem, due to many factors like complex form of the herbal products and inability of traditional methods for standardization and quality control of products [11-13]. Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, definite qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility [15].

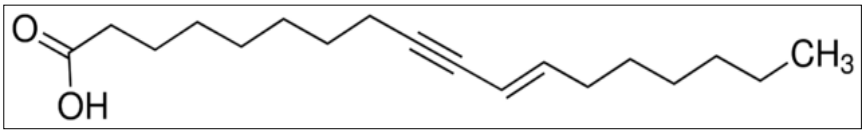


Fig 1: Chemical structure of Xymenynic acid

The aim of this study is to develop and validate HPLC method for determination of xymenynic acid in *S. album* seed extract.

Martials and methods

Chemicals

Analytical grade (AR) solvents such as methanol, chloroform, formic acid and toluene were obtained from Merck Ltd, Bangalore. Standard and sample of xymenynic acid was procured from Sami Labs Ltd. Potency of standard xymenynic acid was 99.38%.

Preparation of standard (STD) solution

The standard solution was prepared by dissolving 20 mg of xymenynic acid in 20 ml methanol in a 25 mL volumetric flask. The resultant solution was sonicated in ultrasonic water bath for 30 min and volume was made up to the mark with methanol. The above solution was then filtered using 0.45µ Syringe filter and was used as standard working solution.

Preparation of the sample solution

The sample solution was prepared by dissolving 20 mg of *S. album* seed extract in 20 ml methanol in a 25 mL volumetric flask. The resultant solution was sonicated in ultrasonic water bath for 30 min and volume was made up to the mark with methanol. The above solution was then filtered using 0.45µ Syringe filter and was used as sample solution.

Mobile phase preparation

The mobile phase was prepared by using methanol and water. About 60 ml of methanol was taken in 100 ml beaker then add 40 ml of water and it was sonicated for 15 minutes using the ultrasonic water bath. The resultant mobile phase was filtered using the vacuum pump.

Chromatographic condition and methods

The chromatographic conditions for analysis of xymenynic acid are mentioned in the table 1 & 2.

Table 1: HPLC System

HPLC System make	Waters
HPLC model	Waters 2998
Software	Waters Empower 3 chromatography software
Pump	Isocratic Pump
Detector	PDA detector
Column	Zorbax C_{18}
Sample injection system	Automatic

Table 2: HPLC Development conditions

Mobile Phase	Methanol: Water (60:40 *v/v*)
Flow rate	1 ml/min
Column temperature	30°C ± 5°C
Sample temperature	25°C ± 5°C
Injection volume	10 µL
Run time	30 min
Detection	PDA at 229 nm

UV Spectrophotometric evaluation

Table 3: UV Spectrophotometric conditions

UV spectrophotometer	Shimadzu
UV spectrophotometer model	Shimadzu UV-1800
Measure mode	Absorbance
Cell path length	1 cm
Wavelength range (nm)	1.0
Scan speed	Medium

Preparation of standard solution

The standard solution was prepared by dissolving 100 mg of xymenynic acid in 100 ml of methanol in a volumetric flask. The resultant solution was sonicated in ultrasonic water bath for 30 min. Then the solution was filtered using 0.45µ Syringe filter. From above solution 5.0 ml transfer into a volumetric flask and dilute up to 50.0 ml with methanol. The resulting solution was used as standard solution.

