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# A validated, precise TLC-densitometry method for simultaneous quantification of mahanimbine and koenimbine in marketed herbal formulations

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## Abstract

**Background** *Murraya koenigii* (L.) Spreng. (Rutaceae) is an important medicinal plant in natural products research for its diverse pharmacological activities. Carbazole alkaloids were the major classes of phytoconstituents obtained from different parts of this plant, such as leaves, stems, and roots. Mahanimbine and koenimbine are two important carbazole alkaloids obtained from the *M. koenigii* plant and are known for their anti-cancer, anti-oxidant, anti-diarrhoeal agents, etc. Standardization plays a vital role in the herbal drug industry for maintaining the quality, purity, safety, and efficacy of herbal formulations, and hyphenated analytical techniques like HPTLC, HPLC, GC–MS, and LC–MS were utilized for this purpose. In the present study, a specific, simple, and rapid semi-automated TLC method was developed to quantify mahanimbine and koenimbine in some marketed herbal formulations, and the same was validated based on (ICH)-Q2-(R1) guidelines.

**Results** This study revealed that the powder formulation ($F_3$) contains the highest amount of mahanimbine (62.32 µg), but the tablet formulation ($F_1$) contains both mahanimbine (41.19 µg) and koenimbine (143.6 µg).

**Conclusion** A simple, specific, and reproducible semi-automated TLC method was developed and validated successfully as per (ICH)-Q2-(R1) guidelines and can be utilized for analysing marketed herbal formulations containing *M. koenigii* powder/extracts.

**Keywords** Mahanimbine, Koenimbine, Carbazole, Densitometry, *Murraya koenigii*

## Background

*Murraya koenigii* plant, commonly known as curry tree in Asia, contains several vital phytoconstituents with diverse pharmacological activities [1]. Mahanimbine and koenimbine are the major carbazole alkaloids obtained from the leaves, roots, and stems of *M. koenigii* [2, 3]. These two phytoconstituents showed promising therapeutic efficacy against different diseases like cancer, obesity, and diabetes while tested in different *in vitro* and *in vivo* models [4]. Mahanimbine inhibited proliferation of lung cancer A549 cells [5], inhibited abnormal growth of the pancreatic cell lines (SW 1190, CFPAC1, BxPC3,

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CAPAN-2, and HPAFII) [6], stimulated memory and learning functions in aged mice [7], and also showed potential anti-diabetic activity on 3T3-L1 cells [8]. Similarly, koenimbine significantly reduced the number and size of breast cancer cell line MCF7 [9] and showed significant inhibition of castor oil-induced diarrhoea (at 50 mg/kg) in the rats [10].

Standardization of herbal formulations helps to set the constant parameters and inherent characteristics to assure safety, efficacy, quality, and reproducibility. According to the definition provided by American Herbal Product Association, "Standardization refers to the body of information and controls necessary to produce material of reasonable consistency [11]." Nowadays, in the Herbal drug industry, standardization is an integral part of quality control for getting suitable raw materials, maintaining the quality and purity of finished products, etc. [12]. Various analytical techniques were utilized for this purpose, such as HPTLC, HPLC, MS, LC–MS, and GC–MS. [13]. HPTLC is a vital separation technique based on the TLC principle, which offers multiple advantages such as minimized exposure to toxic solvents, improved sample application, reduced usage of mobile phase, less analysis time, reduced possibilities of environmental pollution, and higher separation efficiencies [14]. Planar chromatographic methods (TLC and HPTLC) were widely utilized for quality control of herbal formulations. It helps for qualitative, quantitative, and semi-quantitative analysis of phytoconstituents incorporated in different herbal formulations manufactured and marketed by different companies. Several pieces of research proved that thin-layer chromatographic methods successfully ensure the quality and purity of marketed herbal formulations. Abharam A et al. developed a novel HPTLC method for analysing an ayurvedic polyherbal formulation named Pathyashadangam Kwath by employing toluene/ethyl acetate/formic acid (2.5: 2.0: 0.5 v/v/v) as a mobile phase. In this study, andrographolide was used as a marker for standardizing this formulation, and it proved that the presence of this marker may be responsible for its pharmacological activities [15]. Hazra et al. took piperine as a marker for standardizing six polyherbal formulations named Avipattikara, Talisadya, Sringyadi, Sitopaladi, Hingavastaka, and Trikatu and developed a specific and simple HPTLC method by utilizing toluene/ethyl acetate (7:3 v/v) as a mobile phase. This study proved that piperine was identified and quantified in all of the formulations, and it can be concluded that this method can be utilized for routine analysis of piperine in marketed ayurvedic formulations [16]. Kagathara C et al. stated that HPTLC could be a better option for estimating ascorbic acid, quercetin, and curcumin in different herbal formulations. They developed a specific HPTLC method for identification and quantification of the same by employing chloroform/ethyl acetate/formic acid (6:6:2.5 v/v/v) as a mobile phase. From this study, it was observed that these three important phytocompounds were identified and quantified in all of the formulations, and this analytical method can be utilized for quality control of herbal formulations where curcumin, quercetin and ascorbic acid were incorporated [17]. A simple, specific, and rapid HPTLC assay method for analysing tacrolimus ointments was developed by Islam MK et al. which helps to identify and quantify tacrolimus in the same. In this method, toluene–acetonitrile–ethyl acetate–glacial acetic acid (6:2:2:0.1 v/v/v) was used as a mobile phase and it proved the utilization of this method for standardization of marketed tacrolimus ointments [18]. These researches highlighted the importance of planar chromatographic techniques in the standardization of marketed herbal formulations and marker compounds play an important role in this.

