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Original Article

High-Performance Liquid Chromatography Method Development and Validation for Estimation of Mahanimbine in Curry Leaves

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ABSTRACT

Aim: A designed, improved, and validated isocratic High Performance Liquid Chromatography method was used to study mahanimbine. Background: The method is evaluated for linearity, range, system applicability, LOD, LOQ, accuracy, and precision. **Materials and Methods:** For chromatographic separation, an Agilent ZORBAX Bonus RP C_{18} (250x4.6 mm, 5µ) column is employed. The mobile phase contains Methanol: 0.1 % Triethylamine (93:07%). The flow rate is 1.2 ml /min. The method is used to estimate Mahanimbine by using modern extraction techniques. **Results:** Mahanimbine has 4.705 minutes of retention time at 254 nm. The calibration curve was linear at concentrations between 4 and 24 ng/ml (R^2-0.999). Accuracy and precision is found within the limit. The detection and quantification limit are 0.02044 and 0.06194 respectively. The robustness of the developed method is demonstrated. Mahanimbine considerably deteriorated when exposed to water, 0.1N HCl, 0.1N NaOH, oxidizing agent, and UV radiation. **Conclusion:** *Murraya koenigii* is extracted using a different technique, including Soxhlet assisted extraction, Ultrasound assisted extraction, and Accelerated solvent extraction, and mahanimbine was evaluated in those extracts. A new HPLC technique that is precise, effective, and accurate has been developed. To estimate the Mahanimbine in *Murraya koenigii*, the method is successful.

Key words: Mahanimbine; HPLC method development; Forced degradation studies; Extraction of curry leaves

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INTRODUCTION

Mahanimbine, a major bioactive carbazole alkaloid reported to have been isolated from *Murraya koenigii* (L.) Spreng of the Rutaceae family, is known to be 3, 5-dimethyl-3-(4-methylpent-3-enyl)-11H-pyrano [3, 2-a] carbazole (Figure 1).[1-5] The ability of Mahanimbine to stop the growth of cancer cells is demonstrated. Because of its distinctive flavour and perfume, *Murraya koenigii*, commonly known as the curry plant, is a popular spice in Indian kitchens.[6-11] This plant's leaves have been shown to have a significant effect on the central nervous system and have been used to treat epilepsy, as well as for its anti-inflammatory, antioxidant, immunomodulatory, and anti-amnesic properties.[12-15] Mahanimbine has been discovered to have an apoptotic effect on malignant cell lines and to inhibit cell cycle (M-phase). Curry leaves are frequently used to treat diabetes, and there are some studies showing that Mahanimbine has anti-diabetic properties.[16-19] Mahanimbine was determined using a variety of spectroscopic and chromatographic techniques.[20-22]

For the purpose of determining the presence of Mahanimbine in various extracts of *Murraya koenigii* leaves extracted using traditional and non-conventional extraction techniques, a simple, accurate, and reproducible HPLC method has been developed and validated in the current work.

MATERIALS AND METHODS

Chemicals and Reagent

Mahanimbine was purchased from Natural Remedies Pvt. Ltd., HPLC grade Methanol and Triethylamine were procured from Rankem. The high-purity HPLC grade water was used for the study.

Instrumentation

The HPLC system Vanquish was employed. Agilent ZORBAX Bonus RP C_{18} (250 4.6 mm, 5) column was used for the chromatographic separation. Chemicals were weighed using a Vibra HT (Essae) analytical balance. Ultrasonicator was used to degas mobile phase (PCi Analyticals). The "Extra pure" water

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purification equipment from Lab Link was used to obtain HPLC quality water.

Chromatographic conditions

The mobile phase for HPLC analysis was composed of methanol and 0.1% triethylamine (93:07%). The mobile phase was ultrasonically degassed for 10 min prior to use. It underwent vacuum filtration using a 0.22 m filter. A flow rate of 1.2 mL/min was used. A UV detector set to 254 nm was used for the detection. Colomn temperature was set at 25°C.

Preparation of standard stock solution

To prepare stock solutions-I of 1 mg/mL, 1 milligram of Mahanimbine was dissolved in 1 mL of HPLC grade methanol. 100 µL were obtained from Stock 1 and used to bring the volume up to 1 mL with methanol. The prepared stock solution was filtered via a 0.45 m nylon membrane syringe filter.