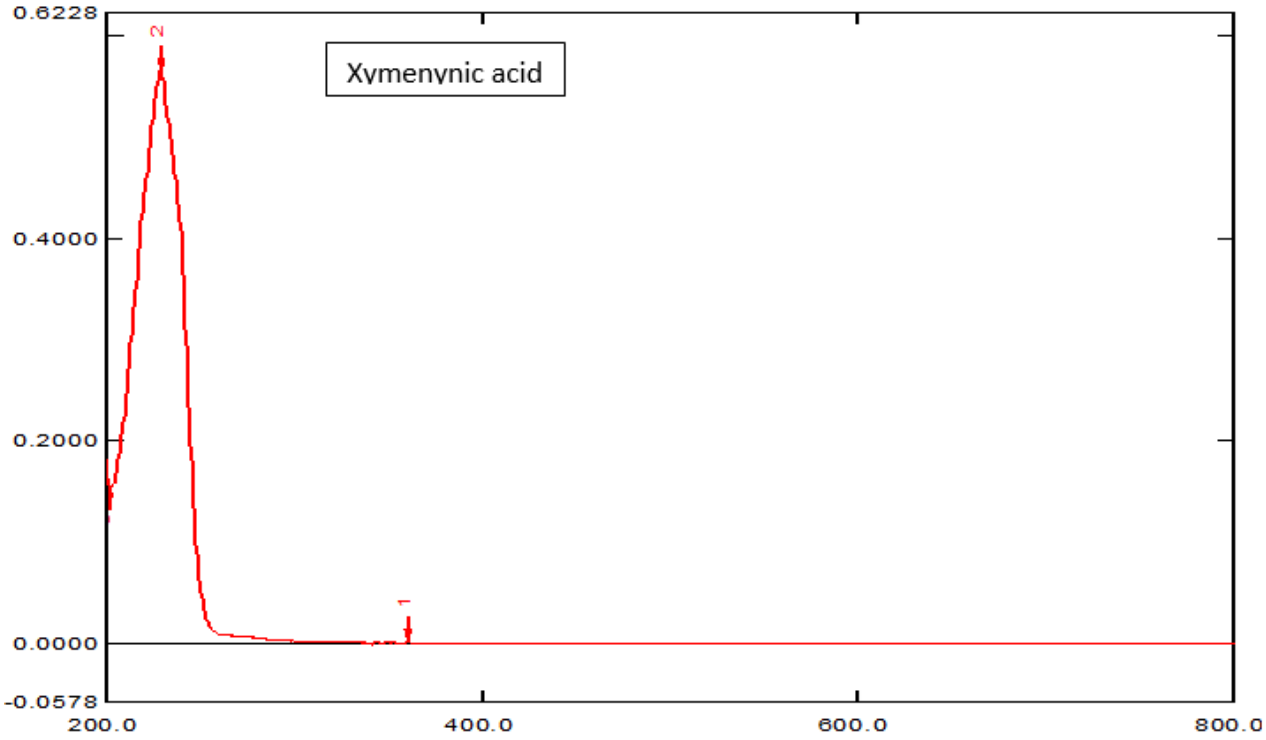
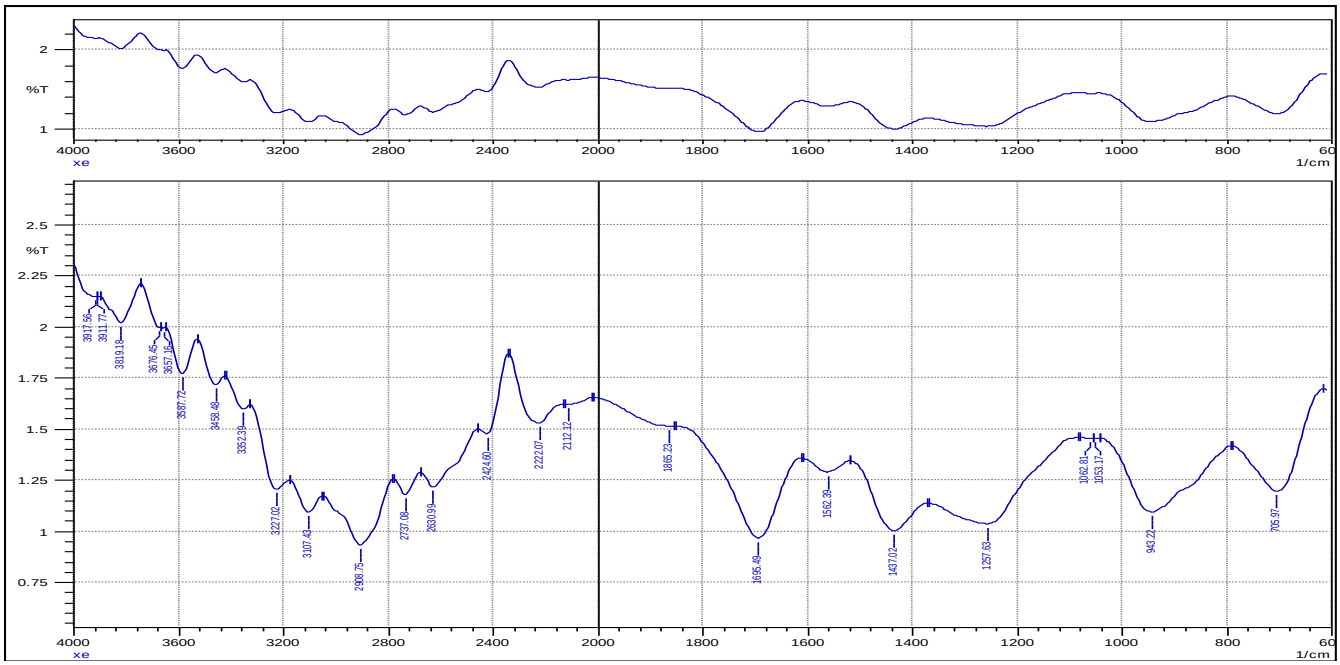