By thorough literature survey, it was found that several analytical techniques were employed to estimate mahanimbine and koenimbine in *M. koenigii* plants. Joshi T et al. utilized a novel UPLC method to determine the natural abundance of the carbazole alkaloids in the *M. koenigii* plant [19]. Viteritti et al. [20] developed an HPLC–MS/MS method to quantify carbazole alkaloids in *M. koenigii* plant. Nandan et al. [21] quantified eleven carbazole alkaloids using a novel UPLC/MS/MS method, including mahanimbine and koenimbine in *M. koenigii* plant collected from six different climatic zones of India. Chatterjee et al. [22] successfully developed a validated qNMR method for quantifying nine important carbazole alkaloids, including koenimbine and mahanimbine. But to the best of our knowledge, there is no analytical method was available for standardizing marketed herbal formulations containing carbazole alkaloids obtained from the *M. koenigii* plant. Hence in the present study, a rapid and simple semi-automated TLC method was established to identify and estimate two essential carbazole alkaloids named mahanimbine and koenimbine (Fig. 1) in some marketed formulations and helps to determine the quality and purity of the formulations. The validation of the established method has been done as per (ICH)-Q2-(R1) guidelines.

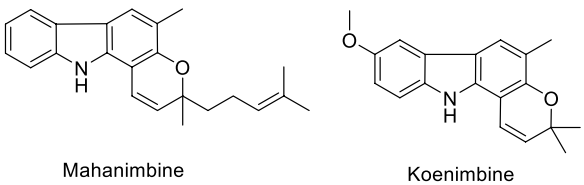



**Fig. 1** Structures of mahanimbine and koenimbine

## Methods

### Instruments

VisionCATS software (version 3.2) is equipped with TLC visualizer 2, Linomat V applicator, and TLC scanner 4 manufactured by CAMAG (Switzerland). TLC silica gel $60F_{254}$ plates were procured from Merck (Germany). The analytical balance and hot air oven were purchased from Sartorius (Germany) and Biotechnics India (India.)

### Reagents and standard substance

Analytical grade Hexane and Ethyl acetate has been procured from Finar (India), and HPLC grade methanol has been purchased from Spectrochem (India). Mahanimbine and koenimbine (purity: > 90% by HPLC) were purchased from Natural Remedies Pvt Ltd. (India).

### Selected marketed formulations for analysis

Three marketed formulations named Merlion Naturals curry leaves extract tablets (packed and marketed by Infinator Pvt. Ltd, Ahmedabad, Gujarat, India), curry leaves capsules (manufactured and marketed by Dr. JPG Organic, Jaipur, Rajasthan, India) and premium curry leaves powder (manufactured by Spag herbals, Hyderabad, Telangana, India) were collected and coded as $F_1$ (for tablet), $F_2$ (for capsule) and $F_3$ (for powder) [23–25]. Out of these formulations, $F_1$ and $F_2$ contain 500 mg of curry leaves powder along with excipients (in Q.S. and the name of the same is not disclosed) and used as a dietary supplement and $F_3$ contains 125 mg of dried curry leaves along with excipients (in Q.S. and the name of the same is not disclosed) and used as a cosmetic. All formulations were stored in a cool environment and protected from direct sunlight.

### Standard and sample preparation

About 5 mg of mahanimbine and koenimbine were dissolved in 50 mL of HPLC grade methanol for preparing standard stock solutions. Further, the working solutions were prepared by diluting the stock solutions with the required quantity of methanol.

For preparing sample solutions from tablets ($F_1$), about five tablets were crushed into powder, and 300 mg of the powder was accurately weighed and macerated with 10 mL of HPLC grade methanol. In the case of capsule ($F_2$), the shells of five capsules were broken and accurately weighed, and 300 mg of powder was macerated with 10 mL of HPLC grade methanol. For preparing sample solutions from powder ($F_3$) (stored in a cool environment and protected from direct sunlight), 300 mg of the powder was accurately weighed and macerated with 10 mL of HPLC grade methanol. Finally, all of the prepared extracts were centrifuged (10 °C, 10,000 RPM). The supernatant fluids were collected and underwent tenfold dilution, which was used for analysis.

### Optimization of the analytical conditions

In the current study, TLC silica gel $60F_{254}$ plates were utilized as a stationary phase. For selecting a suitable mobile phase for analysis, different solvent combinations were tried. By thorough literature survey, it was decided that different combinations of hexane and ethyl acetate in v/v (8:2, 9:1, 9.5:0.5, 7:3) would be used for determining optimal mobile phase composition. Based on the separation pattern obtained from preliminary TLC analysis/observation, a combination of hexane:ethyl acetate (7:3 v/v) was selected as the final mobile phase and utilized throughout the analysis.

### Semi-automated TLC conditions

On each TLC silica gel $60F_{254}$ plate (20 × 10 cm), 4 µL of mahanimbine and koenimbine standards and $F_1$, $F_2$, and $F_3$ were applied at the rate of 150 nL $S^{-1}$ by utilizing a Linomat V applicator. The applied band length was 8 mm, and the application was about 1 cm from the bottom and left edges of the plate. After application, the development of the plates was done in CAMAG twin trough chambers to a distance of 70 mm with the selected mobile phase composition which was previously saturated for 20 min. After that, the developed plates were air-dried, and the fingerprint profile was generated by placing the plates in TLC visualizer 2. Then, the plates were scanned using a TLC scanner 4 at $\lambda = 291$ nm and 285 nm for koenimbine and mahanimbine respectively. The wavelengths were optimized by performing spectrum scanning in the 190–400 nm wavelength range, and the obtained data were compared with the maximum wavelengths mentioned in the literature (mahanimbine-357, 302, 288, and 235 nm; koenimbine-295, 262, and 238 nm) [26]. The conditions for densitometric scanning (VisionCATS version 3.2) were: scanning speed 20 mm/s, data resolution 100 µm/step, and slit dimension 6.00 × 0.45 mm. From densitometric analysis, the retardation factor ($R_f$) for mahanimbine and koenimbine was found satisfactory. For quantitative analysis, the obtained values for the peak areas were utilized.