Method Validation

Method validation was done in line with the standards established by the US Food and Drug Administration and the International Conference on Harmonization (ICH) (USFDA). A number of measures, such as accuracy, precision, robustness, linearity, specificity, LOD, and LOQ, were chosen for the validation tests.

System Suitability

Resolution and repeatability were evaluated to see if the system was suitable. It is frequently used to make sure that the entire system including the instruments, reagents, and column analysts is suitable for the analysis that is being performed. Mahanimbine was tested numerous times at a concentration of 1 ng/mL. The RSD of area and retention time were compared using the ICH recommendations.

Linearity and Range

In order to assess its linearity, the calibration standard curve for Mahanimbine was prepared in HPLC grade methanol. The calibration standard was prepared using six replicates (n=6). Mahanimbine injections into the HPLC were made at concentrations of 4, 8, 12, 16, 20, and 24 ng/mL. The peak areas vs. concentration of each calibration standard were plotted. The linearity was assessed using a linear regression analysis.

Accuracy (% Recovery)

Mahanimbine was injected at three different concentrations (80%, 100%, and 120%), yielding final concentrations of 5, 12, and 23 µg/mL, respectively. This procedure was used to assess the accuracy of the established method in accordance with ICH criteria. A recovery percentage was calculated using the obtained peak area.

Precision

The precision of the developed analytical approach was evaluated using intra-day and inter-day precision studies. Three times every day, the repeatability of three concentrations was assessed (i.e., morning, afternoon an evening). Both intra-day and inter-day precision findings were presented in percentages RSD.

Robustness

Altering an analytical method's parameters and altering the parameters of an analytical method as necessary, its robustness was assessed. Robustness was assessed by looking at how intentionally changing the chromatographic conditions would affect the results. These adjustments were made to examine how they might impact the method. Robustness was assessed using the % RSD and the percent recovery.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The lowest amount of sample that can be detected but not necessarily measured is known as the Limit of Detection (LOD), whereas the smallest amount of sample that can be accurately quantified is known as the Limit of Quantification (LOQ). By injecting a little amount of Mahanimbine, LOD and LOQ were calculated using an HPLC approach that was developed.

Force degradation studies

A forced degradation technique called stress testing is employed to design and validate a stability detection method. Forced conditions lead to a more severe degradation of drugs and drug substances than accelerated conditions do. The ICH recommendations list degrading environments including heat, light, oxidation, acidic, basic, and others. A stability indicating approach needs to be validated with a force degradation study.

Acid and alkali hydrolysis: Mahanimbine (1 mg/mL) was added to 5 mL 0.1N HCl and 0.1 N NaOH, and the mixture was refluxed for 2 hr at 80°C on water bath. After cooling, the sample was neutralized with water before being tested using HPLC method.

Oxidation: A 125 µL aliquot from 1 mg/mL Mahanimbine solution was added to 5 mL of a 10% hydrogen peroxide solution and refluxed for 2 hr at 80°C. The sample was neutralized with water before being analyzed using HPLC.

Thermal conditions: Mahanimbine solution (125 µL) was added to 5 mL HPLC grade pure water, and the mixture was refluxed for 2 hr at 80°C on water bath. After that, the solution was neutralized and HPLC analysis was performed.

Photolytic conditions: One mg of Mahanimbine powder was exposed to UV light (254 nm) for 2 hr. It was then mixed with 1 mL of water and analyzed using the HPLC technique.

Application of proposed HPLC method

The amount of mahanimbine in various *Murraya koenigii* leaf extracts made using various extraction techniques was quantified using the proposed HPLC method. A newly developed HPLC method was used to evaluate the extracted samples. We collected fresh *Murraya koenigii* leaves in Aurangabad, Maharashtra. In a micro tray dryer, the collected leaves were cleaned and dried (S. B. Panchal and Company). To obtain a constant particle size, dried leaves were pulverized in a mixer grinder (Devika mixer grinder) and sieved through a 120-mesh sieve. After that, extraction was done using the obtained powder.

Soxhlet Assisted Extraction (SAE)

A thimble (Borosil, Mumbai, MH, India) containing 10 g of *Murraya koenigii* leaves was placed in a Soxhlet apparatus and extracted with 90% ethanol for 8 hr. A developed HPLC procedure was used to collect, filter, and analyse the extracted material for Mahanimbine. Three sets of *Murraya koenigii* leaves were used for the SAE.

