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Research Article

Determination of Cucurbitacin B in *Trichosanthes dioica* Roxb by HPTLC

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ABSTRACT: **Background:** *Trichosanthes dioica* Roxb, (Cucurbitaceae), is a perennial herb distributed in Asian subcontinent. The pointed gourd is mainly cultivated as a vegetable. The Charaka Samhita prescribes its use in treating jaundice and alcoholism. Ayurvedic literature depicts its use as a diuretic, cardiotoinc, antiulcer and a laxative. Recent research trend on *Trichosanthes dioica* Roxb. has also demonstrated its anticancer potential. **Objective:** The aim of the current work was to develop a new, simple, sensitive, precise, and robust HPTLC method to determine the cucurbitacin content in fruit extracts of *Trichosanthes dioica Roxb* Linn. **Materials and Methods:** *Trichosanthes dioica* Roxb was collected from Jabalpur, India. The dried fruit powder of *Trichosanthes dioica* Roxb. was extracted with methanol in several batches by Soxhlet extraction, and subjected to HPTLC analysis. The method was also assessed for precision, robustness, limit of detection, limit of quantification, accuracy, ruggedness and specificity. **Results:** The present HPTLC method was used to establish a cucurbitacin calibration curve in the concentration range of 40 - 240 ng/spot. Regression analysis showed a good linear relationship with $r^2 > 0.99982$. The method followed the criteria of precision, robustness, limit of detection, limit of quantification, accuracy, ruggedness **and** specificity as per ICH guidelines. **Conclusion:** Statistical analysis of the data demonstrated that our method was selective and reproducible for estimation of cucurbitacin B.

KEYWORDS: *Trichosanthes dioica* Roxb., cucurbitacin B, analytical method development, ICH, HPTLC

INTRODUCTION

Trichosanthes dioica Roxb, (Cucurbitaceae), is a perennial herb distributed in Asian subcontinent. The pointed gourd is mainly cultivated as a vegetable. A number of phytoconstituents like terpenes, saponins, alkaloids, and vitamins have been detected in *T. dioica* extracts. Juice from the leaves of *T. dioica* is used as febrifuge, tonic, in alopecia, and in treatment of enlargement of liver.[1] The ancient traditional medicine book the Charaka Samhita prescribes its use in treating jaundice and alcoholism. Ayurvedic literature depicts its use as diuretic, cardiotonic, antiulcer, laxative etc. Seeds of fruits contain 7-oxidihydrokarounidol-3-benzoate, 24α-ethylcholest-7-enol, 24β-ethylcholest-7-enol. Seeds also contain lectin and peptides. Phytochemicals found plants are alkaloids, glycosides, flavonoids, carbohydrates, fixed oils, steroids, tannins, and phenols.[1] Current research on Trichosanthes dioica Roxb. demonstrates its anticancer potential.[2–5] Cucurbitacins are one of the important classes of terpene present in *Trichosanthes dioica* which exhibit anticancer activity.[6–9]

Cucurbitacins are one of the important tetracyclic triterpenoids. They are generally present in plants which prevent them from predators. In many scientific experiments cucurbitacins have demonstrated antiproliferative and anticancer potential. Apart from this, cucurbitacins have shown antimicrobial, anthelmintic, hepatoprotective and antidiabetic effects.[9] The high-performance thin layer chromatographic (HPTLC) method for quantitation of cucurbitacins from the fruit of *Trichosanthes dioica Roxb* has not been reported. The goal of the present article is to validate and determine the content of cucurbitacins in the fruit extract of *Trichosanthes dioica Roxb* by using the HPTLC method. For this purpose, a new, simple, sen-

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sitive, precise, and robust HPTLC method was developed, to determine the cucurbitacins content in the fruit extract of *Trichosanthes dioica Roxb* Linn. The method was validated for precision, intraday precision, accuracy, limit of detection, and quantitation.

MATERIALS AND METHODS

Plant collection and extraction

Trichosanthes dioica Roxb, collected from Jabalpur, India was authenticated by Dr. A. B. Tiwari, Senior Scientist and Taxonomist, Department of Plant Physiology, Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur (M.P.). A voucher specimen of *Trichosanthes dioica* fruit was submitted (Herbarium file No.-HD/PPHY/301). Analytical grade solvents were used. Standard cucurbitacin B (98% pure) was procured from Sigma Aldrich Chemie (Steinheim, Germany). Silica gel 60F HPTLC pre-coated plates were procured from Merck (Darmstadt, Germany). The dried fruit powder of *Trichosanthes dioica Roxb.* was extracted with methanol (Central Drug House, India) in several batches by using the Soxhlet apparatus. The extract thus obtained was concentrated with the help of a rotary vacuum evaporator.

Preparation of standard solution

A stock solution of cucurbitacin B (1 mg/ml) was prepared by dissolving 10 mg of accurately weighed cucurbitacin B in 10 ml with methanol. The stock solution was further diluted with methanol to give a standard solution of cucurbitacin B(40 µg/ml). This concentration was used as the working standard for the HPTLC method.