### Method validation

Method validation is an integral part of any analytical experiment/procedure for getting accurate and reproducible results. This process is performed by checking the following parameters: linearity, LOQ, LOD, accuracy (recovery), precision (intra- and inter-day precision), reproducibility, and robustness.

### Linearity

In any analytical method, linearity is an important parameter that describes its ability to get test results directly proportional to the content (concentration) of analyte present in the sample [27]. For quantifying the analytes present in the formulations, dilution of the standard stock solutions has been done to get linearity of standard solutions (considering the quantitation limit) containing koenimbine and mahanimbine in the concentration range of 50–450 and 100–400 µg/mL respectively keeping the applied volume 4 µL constant. The standards were applied in triplicate to generate the calibration curve. The correlation coefficient ($R^2$), intercept, and slope of the calibration curves were estimated to obtain the method linearity.

### Detection limit (LOD)

The detection limit (LOD) is defined as the lowest amount of the analyte detected in the prepared sample. LOD can be calculated by using the following equation:

$$\text{LOD} = 3.3 \times \text{standard deviation of the response} / \text{slope of the calibration curve}$$

### Quantitation limit (LOQ)

The quantitation limit (LOQ) is defined as the lowest amount of the analyte quantified with sufficient accuracy and precision. It can be calculated by using the following equation:

$$\text{LOQ} = (10 \times \text{standard deviation of the response}) / \text{slope of the calibration curve}$$

### Accuracy (recovery)

The accuracy of any analytical procedure is defined as the degree of agreement between the values considered to be true and the amount of the substance in the test samples obtained from the analysis [27, 28]. For estimating the percentage of recovery of mahanimbine and koenimbine in $F_1$, about 300 mg of powdered drug and 32 µg (80%), 40 µg (100%), 48 µg (120%) of mahanimbine standards and 112 µg (80%), 140 µg (100%) and 168 µg (120%) of koenimbine standards were mixed and further diluted with required quantity of HPLC grade methanol. In the case of $F_2$ and $F_3$, about 300 mg of powdered drugs and 40 µg (80%), 50 µg (100%), 60 µg (120%), 48 µg (80%), 60 µg (100%) and 72 µg (120%) of mahanimbine and 112 µg (80%), 140 µg (100%) and 168 µg (120%) of koenimbine standards were mixed and diluted with required quantity of HPLC grade methanol. After that, the percentage of recoveries was calculated and documented.

### Precision

The precision of any analytical method was defined as the degree of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [27, 28]. In the present study, inter-day precision studies of the developed method have been done by applying the standard solutions in triplicate at three different concentration levels three times at 72 h intervals, and for performing intra-day precision studies, the standard solutions were applied in triplicate at three different concentration levels three times within two hour intervals on the same day.

### Reproducibility

The developed method was validated for the reproducibility of both for application (repeatability of application) and scanning (repeatability of scanning) [29]. The experiments were performed in triplicate by different analysts in different laboratories of NIPER Hyderabad, and it was observed that the %RSD values were obtained between ± 5. Hence, it can be concluded that the developed method is reproducible.

### Robustness

Robustness study needs to be done during method development, and it helps to determine the reliability of an analysis concerning deliberate variations in method parameters. In the present study, the following variations have been done to check the robustness of the developed method; different volumes of mobile phase compositions were utilized (Hexane: Ethyl acetate = 7:3, 8:2; 9:1); the laboratory temperature set to 22 °C, 25 °C, and 30 °C; humidity—35% RH, 55% RH, and 75% RH, varying the wavelengths for scanning (293, 291 and 289 nm) and changing distance for development of TLC plates (78, 76 and 74 mm), etc. It was observed that the developed method gave reproducible results in all varying conditions.

## Results

Three different marketed formulations (tablet, capsule, and powder) were collected from different distributors and coded as $F_1$, $F_2$, and $F_3$. The stock solutions of the standards were prepared by dissolving 5 mg of both standards in 50 mL of HPLC grade methanol and the working solutions were prepared by diluting the stock solutions with the required quantity of methanol. In the present study, TLC silica gel $60F_{254}$ plates were used as a

stationary phase, and a combination of hexane:ethyl acetate (7:3) was utilized as a mobile phase. The developed fingerprinting profile showed that the mahanimbine and koenimbine were present in the selected formulations (Fig. 2). Both phytocompounds were densitometrically detected at $\lambda = 285$ and 291 nm, respectively. The absorption spectrum and the peak areas were recorded and documented for analysis. From spectrum data, it was observed that in the wavelength range of 190–400 nm, absorption maxima for mahanimbine and koenimbine were observed at 285 and 291 nm (Fig. 3). So, these two wavelengths were selected for further quantification. The specificity and peak purity of the method were determined by comparing the spectra of mahanimbine and koenimbine in the selected formulations with the standards. The peaks obtained by densitometric scanning were easily identifiable, symmetrical, and resolved well. From densitometric scanning, the obtained $R_f$ values for mahanimbine and koenimbine were 0.48 and 0.60, respectively (Figs. 4 and 5).