Ultrasound Assisted Extraction (UAE)

Using an ultrasonic bath (Labman Scientific Instruments Ltd.,) 10 g of *Murraya koenigii* leaf powder were extracted for a predetermined amount of time (5, 10 and 15 min). The powder was poured into a beaker with a volume of extraction solvent (1:10), and it was then sonicated for 15 min. After filtering the solution, a sample was collected, and its Mahanimbine content was assessed using an HPLC method.

Accelerated Solvent Extraction (ASE)

An accelerated solvent extractor was used for the extraction (Buchi, Speed extractor E-914). A particular quantity of diatomaceous earth was added to 5 g of powdered drug before being added to stainless steel cells. Different temperature, pressure, cycle count, and hold duration parameters were used during the extraction process.

RESULTS AND DISCUSSION

Optimization of RP-HPLC Method

The peak of Mahanimbine was well defined and free of tailing under the required experimental conditions as indicated in Table 1, with a short retention time of 4.705 min as shown in Figure 2.

System suitability

The system suitability parameters, such as retention time, theoretical plates and peak area were calculated and compared with the standard values. The results are shown in Table 2.

Method validation

Linearity and Range

Least squares linear regression analysis of the calibration curve was established for determination of linearity. The calibration curves were linear over the concentration range of 4-24 ng/mL. Peak areas of Mahanimbine was plotted against respective concentration (Figure 3). Correlation coefficient was found to be 0.999. The regression equation was,

$$Y = 24324x + 6653.33 \ (r^2 = 0.9991)$$

Table 1: **The optimized chromatographic conditions.**

Separation variable	Optimized conditions
Chromatography	Vanquish UHPLC
Column	Agilent ZORBAX Bonus RP C18 (250 × 4.6 mm, 5µ)
Mobile phase	Methanol: 0.1% Triethylamine (93:07%).
Flow rate	1.2 mL/min
Total Run Time	15 min
Temperature	25°C
Detection wavelength	254 nm
Retention time	4.705

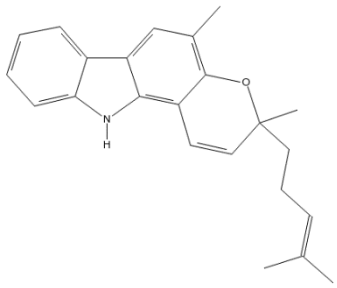

Figure 1: Chemical Structure of Mahanimbine.

Table 2: **System suitability parameters for Mahanimbine.**

Sl. No.	Parameter	Acceptance criteria	Results	
			Mahanimbine	%RSD
1	Retention Time	%RSD ≤ 2%	4.705	0.0402
2	Peak Area	%RSD ≤ 2%	126172.4	0.094775
3	Theoretical plates	≥ 2000	9102.6	0.967711

The peak area against concentration is shown in Table 3.

Accuracy

The accuracy of developed method was determined by recovery experiments which were carried out by spiking aliquots of Mahanimbine with different concentration range at three levels. The % relative standard deviation and percent recovery were calculated as depicted in Table 4.

Precision

The precision of method was studied at 2 levels intraday (repeatability) and interday precision (intermediate precision). The repeatability was performed by testing three different solutions of Corosolic acid solution at 6, 9 and 14 µg/mL on the same day and three sample solution also evaluated at three different days. Results were reported in terms of RSD (shown in Table 5).

Table 3: Linearity of Mahanimbine.

Sl. No.	Conc. (ng/mL)	Peak Area
1	4	99400
2	8	199580
3	12	299380
4	16	397460
5	20	501720
6	24	582560
7	Slope	24324
8	y-intercept	6653.3
9	r^2	0.999

Robustness

Evaluation of robustness is depending on the assay method, type of procedure conditions etc. It was tested by minor changes in chromatographic conditions as depicted in Table 6.

Force degradation studies

The forced degradation study of Mahanimbine was performed. Significant degradation was observed when exposed to water, acid (0.1N HCl), base (0.1N NaOH), oxidizing agent (10% H_2O_2), and UV light. Percent degradation under different conditions is

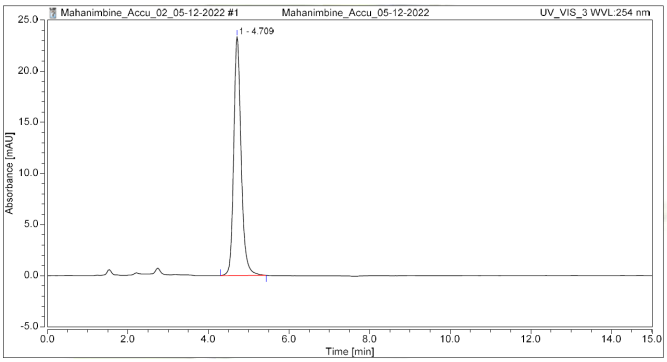

Figure 2: HPLC chromatogram of Mahanimbine standard.