Fig 2: UV spectra of xymenynic acid

In UV spectrophotometric evaluation methanol was used as blank solution and lambda max of xymenynic acid was found at 229 nm.

FTIR Study of Xymenynic acid



705.97 943.22 1053.17 1062.81 1257.63 1437.02 1562.39 1695.49 1865.23 2112.12 2222.07 2424.60 2630.99 2737.08 2908.75 3107.43 3227.02 3352.39 3458.48 3587.72 3657.16 3676.45 3819.18 3911.77

Fig 3: FTIR of xymenynic acid

Table 4: FTIR Interpretations

Sr. No	Functional groups	Observed frequency (cm^{-1})	Vibration Mode
1	-COOH	1257.63	Stretching
2	$-CH_3$	1437.02	Stretching
3	$-CH_2$	1562.39	Stretching
4	C≡C	2222.07	Stretching
5	-OH	3227.02	Stretching

Validation of HPLC Method [16].

The objective of validation of an analytical procedure is to demonstrate that the method is suitable for analysis of desired sample. HPLC method of xymenynic acid was validated as per the guidelines of the International Conference on Harmonization (ICH) Q2 (R1) for the parameters like linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

Specificity

The specificity of the method was studied by assessment of peak purity of xymenynic acid using the Waters empower software and diode array detector and represented in terms of purity angle, purity threshold, and purity.

Linearity

The linearity of an analytical procedure is its ability to obtain test results within a given range, which are directly proportional to the concentration of the analyte in the sample.

Accuracy

The accuracy of an analytical procedure is the closeness between the conventional true value or an accepted reference value and the value found. The accuracy of the method was determined by application of the standard addition method.

Precision

Precision is the nearness of values between a series of measurements obtained from multiple sampling of the same sample under the prescribed conditions. Precision was determined as the intraday and interday difference of results from the analysis of five different standard solutions. Intraday precision was determined by triplicate analysis of each solution on a single day.

Robustness

Robustness of the method was determined by slight deviation in the method parameters. The parameters selected were deviation in column chemistry, wavelength, column temperature, flow rate, and mobile phase gradient. The retention time of standard and sample, respectively, was determined and % RSD using system suitability parameters was observed.

Recovery studies

The accuracy of the method was determined from recovery studies by adding known amount of each standard at 80%, 100%, and 120% levels to the pre-analyzed sample followed by replicate quantitative analysis using proposed method.

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of the calibration curve at low concentration levels according to ICH guidelines.

LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantified. LOQ of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantified. LOD and LOQ were calculated by using the following equations.

$LOD = 3.3\ \sigma / S$

$LOQ = 10\ \sigma / S$

σ = standard deviation of response

S = slope of calibration curve

Results and Discussion

Specificity

The specificity of the method for Assay was demonstrated by injection of following solutions into the HPLC system as blank and standard test Solution. By comparing the chromatograms of the blank, standard and test solution it was observed that there is no peak co-eluted and interfering peak was observed from blank at the retention time of xymenynic acid.

