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Chromatographic Fingerprinting and Quantitative Analysis of Marker in the Extract of *Gloriosa superba* Tubers Collected from Some Region of Chhattisgarh

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ABSTRACT

Background: *Gloriosa superba* (Family: *Liliaceae*) is commonly known as Kalihari in India and has been used by several indigenous communities to treat a snake bite, skin diseases and joint pain. It has been also scientifically reported for many pharmacological activities such as hypoglycaemic, hepatoprotective, anticancer, anti-inflammatory. Present work is an effort to develop validated HPTLC method for the detection and quantification of chief constituent in the tuber extract of *Gloriosa superba*. **Methods:** HPTLC analysis of tuber extract has been performed on Silica gel 60 F_{254} (10 cm×10 cm) plates with mobile phase consisting toluene, ethyl acetate and diethylamine (02:08:02, v/v/v). Densitometric scanning of the plate was performed at 371nm by using CAMAG TLC scanner III equipped with visionCATS version 2.4.17207.2 (CAMAG) and developed method was also validated for accuracy, precision and robustness as per ICH guidelines. **Results:** present work has confirmed the rich content of colchicine in tuber extract of *Gloriosa superba*. The calibration curve was linear in the selected range of 0.4-1.2 µg/spot and regression equation found to be y = 0.0285x + 0.0074. the correlation coefficient (r) was 0.9978 for the regression equation. The LOQ and LOD was 0.170 µg /spot and 0.056 µg /spot respectively. The average recovery of colchicine at three levels was 99.5, 98.6 and 99.6 %, which indicated the remarkable reproducibility of the method. **Conclusion:** findings revealed that the developed method is simple, precise, and accurate for quantitative analysis of *Gloriosa superba*; and it might be useful for quality control of herbal medicine.

Key words: Chromatography, Kalihari, Quantitative, HPTLC, *Liliaceae*.

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INTRODUCTION

Gloriosa superba is enormously valued in Indian folk medicine and its tremendous medicinal potential has been well mentioned in the Ayurveda for the treatment of various ailments.[1] It is usually known as "kalihari" in India, belonging to family *Liliaceae*, deciduous climbing shrub with ***Appealing*** wavy-edged yellow and red flowers.[2-5] Leaves are ovate, lanceolate and twist at the tip that empowers the plant to climb 10 feet or higher; the stem is 1-2 meter high; tuber are perennial and V or L shaped, roots are fibrous; flowers are large singular and axillary.[6-8] It is widely dispersed around most tropical and subtropical nations such as Africa, India, Sri Lanka, Bangladesh, Myanmar and Malaysia.[9,10] As a folkloric medicine, seed and tuber part of *Gloriosa superba* has been used in the tribal region of different countries to treat diseases like chronic ulcer, leprosy, intestinal worm, joint pain, snake bite, skin diseases etc.[6,11-14] It has been also claimed for several therapeutic potentials by many *in-vitro* and *in vivo* screening for hypoglycaemic, anticancer, hepatoprotective, anthelmintic, anti-inflammatory, analgesic, antimicrobial, antivenom, and antifungal activity.[13,15-22] In last few years, it has been mostly researched for its chemotherapeutic nature against various carcinomas such as Lung cancer (A549) cell line, breast cancer (MCF-7 and MDA-MB231) cell line, pancreatic carcinoma (PANC-1) cell line and bacteria.[21,23] Results of antimicrobial and anticancer studies were significant, which suggested that the medicinal properties of *Gloriosa superba* could be attributed to its precious alkaloidal content. This plant contains a number of alkaloidal compounds, which are mainly colchicine, colchicoside, and other colchicine derivatives.[24] Nowadays, the various advanced analytical techniques (HPLC, GCMS and NMR-spectroscopy) has also contributed to identify some other colchicine derivatives such as N-formyl-N-deacetylcolchicine. 3-demethylcolchicine, β and γ-lumicolchicine.[25-28]