Sample preparation

An amount of 100 mg of methanolic extract of dried fruit powder of *Trichosanthes dioica* Roxb. Was accurately weighed into a 50 ml volumetric flask. It was dissolved in 10 ml of methanol by sonication. A volume of 2 ml of this solution was pipetted out in a 10 ml volumetric flask and a further 8 ml of methanol was added to give a total volume of 10 ml. The stock solution of the sample, having concentration of 0.4 mg/ml (0.4 µg/µl) was prepared thus. This concentration was used for the estimation of cucurbitacin B from the dried fruit powder of the plant material. All samples were passed through a 0.22 µ membrane filter from Millipore (Malsheim, France).

Instrumentation and chromatographic conditions

HPTLC aluminium plates pre-coated with silica gel $F60_{254}$ (10 X 10 cm) with 200 µm thickness (E. Merck, Germany) were used as the stationary phase in this study. The plates were pre-washed with methanol and activated at 110° C for 10 minutes prior to chromatography. The samples were spotted in the form of bands, of 8 mm, with the help of a Camag 100 microliter syringe using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 100 nl s^{-1} was employed and the space between two bands was 12 mm. The slit dimension was kept at 6 mm X 0.45 mm, with a scanning speed of 20 mm/second, and a data resolution of 100 µm/step was employed. The composition of the mobile phase was toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2). The linear ascending development was carried out in a twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 minutes at room temperature (25 ± 2°C). The length of the chromatogram run was 80 mm. Subsequently, the plate was allowed to dry at room temperature. The separated bands on the HPTLC plates were scanned over the wavelength range of 200 - 400 nm. The source of radiation utilized was the tungsten lamp. The maximum absorbance was found at 289 nm. The images were captured on Camag reprostar 3 with win-CATS software 4.05.

Calibration curve of cucurbitacin B

A stock solution of cucurbitacin B (0.4 µg/µl) was prepared in methanol. Different volumes of standard solution were spotted on the HPTLC plate, to obtain amounts of 40, 80, 120, 160, 200, 240 ng/spot of cucurbitacin, respectively. The data of the peak areas plotted against the corresponding concentrations were treated by least-square regression analysis.

Method validation[10,11]

Precision repeatability of the sample application and measurement of the peak area were carried out using six replicates of the same spot (120 ng $spot^{-1}$ of Cucurbitacin) and were expressed in terms of percent relative standard deviation (%R.S.D.) and standard error (S.E.). The intra- and inter-day variation for the determination of cucurbitacin B was carried out at three different amounts of 80, 120, and 160 ng $spot^{-1}$.

Robustness of the method

By introducing small changes in the mobile phase composition, mobile phase volume, duration of mobile phase saturation, and activation of the pre-washed TLC plates with methanol, the effects on the results were examined. Robustness of the method was performed in triplicate at a amount of 120 ng $spot^{-1}$ and the %R.S.D and S.E. of peak areas was calculated.

Limit of detection and limit of quantification

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times and the signal-to-noise ratio was determined. The LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were experimentally verified by diluting the known concentrations of cucurbitacin B until the average responses were approximately thrice or 10 times the standard deviation of the responses for six replicate determinations.

Accuracy

The accuracy of the method was determined by the addition of standard compounds in the samples at three different levels (75, 100, and 125 % of the peak respectively), and the mixture was analyzed under optimized conditions. The accuracy was calculated from the test result as the percentage of analytes recovered by the assay.

Ruggedness

A solution of concentration 200 ng spot^{-1} was prepared and analyzed on day 0 and after 6, 12, 24, 48, and 72 hours. Data were treated for % R.S.D., to assess the ruggedness of the method.

Specificity

The specificity of the method was ascertained by analyzing the standard drug and extract. The spot for cucurbitacin B in the sample was confirmed by comparing the Rf values and spectra of the spot with that of the standard. The peak purity of the Cucurbitacin was assessed by comparing the spectra at three different levels, namely, the peak start (S), peak apex (M), and peak end (E) positions of the spot.

RESULTS AND DISCUSSION

Development of the optimum mobile phase

TLC procedures were employed to develop the mobile phase of extract. Standard and test samples were spotted on the TLC plates, and a number of solvent systems were utilized to achieve a good separation. Ultimately, Ethyl acetate- methanol-water in ratio 81:11:8 was utilized which gave a good resolution for the standard with Rf= 0.70. Well-defined spots were obtained when the chamber was saturated with the mobile phase for 30 minutes, at room temperature. The R f value of the plant extract was compared with the standard. The HPTLC plate pictures are shown a little later in the text, with the selected solvent system.

Calibration curves of cucurbitacin B

The calibration curve was developed for cucurbitacin at a specific Rf value. The present HPTLC method has shown a calibration curve in the concentration range of 40–240 ng/spot for cucurbitacin. Regression analysis showed a good linear relationship with $r^2 > 0.99982$ for cucurbitacin. The standard deviation of intercept has been found to be less than 2%. No significant difference has been observed in the slopes of the standard curves.