The lambda max of xymenynic acid was found to be 228.9 nm as shown in figure 2.

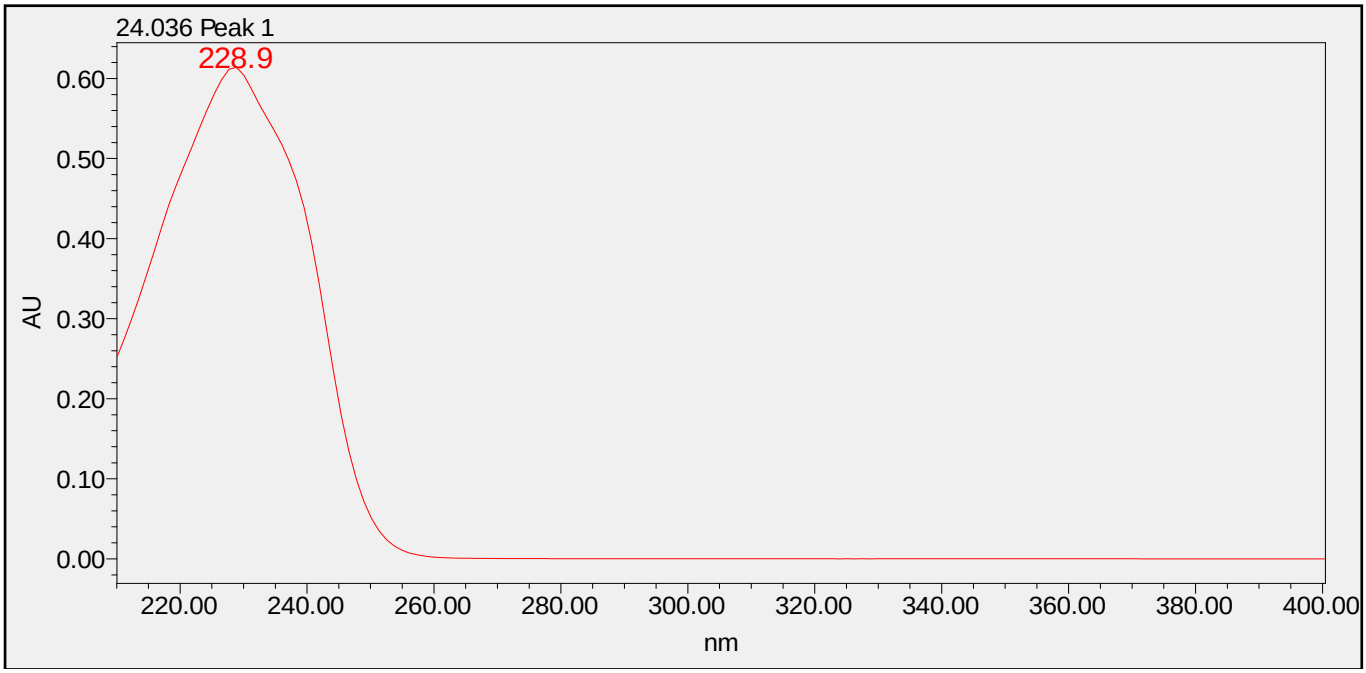


Fig 4: HPLC spectra of xymenynic acid

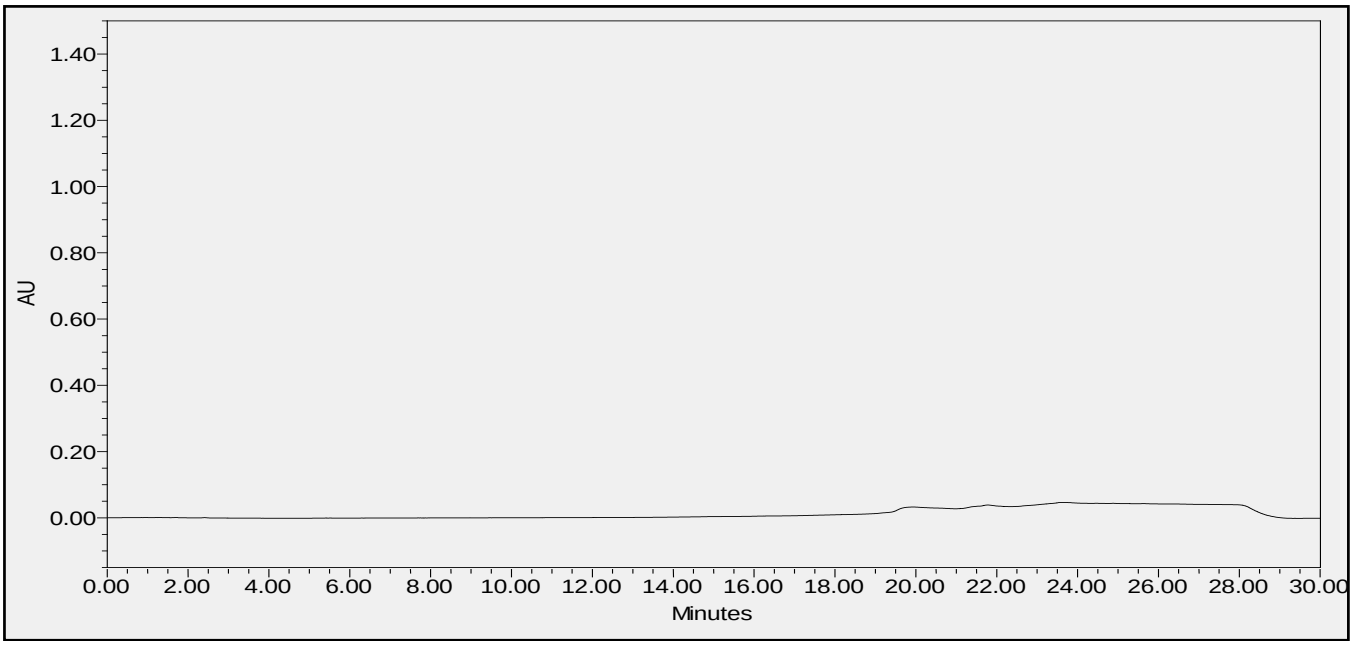


Fig 5: HPLC chromatogram of blank

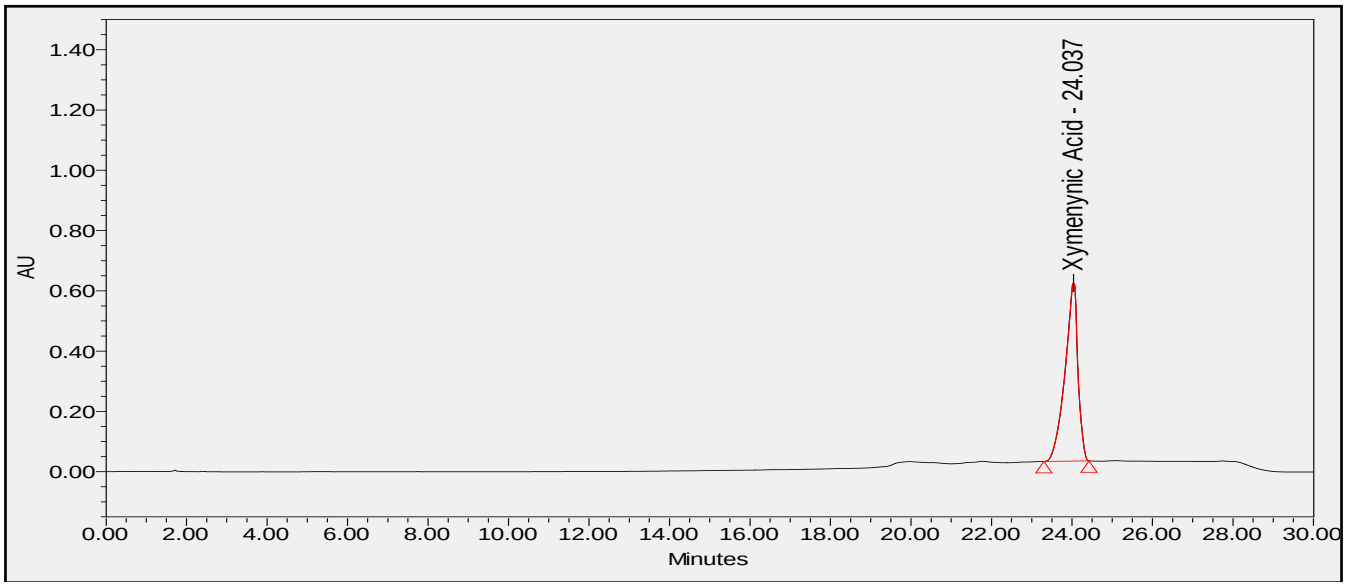