Gloriosa superba is part of the traditional medicinal system of India and its therapeutic potential has also proven in different pharmacological screening. Hence, for its therapeutic use as herbal medicine, there is great need of a precise validated method for qualitative and quantitative determination of its marker constituents.[29] Therefore, the present work is intended to develop a chromatographic profile for the quantification of marker compound in tuber extract of *Gloriosa superba* by using a validated HPTLC method.[30]

MATERIALS AND METHODS

Plant material and chemicals

The tuber part of plant *Gloriosa superba* was collected during the month of September from some region of Korba (Chhattisgarh, India). Tubers were cleaned with water and dried under the shade then coarsely powdered and stored in airtight containers for further study.[31,32] The plant was identified and authenticated by Dr. N.K. Dubey, Department of Botany, Banaras Hindu University, Varanasi (U.P.). Herbarium specimen of *Gloriosa superba*, bearing voucher specimen number *Lilia.* 2018/02 was deposited in the Department of Botany, Banaras Hindu University, Varanasi.

All the reagents, solvents, and chemicals utilized in the estimation were an analytical grade.[33] Colchicine was used as a standard drug, obtained from Sigma Aldrich (Mumbai, India).[34]

Preparation of plant extract

Pulverized tubers were extracted with ethanol using a Soxhlet apparatus.[35] The extracts were then evaporated to dryness to obtain alcoholic extract.[36,37]

Phytochemical screening

Tuber extract was analysed for preliminary phytochemical analysis by utilizing different reagents such as Mayer, Dragendroff, Wagner, etc.[38,39]

Preparation of stock, working standard and sample Solutions

A stock solution of standard (1000 µg/ml) was prepared by dissolving 25 mg colchicine in 15 ml of methanol in a 25 ml volumetric flask then sonicated for 10 minutes. After sonication volume made up to 25 ml with methanol and filtered by using Whatman filter paper no. 41 (E. Merck, Mumbai, India). From the dilution of stock solution, working standard solutions were prepared to achieve the concentration range of 0.4-1.2 µg/spot on the HPTLC plate.[40,41]

The sample solution was prepared by dissolving 25 mg tuber extract of *Gloriosa superba* in 15 ml of methanol in a 25 ml volumetric flask and sonicated for 10 minutes. After sonication, the volume made up to 25 ml to obtain 1000 µg/ml concentration. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India), and the filtrate solution was utilized as the sample solution for assay.[42]

High-performance thin-layer chromatographic analysis

High-performance thin layer chromatography was carried out using 10 cm × 10 cm aluminium plates coated with silica gel 60 F_{254} (Merck, Germany). Length of 8 mm bands of colchicine (standard) and tuber extract (Sample) were spotted at 10 mm from the bottom edge and 15 mm from the side edge of HPTLC plate by CAMAG (Muttenz, Switzerland) Linomat V sample applicator with a 100 µl Hamilton (USA) syringe.[43] Development of chromatogram was done in CAMAG glass twin- trough chamber saturated with mobile phase comprising toluene: ethyl acetate: diethylamine (02:08:02, v/v/v) at room temperature (28±2°C). Then plates were allowed to dry in the air for further scanning process.[41]

Densitometric evaluation of the chromatograms

The developed chromatogram was fixed on CAMAG TLC Scanner III for densitometric evaluation using visionCATS software (version 2.4.17207.2). The scanner operating parameters were set as follows: Mode: absorption/reflection; slit dimension: 5×0.45 and 20 mm/s scanning speed at optimized wavelengths of 371 nm.[44]

Linearity, limits of quantification and detection

The Calibration curve was plotted as per peak area obtained for a selected range of concentrations (0.4-1.2 µg/spot). The regression equation and correlation coefficient (r) were obtained from the calibration curve.[45,46] The limit of quantification (LOQ) and limit of detection (LOD) were calculated by the equations:

$$LOD = 3.3\times(SD/S) \text{ and } LOQ = 10\times(SD/S)$$

Where SD is the standard deviation of the peak areas of standard drug, S is the slope of the corresponding calibration curve.[47-49]