Further, the developed method was validated as per the guidelines mentioned by (ICH)-Q2-(R1). To determine the linearity of the developed method, 4 µL of standard solutions of increasing concentration (50–450 µg/mL for koenimbine and 100–400 µg/mL for mahanimbine, respectively) were applied on TLC silica gel $60F_{254}$ plates in triplicate. The calibration curve was generated (Fig. 6, Tables 1 and 2), and a linear relationship was established for mahanimbine and koenimbine in the concentration range of 100–400 and 50–450 µg/mL (Table 3). From the calibration curve, the obtained equations were $Y = 3.3614x + 95.5$ and $Y = -0.0069x^2 + 8.2023x + 172.76$, correlation coefficients ($R^2$) = 0.9985 and 0.9998, coefficient of variances (CV) = 0.34% and 0.89%, respectively (Fig. 6, Table 3). The obtained $R^2$-value indicates a strong correlation between the concentrations of phytocompounds and peak areas.

The LOQ and LOD values were calculated, and the obtained values were 18.44 ng/spot, 31.57 ng/spot, for koenimbine and 32.81 ng/spot, 72.81 ng/spot for mahanimbine, respectively (Table 3). Inter-day and intra-day precision studies have been done for the developed method; for intra-day, the studies were done in triplicate at three hrs time intervals; for inter-day, it was also done in triplicate at three consecutive days (Tables 4 and 5). The developed method was evaluated for recovery (accuracy) studies. For this purpose, the selected formulations and the standards were mixed and diluted with the required quantity of HPLC grade methanol. The calculated % of recovery for mahanimbine was: 96.80 ($F_1$), 98.47% ($F_2$), and 95.13% ($F_3$), and for koenimbine was: 97.61% ($F_1$), respectively (the % recovery was found to be between 95 and 105%; hence, the developed method was accurate) (Tables 6, 7, 8 and 9). The recovery analysis of koenimbine was not done in $F_2$ and $F_3$ as in $F_3$, it was not identified, and in case of $F_2$, though, it was identified but not quantified properly (quantification value comes under LOQ). Robustness studies were also performed by altering the method parameters, and there is no significant change observed in the obtained results (Tables 10 and 11).

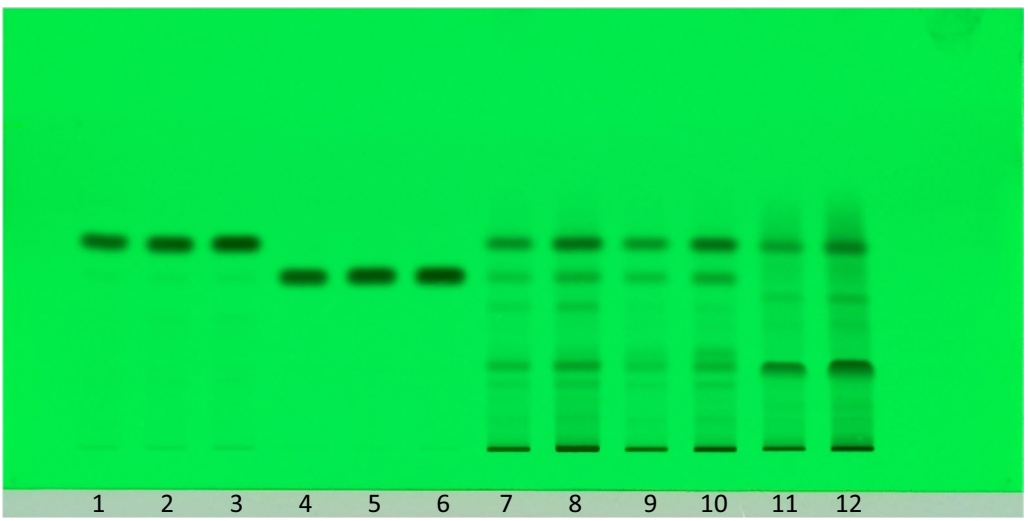
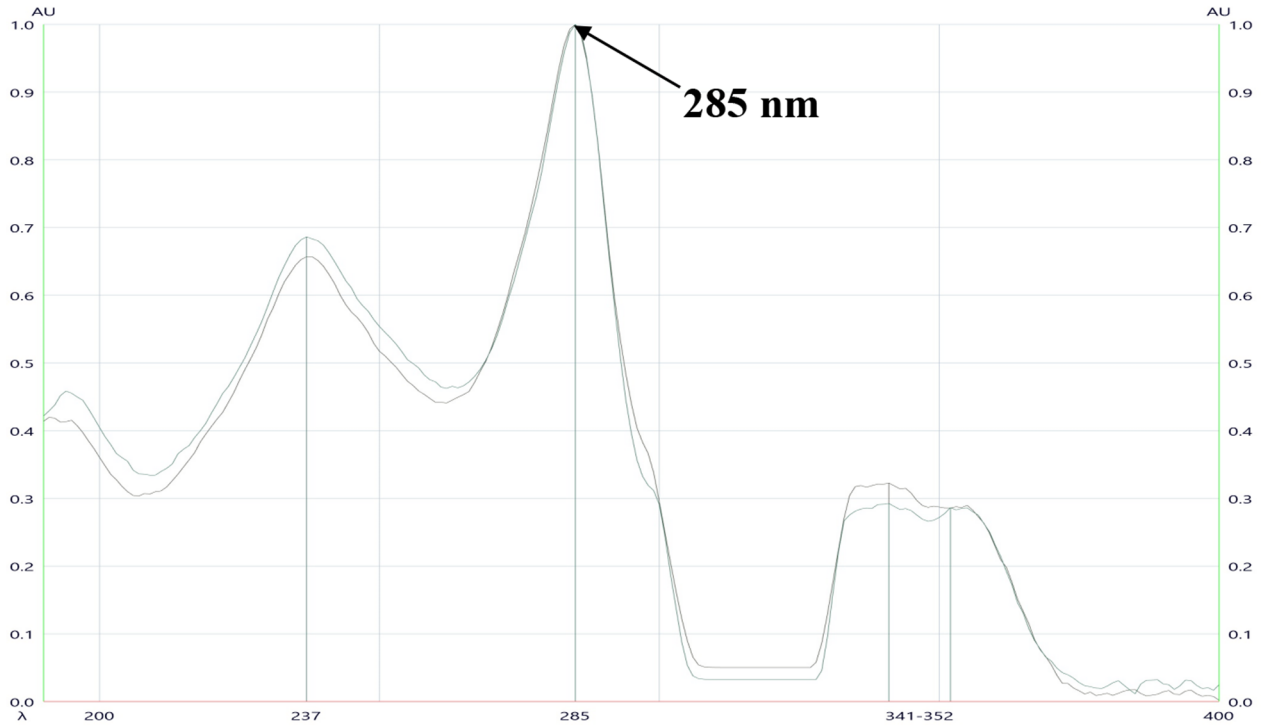
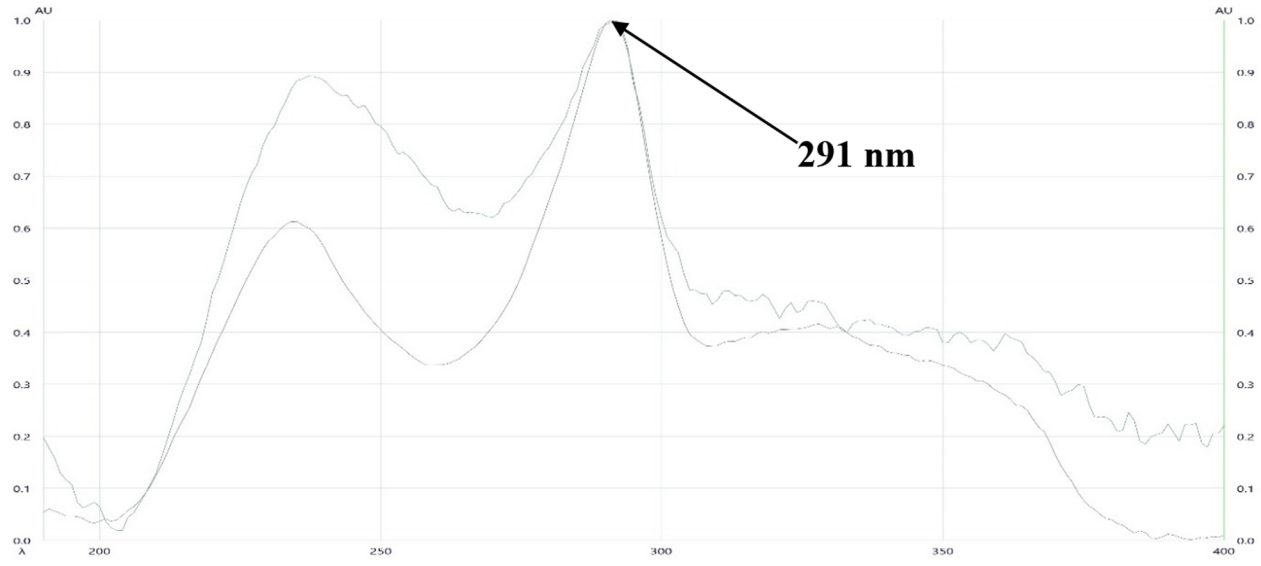