Fig 6: HPLC chromatogram of standard

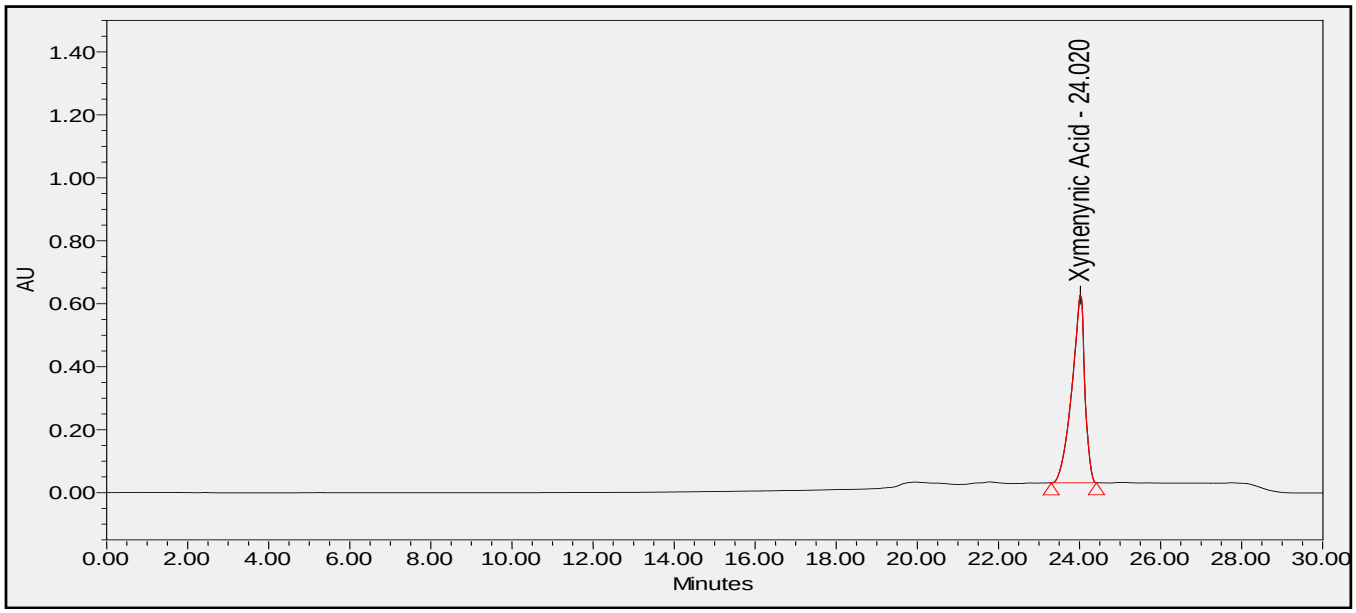


Fig 7: HPLC chromatogram of sample

Table 5: Specificity for HPLC

Sr. No.	Sample Name	Analyte Name	Purity Flag	Specificity
1.	Sample	Xymenynic extract	No	Specific
2.	Standard	Xymenynic acid	No	Specific
3.	Blank	No Peak	-	-

System precision

System precision was evaluated from five replicate injections of standard as per proposed method. Average and relative standard deviation from the six injections are given in the table 6. The relative standard deviation was observed within limits and which indicates the precision of the system.