Accuracy

The Accuracy of the method was determined by recovery studies using method of standard addition. Recovery studies were performed by addition of colchicine (standard) in a pre-analyzed sample (tuber extract) at three different levels, then tested using developed HPTLC method that mentioned earlier. Thereafter, percent recovery and RSD were calculated (Table 2).[50]

Precision

Intraday and inter-day precision have been determined by application and analysis of peak area of each selected three concentrations (0.4, 0.6, 0.8 µg/spot) of standard (colchicine) in five different times in a day and in five different days by using the proposed method. Then as per the peak area of five replicates of each concentration, percent RSD was calculated (Table 3).[51]

Robustness

Robustness of the proposed HPTLC method was detected for selected concentration of 0.4 µg/spot by changing the proportion of solvents of mobile phase for chromatographic determination of colchicine. Robustness were calculated in the term of %RSD.[28]

RESULTS

Phytochemical screening

Phytochemical screening indicated the presence of Alkaloid, carbohydrate, Glycoside, Sterols in *Gloriosa superba* tubers.

HPTLC Analysis

HPTLC analysis of tuber extract of *Gloriosa superba* has been performed after selecting the optimized solvent system for better separation of constituents. The combination of toluene, ethyl acetate and diethylamine (at the proportion of 02:08:02, v/v/v) has been selected after testing different proportions of solvents for the development of chromatogram. The selected mobile phase exhibited considerable separation of constituents of the ethanolic extract of the tuber. For the development of chromatogram, saturation time and volume of the mobile phase were 25 minutes and 25mL respectively. The developed chromatogram was visualized at 254 nm (Figure 1). The bands were compact, sharp, symmetrical, and obtained at R_f 0.16 ± 0.010. The densitometric evaluation was conducted at 371 nm for tuber extract and colchicine (Figure 2 and Figure 3).[52]

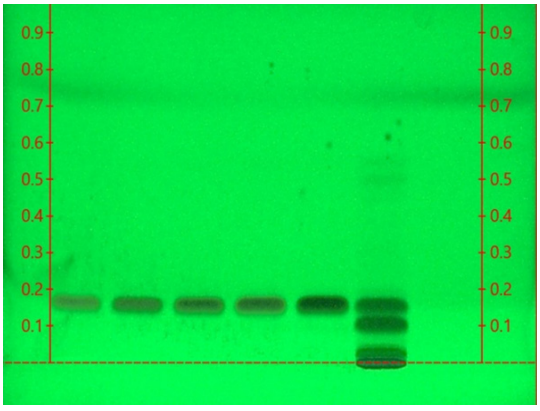

Figure 1: Chromatogram of tuber extract of *Gloriosa superba* at wavelength of 254 nm.

Colchicine content was also found 3.7 (% w/w) in the extract, which indicated its rich content in the tuber part of *Gloriosa superba*.

Linearity, limits of quantification and detection

The calibration curve was established for the selected standard concentration range of 0.4 to 1.2 µg /spot (Figure 4). Linearity has been found in the plotted curve of peak area against concentration and regression equation found to be y = 0.0285x + 0.0074. The correlation coefficient (*r*) was 0.9978 for the regression equation. The limit of quantification and limit of detection was 0.170 µg /spot and 0.056 µg / spot respectively (Table 1).[48]

Accuracy

The accuracy of the method has been determined in terms of percent recovery, which is obtained by HPTLC analysis after the addition of standard at three levels. The recovery values were obtained in the range 98.6–99.7% (Table 2), showing the reliability, and accuracy of the method.[53]

Precision

It has been determined in terms of percentage relative standard deviation for intra-day and inter-day precision analysis (depicted in Table 3). Percent RSD for Intraday precision was between 0.134-0.645 and for Inter-day precision, Percent RSD was 0.172-0.501.[53]

Robustness

Robustness has represented in term of SD and % RSD was calculated for colchicine. Percent RSD was found between 0.39-0.59, which indicated the robustness of the method (Table 4). [28]

Table 1: Method validation parameters for the quantification of colchicine by HPTLC method.