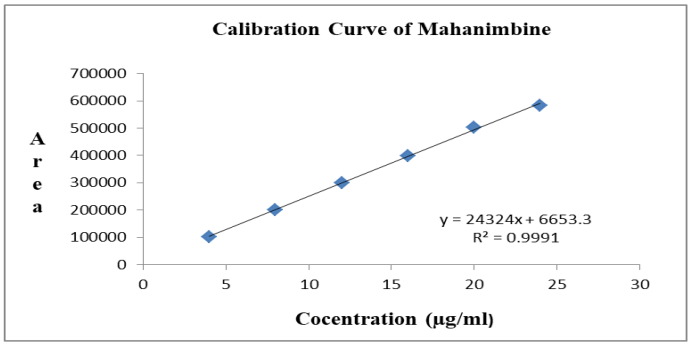

Figure 3: Calibration curve for Mahanimbine.

Table 4: Linearity of Mahanimbine.

Sl. No.	Sample	Spiked level	Theoretical Conc. (µg/mL)	Accuracy (Diff. %)					
				Intra-run (*n*=9)			Inter-run (*n*=9)		
				Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
1	LQC	80%	5	0.5249	0.7039	0.5868	2.64	0.2353	1.01
2	MQC	100%	12	0.5391	0.8015	0.0651	-2.62	1.56	2.71
3	HQC	150%	23	0.0620	-0.1203	0.5962	-2.6	-2.60	-2.58

Table 5: Intra and Inter-day precision data for Mahanimbine.

Sl. No.	Sample	Nominal Conc. (µg/mL)	Precision (% CV)					
			Intra-run (*n*=9)			Inter-run (*n*=9)		
			Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
1	LQC	6	0.0830	0.7502	0.0692	3.33	2.21	1.53
2	MQC	9	0.8781	0.0096	0.0132	2.94	3.66	1.99
3	HQC	14	0.1309	0.0087	1.80	3.44	3.17	2.11