**Fig. 2** Developed HPTLC fingerprint profile. Track (1–3): mahanimbine standard, Track (4–6): koenimbine Standard, Track (7–8): $F_1$ (Tablet), Track (9–10): $F_2$ (Capsule), Track (11–12): $F_3$ (Powder)




**(a)**




**(b)**

**Fig. 3** Spectrum data of **a** mahanimbine and selected formulations at 285 nm **b** koenimbine and selected formulations at 291 nm

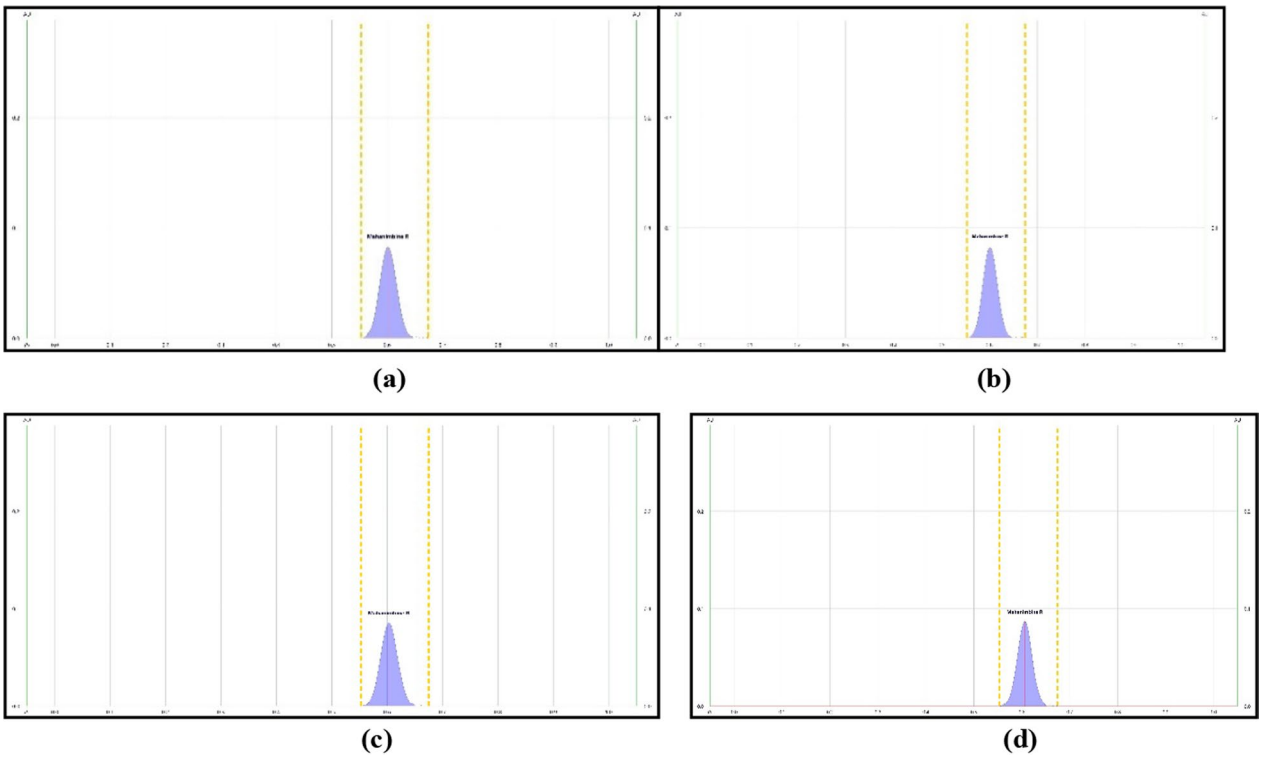
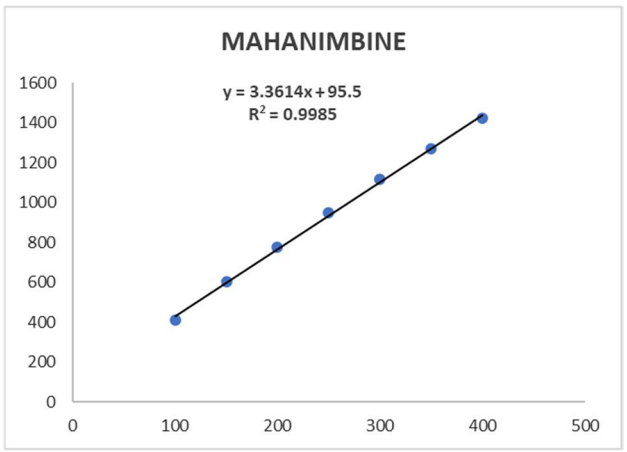


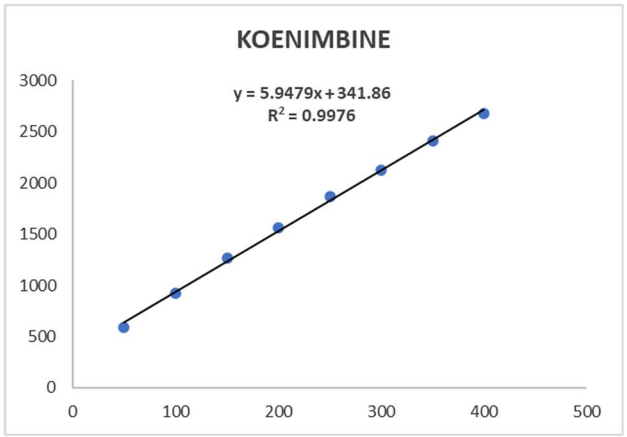

**Fig. 4** HPTLC chromatogram at 285 nm. **a** mahanimbine standard **b** $F_1$ (Tablet) **c** $F_2$ (Capsule) **d** $F_3$ (Powder)


**Fig. 5** HPTLC Chromatogram at 291 nm. **a** koenimbine standard **b** $F_1$ (Tablet)

After that, the developed method was successfully employed to estimate mahanimbine and koenimbine in the selected marketed formulations ($F_1$, $F_2$, and $F_3$). From this study, mahanimbine was identified and quantified in all of the formulations, but koenimbine was only quantified in $F_1$ though it was identified in both $F_1$ and $F_2$ as the quantified value in $F_2$ comes under LOQ and this identification was done by comparing the $R_f$ values (0.60 and 0.48), and absorption maxima (285 and 291 nm) of the standards. The quantity of both phytocompounds in the formulations was calculated based on the peak areas obtained from the chromatogram. The amount of mahanimbine in $F_1$, $F_2$, and $F_3$ was found to be 41.19 µg, 53.24 µg, and




(a)




(b)

**Fig. 6** Calibration curve of **a** mahanimbine standard **b** koenimbine standard

**Table 1** Linear regression data of mahanimbine

| Sl No | Conc. in ppm | Mean ± RSD |
|---|---|---|
| 1 | 100 | 420 ± 2.38 |
| 2 | 150 | 602 ± 1.99 |
| 3 | 200 | 777 ± 2.18 |
| 4 | 250 | 951 ± 1.15 |
| 5 | 300 | 1116 ± 1.25 |
| 6 | 350 | 1272 ± 1.25 |
| 7 | 400 | 1421 ± 1.4 |

62.32 µg, and for koenimbine, it was 143.6 µg, respectively (Table 12). It indicates that the highest quantity of mahanimbine was found in $F_{3,}$ but in $F_{1,}$ both the phytocompounds were present.