Parameters	Results
Linearity range (µg/spot)	0.4-1.2 µg/spot
Regression equation	y = 0.0285x + 0.0074
Correlation coefficient (r^2)	0.9978
Slope	0.0285
Intercept	0.0074
LOD	0.056 µg /spot
LOQ	0.170 µg /spot

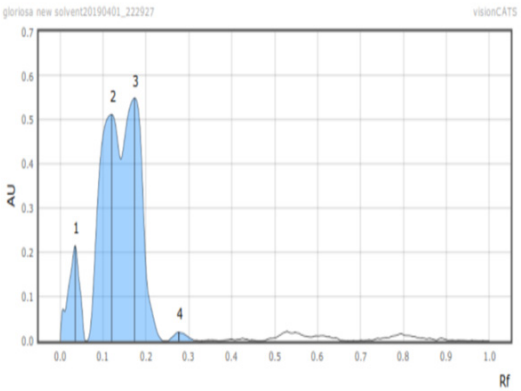

Figure 2: Densitogram of tuber extract of *Gloriosa superba*.

DISCUSSION

In the last few decades, herbal medicine showed the tremendous therapeutic capacity to cure and management of chronic diseases, which enhance the reliability and use of herbal medicine. Hence, it is very essential to develop precise methods using analytical techniques to control the quality of herbal medicine.[54] In this recent era, Classic analytical techniques become more advanced, and new modern analytical techniques have invented for quantitative analysis of medicines. These techniques have been given great contributions to the development of an accurate, sensitive and reliable method for the identification and quantitative analysis of marker compounds in herbal medicine.[55] Among these techniques, High-performance thin layer chromatographic (HPTLC) technique is one of the most preferred analytical tools for quality and safety assessment of herbal medicines.[56] Hence, there are many validated analytical methods were developed by using HPTLC for numerous herbs such as *Amaranthus spinosus, Artemisia annua, Withania somnifera, Wattakaka volubilis* etc.; which revealed reliability and importance of HPTLC in the standardization of herbal medicine.[45,50,57,58]

In the present study, a validated method has been developed for the estimation of marker compound (colchicine) in *Gloriosa superba* by using HPTLC. The developed method revealed that the mobile phase comprising Toluene: Ethylacetate: Diethylamine (02:08:02, v/v/v) showed clear separation of marker compounds of tuber extract. A linear calibration curve has found for colchicine in the range of 0.4-1.2 µg/spot with a correlation coefficient of 0.9978. LOD and LOQ for colchicine were 0.056 µg /spot and 0.170 µg /spot, which showed adequate sensitivity

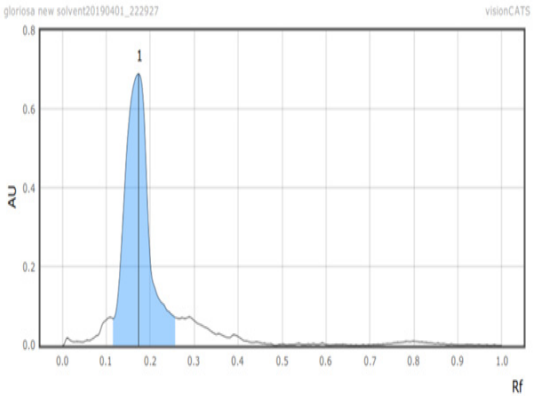

Figure 3: Densitogram of standard (Colchicine).