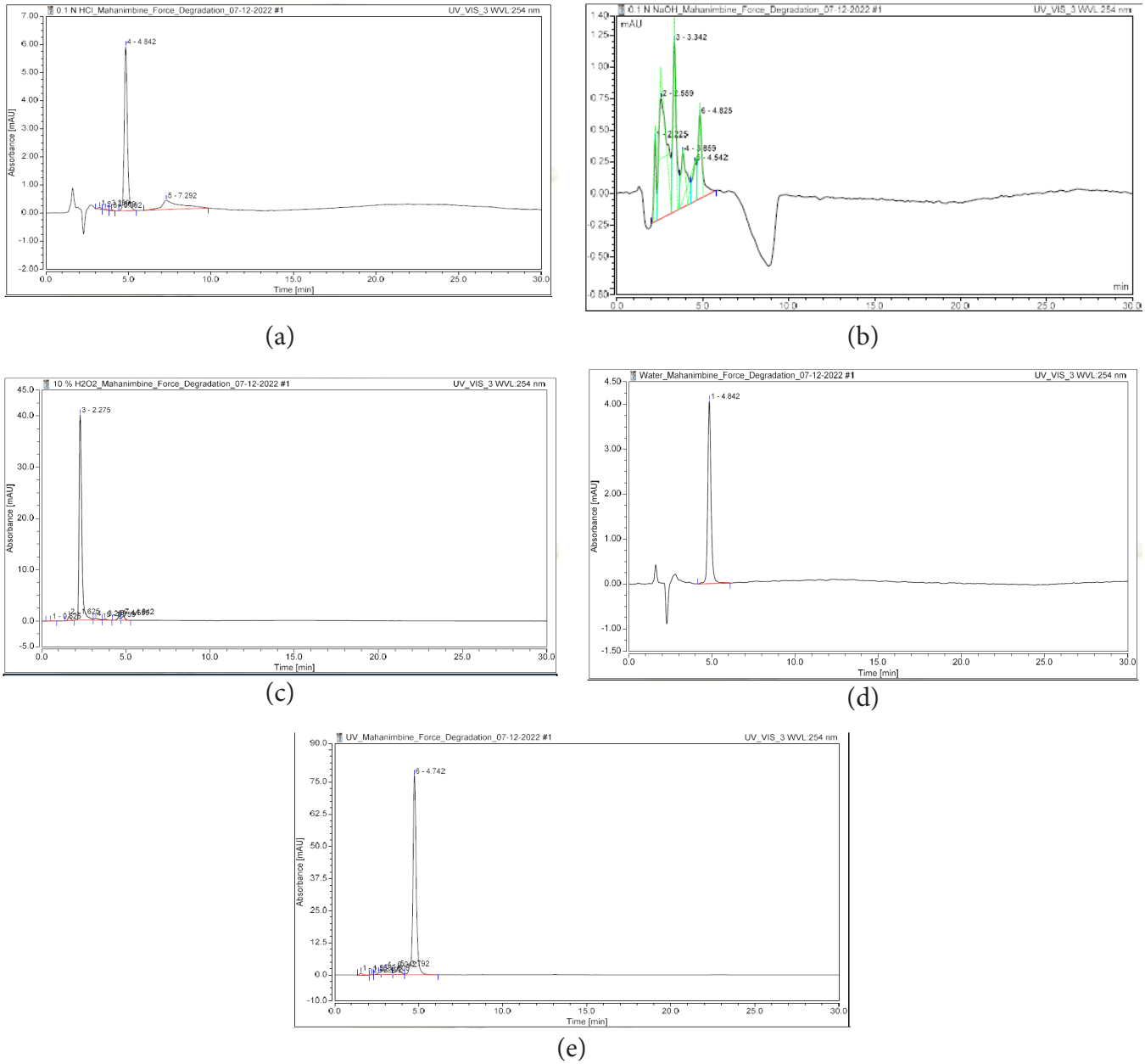

Figure 4: Forced degradation study representing Mahanimbine behavior in 0.1N HCl (a), 0.1 N NaOH (b), 10% H_2O_2 (c), Water (d), Exposed to UV light (e).

(a)

Table 6: Robustness study for Mahanimbine.

Sl. No.	Parameter	Setting	Mahanimbine			
			RT	% RSD	Amount (ng)	% RSD
1	Wavelength (nm)	253	4.772	0.0121	11.81	1.79221
		254	4.774	0.01209	12.01	0.24979
		255	4.776	0.01209	12.3573	4.37243
2	Mobile phase flow rate (mL/min)	1.3	4.777	0.03197	11.9133	0.41406
		1.2	4.775	0.03199	12.002	0.09278
		1.4	4.771	0.0121	11.6867	1.71206
3	Mobile phase composition (% v/v)	92:08	4.779	0.02092	11.68	1.62671
		93:07	4.775	0.02094	11.94	0.36507
		94:09	4.771	0.02096	11.65	2.5794