Method validation

Repeatability of the procedure was validated in system and method precisions. System precision was measured for six spots of the cucurbitacin. The results of the precisions were expressed in % R.S.D and are summarized in Table 1. System and method precisions were found to be less than 2%. Intraday precision was determined by spotting the three different concentrations of cucurbitacin, six times a day. Intraday precisions results were expressed in % R.S.D. and dried fruit extract was found to be less than 2%. The accuracy was determined by the standard addition technique. Known amounts of the reference compound in three different levels (75, 100, 125%) were added to the sample (fruit extract) and chromatography was conducted under optimized conditions. The accuracy was then calculated from the test results as the percentage of analytes recovered by the assay. The results indicated that the accuracy of the method was very good, as supported by the recovery of 101.39 - 104.87% (Table 2).

Table 1: Intra and Inter day precision of HPTLC method.

Amount (ng/spot)	Intra-day precision		Inter-day precision	
	Mean±SD	RSD	Mean±SD	RSD
80	1219.45±1.12	0.08	1220.36±1.10	0.08
120	1939.78±1.20	0.06	1940.11±1.37	0.08
160	2668.32±1.12	0.04	2669.45±1.52	0.05

Table 2: Recoveries study data of HPTLC studies.

Excess Standard Added (%)	Amount of standard added (ng)	Amount of standard drug determined (ng)	Recovery (%)	% R.S.D.	S.E.
75	175	170.45	95.98	1.27	0.509
100	200	195.67	96.07	0.35	0.136
125	225	217.28	94.32	0.85	0.345

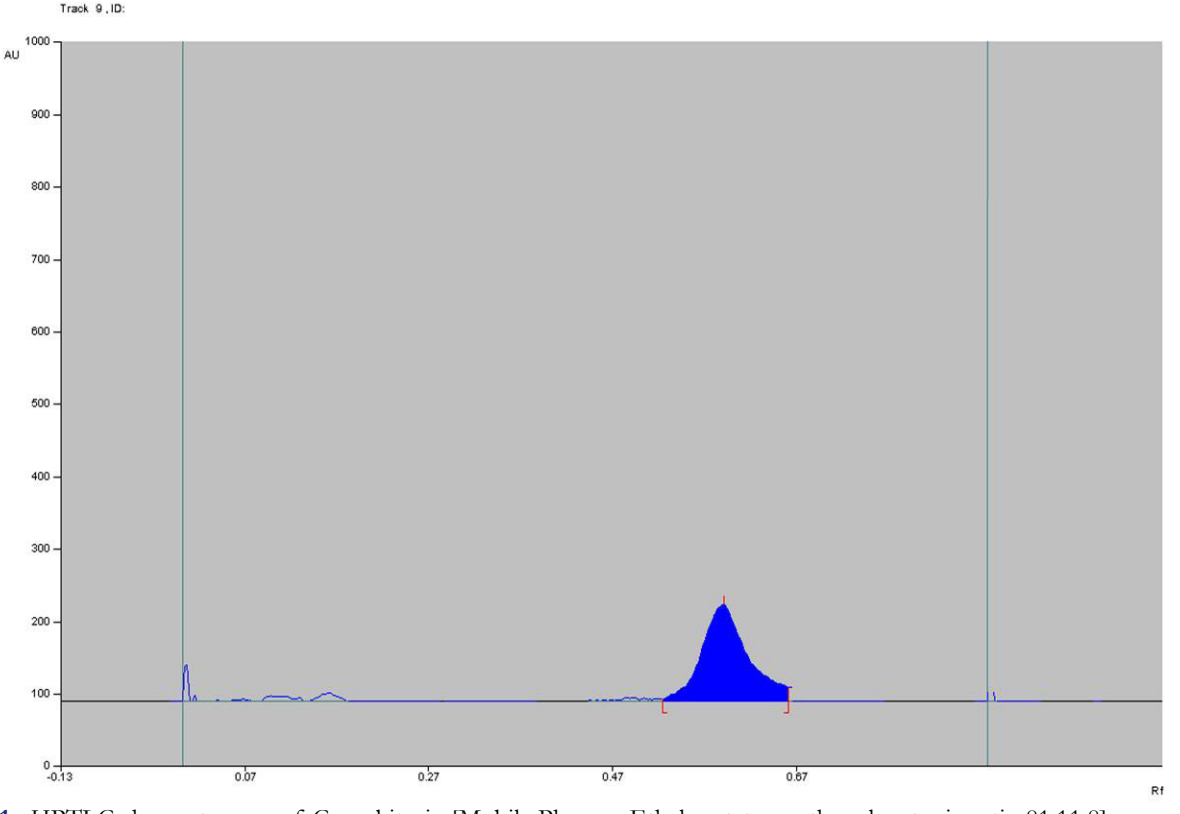

Figure 1. HPTLC chromatogram of Cucurbitacin [Mobile Phase = Ethyl acetate- methanol-water in ratio 81:11:8]

CONCLUSION

HPTLC method was developed and validated for the simultaneous determination of cucurbitacin B. The method was found to be simple, rapid, accurate, specific, and robust for the analysis of cucurbitacin B in *Trichosanthes dioica* Roxb, which could be adopted by any laboratory for the quality control of crude drugs and formulations that contains cucurbitacin B as active markers or *Trichosanthes dioica* Roxb as an ingredient.

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