**Table 2** Linear regression data of koenimbine

| Sl No | Conc. in ppm | Mean ± RSD |
|---|---|---|
| 1 | 50 | 558 ± 1.79 |
| 2 | 100 | 931 ± 2.14 |
| 3 | 150 | 1254 ± 1.19 |
| 4 | 200 | 1542 ± 1.16 |
| 5 | 250 | 1781 ± 0.89 |
| 6 | 300 | 2010 ± 0.69 |
| 7 | 350 | 2189 ± 0.91 |
| 8 | 400 | 2364 ± 0.59 |
| 9 | 450 | 2461 ± 0.6 |

**Table 3** Method validation parameters

| Sl No | Parameters | Mahanimbine | Koenimbine |
|---|---|---|---|
| 1 | Wavelength | 285 nm | 291 nm |
| 2 | Linearity range concentration (in µg/ml) | 100–400 | 50–450 |
| 3 | Coefficient of variances (CV) | 0.34% | 0.89% |
| 4 | Correlation coefficients ($R^2$) | 0.9985 | 0.9998 |
| 5 | Limit of detection (ng/spot) | 32.81 | 18.44 |
| 6 | Limit of quantitation (ng/spot) | 72.81 | 31.57 |
| 7 | Reproducibility | Reproducible | Reproducible |

**Table 4** Intra- and inter-day precision data of mahanimbine

| Conc. in ppm | Mean ± RSD |
|---|---|
| Intra-day | |
| 180 | 510 ± 1.96 |
| 260 | 564 ± 2.48 |
| 340 | 636 ± 2.83 |
| Inter-day | |
| 240 | 710 ± 1.4 |
| 300 | 760 ± 2.63 |
| 360 | 825 ± 1.81 |

## Discussion

*M. koenigii* is an important medicinal plant and several herbal formulations were available in the market where *M. koenigii* leaf powders or extracts were incorporated, and these formulations were used as dietary supplements, cosmetics, etc. But there is no specific analytical method was reported for standardizing the same and marker-based standardization by utilizing planar chromatographic methods can be a better option for

**Table 5** Intra- and inter-day precision data of koenimbine

| Conc. in ppm | Mean ± RSD |
|---|---|
| Intra-day | |
| 140 | 590 ± 1.69 |
| 220 | 645 ± 2.32 |
| 300 | 705 ± 0.70 |
| Inter-day | |
| 200 | 770 ± 1.29 |
| 260 | 820 ± 1.21 |
| 320 | 870 ± 2.29 |

**Table 6** Accuracy (recovery) data of mahanimbine obtained from $F_1$

| Formula | Area obtained from software | % of recovery |
|---|---|---|
| 3 bands of 100% sample | 0.002575741<br>0.005378757<br>0.005765268 | 96.806837 |
| 3 bands of 100% Sample + 80% Standard | 0.007342609<br>0.008868828<br>0.008646599 | |
| 3 bands of 100% Sample + 100% Standard | 0.009309263<br>0.009584616<br>0.009561901 | |
| 3 bands of 100% Sample + 120% Standard | 0.01010137<br>0.010271852<br>0.010140663 | |
| 2 bands of 80% Standard | 0.007182594<br>0.004321403 | |
| 2 bands of 100% Standard | 0.004206004<br>0.004546618 | |
| 2 bands of 120% Standard | 0.00511068<br>0.005268287 | |

this. Hence, there is a need to develop a suitable analytical method that helps to perform qualitative and quantitative analysis of two major marker compounds of *M. koenigii* named mahanimbine and koenimbine in the marketed herbal formulations for routine quality control analysis. Hence, in the current study, three marketed formulations manufactured by different companies (coded as $F_1$, $F_2$, and $F_3$) were collected, and quantitative estimation of mahanimbine and koenimbine was done in these formulations by using a semi-automated TLC method. Koenimbine ($R_f$ value 0.60) and mahanimbine ($R_f$ value 0.48) were identified in all of the formulations at the wavelengths of 291 and 285 nm, but only mahanimbine was quantified in all of the formulations, and koenimbine was quantified only in $F_1$. For performing this analysis, different mobile phases were employed, but based on the separation pattern, hexane:ethyl acetate (7:3 v/v) was selected for final

**Table 7** Accuracy (recovery) data of mahanimbine obtained from $F_2$

| Formula | Area obtained from Software | % of recovery |
|---|---|---|
| 3 bands of 100% sample | 0.00044788<br>0.011267132<br>0.009436818 | 98.47378217 |
| 3 bands of 100% Sample + 80% Standard | 0.008830368<br>0.010630508<br>0.014043002 | |
| 3 bands of 100% Sample + 100% Standard | 0.014783529<br>0.015096105<br>0.014532022 | |
| 3 bands of 100% Sample + 120% Standard | 0.01413927<br>0.015060204<br>0.015253995 | |
| 2 bands of 80% Standard | 0.01569976<br>0.004768991 | |
| 2 bands of 100% Standard | 0.004737254<br>0.005804689 | |
| 2 bands of 120% Standard | 0.005710901<br>0.006967575 | |

**Table 8** Accuracy (recovery) data of mahanimbine obtained from $F_3$

| Formula | Area obtained from Software | % of recovery |
|---|---|---|
| 3 bands of 100% sample | 0.002068801<br>0.003960302<br>0.004055559 | 95.13510953 |
| 3 bands of 100% Sample + 80% Standard | 0.005123489<br>0.006076501<br>0.006129103 | |
| 3 bands of 100% Sample + 100% Standard | 0.006913892<br>0.007452141<br>0.007627958 | |
| 3 bands of 100% Sample + 120% Standard | 0.008180758<br>0.008607295<br>0.008389677 | |
| 2 bands of 80% Standard | 0.005785998<br>0.003193103 | |
| 2 bands of 100% Standard | 0.002769779<br>0.003819682 | |
| 2 bands of 120% Standard | 0.004226519<br>0.00443158 | |

quantification. This method passed all the parameters of analytical validation as per the guidelines prescribed in (ICH)-Q2-(R1) and gave reproducible results. The peak shapes of mahanimbine and koenimbine were found to be good. Hence, the developed semi-automated TLC method can be utilized for the routine quality control analysis of marketed herbal formulations of *M. koenigii* effectively, and its advantages are specificity, accuracy, and simplicity.