Table 6: System precision

Injection No.	Peak Area of xymenynic extract
1	13724406
2	13717074
3	13625827
4	13594409
5	13588965
Mean	13641217
% RSD	0.46

Method precision

The six test solutions were prepared separately. Each was analyzed as per proposed procedure. The average % assay and RSD for xymenynic acid was calculated (Table 7). The % RSD sample was < 2.0% indicates that the method has an acceptable level of precision.

Table 7: Method precision

Sr. No	Amount Taken (µg/ml)	Area	Amount found	% Assay
1	10.08	13653334	0.9860	98.60
2	10.01	13614449	0.9901	99.01
3	10.01	13601105	0.9891	98.91
4	10.09	13694839	0.9881	98.81
5	10.10	13726048	0.9893	98.93
6	10.04	13770357	0.9984	99.84
			Mean	99.01
			SD	0.42874
			% RSD	0.43

Intermediate precision

The intermediate precision was determined by comparison of two independent analysis on 2 different days. The data of the first day was taken from the analysis of Method precision. The relative standard deviation from day fist and day second analysis should be within limits. The overall % RSD for % of xymenynic acid from day-1 and day-2 was observed < 2.0%. The % RSD of % assay xymenynic acid from six determinations was within acceptance criteria for day fist and day second.

Table 8: Intermediate Precision

Sr. No	Amount Taken (µg/ml)	Area	Amount found	% Assay
1	10.03	13647739	0.9905	99.05
2	10.06	13581170	0.9828	98.28
3	10.07	13657947	0.9873	98.73
4	10.04	13605786	0.9865	98.65
5	10.05	13564206	0.9825	98.25
6	10.01	13508069	0.9824	98.24
			Mean	98.53
			SD	0.3342
			% RSD	0.34

Linearity

The calibration curve for xymenynic acid was found to be linear from 50-150ppm concentration range, as shown in the figure. The Correlation coefficient (R^2) for Xymenynic acid was found to be 0.9984 and the equation of line was y = 18910x + 5048.7

Table 9: Linearity

% Level	Conc. of Xymenynic acid (µg/ml)	Average Peak area of xymenynic acid
50	200	6557648
75	400	9879505
100	600	13898374
125	800	17190801
150	1000	21191568
(Correlation coefficient) R^2		0.9984
Slope of Regression line		18910