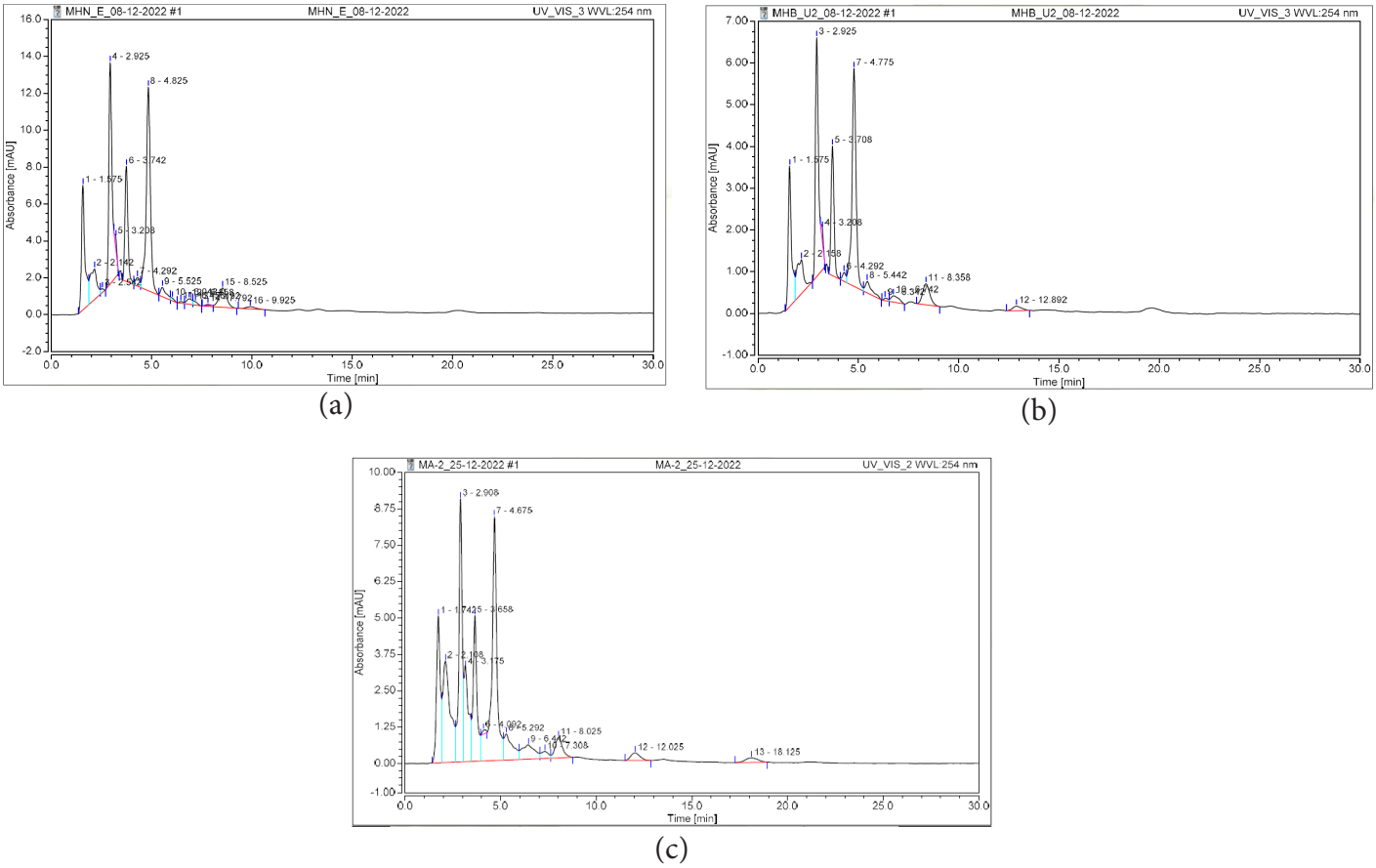

Figure 5: HPLC Chromatogram of SAE (a), UAE (b) and ASE (c).

Table 7: Summary of stressed degradation studies of Mahanimbine.

Drug	% of degradation				
Mahanimbine	Acid	Base	Oxidation	Water	UV
	19.05	82.24	89.20	83.92	10.47

Table 8: Mahanimbine content in different extracts.

Sl. No.	Extraction Method	Mahanimbine (mg/gm)
1	SAE	0.012
2	UAE	0.011
3	ASE	0.018

summarized in Table 7 and Chromatograms are shown in Figure 4.

Application of proposed HPLC method

Murraya koenigii leaves were extracted by SAE, UAE, and ASE, and the amount of mahanimbine was determined using a newly designed HPLC method. In Table 8, the content of mahanimbine is shown. Mahanimbine was extracted with the highest amount. Figure 5 displayed the extract HPLC chromatograms.

CONCLUSION

A verified HPLC method was developed after investigation on mahanimbine. In the presence of acid, basic, oxidation, and UV light, studies on force degradation were conducted. The technique was found to be exact, linear, accurate, and reliable. It was discovered that the validation's findings fell within accepted limits. The proposed HPLC approach proved successful in estimating the presence of mahanimbine in *Murraya koenigii* extracts.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LOD: Limit of detection; **LOQ:** Limit of quantification; **HPLC:** High performance liquid chromatography; **UV:** Ultraviolet; **HCL:** Hydrochloric acid; **NaOH:** Sodium hydroxide; **H_2O_2:** Hydrogen peroxide; **ICH:** International Conference on Harmonization; **USFDA:** United State Food and Drug Administration; **RT:** Retention time; **RSD:** Relative standard deviation; **CV:** Coefficient of variation; **LQC:** Lower quality control; **MQC:** Middle quality control; **HQC:** Higher quality control.

SUMMARY

The proposed work explored the HPLC method development and validation of mahanimbine. The results obtained from given research work will be useful for quality assessment of *Murraya koenigii* and also optimization of extraction methodologies for extraction of mahanimbine from leaves of *Murraya koenigii*.

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