**Table 9** Accuracy (recovery) data of koenimbine obtained from $F_1$

| Formula | Area obtained from Software | % of recovery |
|---|---|---|
| 3 bands of 100% sample | 0.002068801<br>0.003960302<br>0.004055559 | 97.61435044 |
| 3 bands of 100% Sample + 80% Standard | 0.005123489<br>0.006076501<br>0.006129103 | |
| 3 bands of 100% Sample + 100% Standard | 0.006913892<br>0.007452141<br>0.007627958 | |
| 3 bands of 100% Sample + 120% Standard | 0.008180758<br>0.008607295<br>0.008389677 | |
| 2 bands of 80% Standard | 0.005785998<br>0.003193103 | |
| 2 bands of 100% Standard | 0.002769779<br>0.003819682 | |
| 2 bands of 120% Standard | 0.004226519<br>0.00443158 | |

**Table 10** Data of robustness studies of mahanimbine

| Volumes | Mean area ± RSD |
|---|---|
| Mobile phase compositions (Hexane: Ethyl acetate, v/v) | |
| 6.9:3.1 | 695 ± 2.20 |
| 7.1:2.9 | 698 ± 1.90 |
| 7:3 | 701 ± 2.42 |
| Temperature (°C) | |
| 22 ± 2 | 702 ± 2.80 |
| 25 ± 2 | 706 ± 2.00 |
| 27 ± 2 | 704 ± 2.65 |
| Humidity (% RH) | |
| 45 ± 5 | 704 ± 2.35 |
| 55 ± 5 | 708 ± 2.70 |
| 65 ± 5 | 706 ± 2.50 |
| Distance for development of TLC plate (mm) | |
| 78 | 720 ± 2.10 |
| 76 | 726 ± 1.80 |
| 74 | 723 ± 2.20 |
| Wavelength (nm) | |
| 287 | 715 ± 1.40 |
| 285 | 725 ± 1.70 |
| 283 | 720 ± 1.90 |

## Conclusion

The current study proved that the developed HPTLC method was precise, specific, simple, and accurate in estimating mahanimbine and koenimbine in selected marketed herbal formulations. This study revealed that mahanimbine is identified and quantified in all formulations, but koenimbine is only identified and quantified in tablet formulation. The performed quantitative and qualitative analysis of the content of mahanimbine and koenimbine can help to maintain the quality of the marketed herbal formulations containing *M. koenigii* extracts/powders. The developed method has been validated as per the guidelines mentioned in (ICH)-Q2-(R1), which confirmed this method's accuracy, precision, and reliability.

**Table 11** Data of robustness studies of koenimbine

| Volumes | Mean area ± RSD |
|---|---|
| Mobile phase compositions (Hexane: Ethyl acetate, v/v) | |
| 6.9:3.1 | 756 ± 1.32 |
| 7.1:2.9 | 760 ± 1.40 |
| 7:3 | 764 ± 2.10 |
| Temperature (°C) | |
| 22 ± 2 | 758 ± 2.30 |
| 25 ± 2 | 762 ± 2.10 |
| 27 ± 2 | 760 ± 1.95 |
| Humidity (% RH) | |
| 45 ± 5 | 768 ± 1.20 |
| 55 ± 5 | 776 ± 2.20 |
| 65 ± 5 | 772 ± 1.80 |
| Distance for development of TLC plate (mm) | |
| 78 | 764 ± 2.40 |
| 76 | 770 ± 2.10 |
| 74 | 767 ± 2.30 |
| Wavelength (nm) | |
| 293 | 774 ± 1.60 |
| 291 | 782 ± 2.10 |
| 289 | 774 ± 2.20 |

All the experiments were performed in triplicate

**Table 12** Quantity of mahanimbine and koenimbine in $F_1$, $F_2$, and $F_3$ (in 30 mg)

| Sl No | Name of the formulations | Mahanimbine (µg) | Koenimbine |
|---|---|---|---|
| 1 | $F_1$ | 41.19 | 143.6 µg |
| 2 | $F_2$ | 53.24 | Not quantified |
| 3 | $F_3$ | 62.32 | Not quantified |

**Abbreviations**

| | |
|---|---|
| TLC | Thin-layer chromatography |
| HPTLC | High-Performance Thin-Layer Chromatography |
| LOD | Detection limit |
| LOQ | Quantitation limit |
| $R^2$ | Correlation coefficient |
| CV | Coefficient of variance |
| LC–MS | Liquid Chromatography–Mass Spectrometry |

| | |
|---|---|
| $R_f$ | Retardation factor |
| mg | Milligram |
| ICH | International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use |
| mL | Millilitre |
| v/v | Vol/vol |
| HPLC | High-performance Liquid chromatography |
| UPLC | Ultra-performance Liquid chromatography |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| GC–MS | Gas Chromatography–Mass Spectrometry |
| qNMR | Quantitative NMR |
| cm | Centimetre |
| µL | Microlitre |
| MS | Mass spectrometry |
| nm | Nanometre |
| mm | Millimetre |
| nm/s | Nanometre/second |
| nm/step | Nanometre/step |
| µm/step | Micrometre/step |
| min. | Minute |
| hrs | Hours |

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**Author contributions**
NM, RA, and KM prepared the methodology, performed the experiments, and conducted the statistical analysis. The manuscript draft was prepared by NM, KM, RA, and RS. RJS and VRK supervised and edited the manuscript. The final version of the manuscript was approved by all of the authors.

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The data sets utilized and analysed during the present study can be available upon reasonable request.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
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**Competing interests**
The authors declare that they have no competing interests.

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