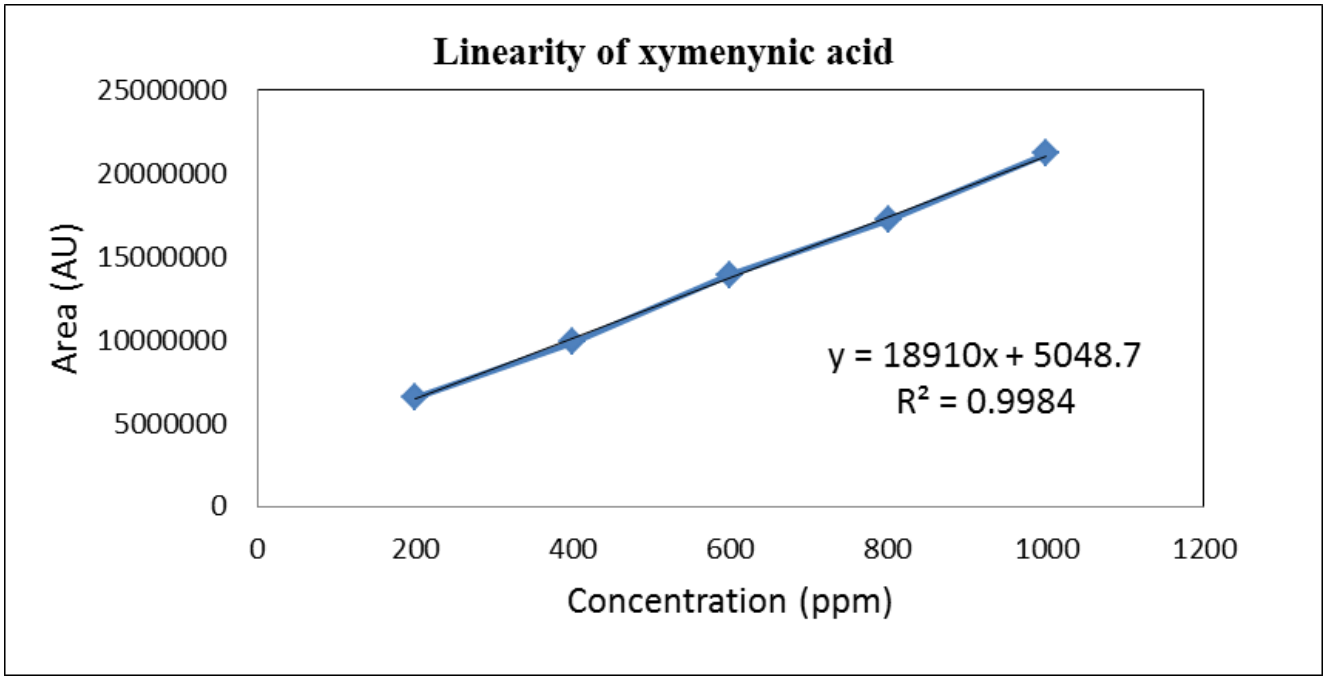


Fig 8: Calibration curve of xymenynic acid

Robustness

The influence of slightly changed parameters of the chromatographic conditions was tested according to ICH guidelines to demonstrate sufficient robustness of the method. The tests are carried out by injecting Diluent and standard solution by varying each of the parameters of chromatography mentioned in Table 9 & 10. The % RSD and system suitability parameters for results obtained with varied chromatographic conditions were within the limits. Hence, the method is robust. (Table 10&11)

Table 10: Robustness parameter

Sr. No	Parameters	Working parameter	- Changes	+ changes
1	Flow	1mL/minute	0.9mL/minute	1.1mL/minute
2	Temperature	25°C	20°C	30°C
3	Wavelength	229 nm	225 nm	235 nm

Table 11: Robustness parameter

Robustness parameter		% RSD	Peak tailing	Theoretical plates
Wavelength (nm)	228	0.44	0.77	26459
	229	0.43	0.76	26756
	230	046	0.77	26674
Temperature (°C)	25	0.44	0.77	26992
	30	0.49	0.76	26562
	35	0.40	0.76	26258
Flow (mL/min)	0.9	0.41	0.78	26489
	1.0	0.39	0.77	26085
	1.1	0.42	0.78	26440

Accuracy

The accuracy was determined from recovery studies. A known but varying amount of Standard xymenynic acid was spiked into reanalyzed test solution at 80%, 100% and 120% recovery levels of working standard in triplicate. The spiked test solution was analyzed according to the proposed procedure. The percentage recoveries were calculated against respective levels and mentioned in Table 12.

Table 12: Recovery

Recovery level	Sample wt.	STD wt. (Spiked)	Actual recover Area	Amount Recover	% Recovery	Avg. % Recovery
80% - 1	10.1	8.02	10936299	8.05	100.37	101.25
80% - 2	10.1	8.03	10900339	8.05	100.21	
80% - 3	10.1	8.01	11184791	8.26	103.18	
100% - 1	10.1	10.05	13874158	10.18	101.31	100.37
100% - 2	10.1	10.10	13863590	10.15	100.50	
100% - 3	10.1	10.06	13688388	9.99	99.31	
120% - 1	10.1	12.01	15954107	11.78	98.06	99.15
120% - 2	10.1	12.06	16221255	11.94	99.01	
120% - 3	10.1	12.02	16360494	12.07	100.39	

Stability of standard and sample solution

The % RSD for all validation parameters of xymenynic acid was found to be less than 2.0s%. Thus, the standard and sample solutions were stable up to 25 hours at room temperature.

Conclusion

A validated HPLC method has been developed for the determination of xymenynic acid. The proposed method of analysis is rapid, selective simple, precise, accurate, reproducible, specific, less time consuming and cost effective. The analysis proved that the method is suitable for the analysis of xymenynic acid. This method will help the manufacturers and analysts in determining the quality and standardization of xymenynic acid.

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