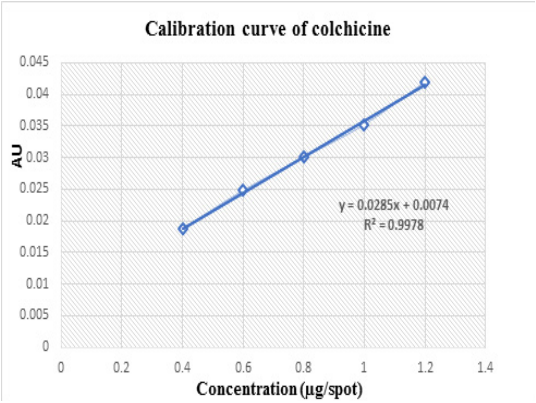

Figure 4: Calibration curve of colchicine.

Table 2: Results of recovery study (n=5).

Amount of colchicine in prenasalized sample (in µg)	Percentage spike (%)	Added Amount of colchicine (in µg)	Total measured amount of colchicine in mixture (in µg)	Amount of colchicine found in mixture (in µg)	RSD (%)	Recovery (%)
37.9	0	0	37.9	37.8	0.50	99.7
37.9	50	18.9	56.8	56.7	0.37	99.5
37.9	100	37.9	75.8	75.3	0.39	98.6
37.9	150	56.8	94.7	94.5	0.08	99.6

RSD=Relative Standard Deviation

Table 3: Results of intra-day and inter-day precision study (*n*=5).

Concentration µg/spot	Intra-day		Inter-day	
	Average conc. Found ± SD	RSD (%)	Average conc. Found ± SD	RSD (%)
0.4	0.398 ±0.0025	0.645	0.397 ±0.0019	0.501
0.6	0.608 ±0.0032	0.532	0.604 ±0.0027	0.459
0.8	0.793 ±0.0010	0.134	0.793 ±0.0013	0.172

RSD= Relative Standard Deviation, SD=Standard Deviation

Table 4: Results of robustness study (*n*=5).

Concentration (µg/spot)	Mobile phase ratio		Results	
	Original	Used	SD	% RSD
0.4	02:08:02	03:07:02	0.0023	0.59
		02:08:02	0.0015	0.39
		01:09:02	0.0018	0.46

RSD= Relative Standard Deviation, SD=Standard Deviation

of the proposed method. The content of colchicine was quantified in tuber extract and found to be 3.7 (% w/w), which attributed to the pharmacological activity of tubers of *Gloriosa superba*. Accuracy of the method has also investigated by standard addition method by spiking the amount of colchicine at 50, 100 and 150%. In the accuracy study, the percent recoveries were between 98.6-99.7%, which indicated adequate reliability, repeatability, and accuracy of the method.[59] The percent RSD obtained for intraday and inter-day precision were in between 0.134-0.645, which is significant as per ICH guidelines and suggested that the method is precise. In robustness study, low present RSD was indicated that the proposed method is robust. Our findings revealed that the proposed method is precise, sensitive, suitable and a need for quantitative analysis of *Gloriosa superba***.**

CONCLUSION

Gloriosa superba is one of the very important herb, that traditionally used by indigenous cultures of different countries to cure joint pain, dermatological diseases, and snakebite.[6] Its therapeutic efficacies such as antidiabetic, anticancer, hepatoprotective and antimicrobial, etc., have been also proven by scientists.[15-17,21] Thus, it is a great need of its standardization by analytical techniques to enhance Better and safe utilization of its constituents in the health care system. Present work is an effort to develop a simple and precise method, to determine and quantify chief constituents of the tuber part of *Gloriosa superba*. It has been found that present work can be useful for analytical research, routine analysis, and standardization of plant crude extract to control its qualitative and quantitative parameters.[44]

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CONFLICT OF INTEREST

There is no conflict of interest.

ABBREVIATIONS

HPTLC: High-performance thin-layer chromatography; **HPLC:** High-Performance Liquid Chromatography; **GC-MS:** Gas Chromatography Mass Spectrometry; **NMR:** Nuclear magnetic Resonance; **ICH:** International Conference on Harmonization; **R_f:** Retention factor**; ml:** Millilitre; **mg:** Milligram; **µg:** Microgram; **r:** Correlation coefficient; **°C:** Degrees Centigrade; **cm:** Centimetre; **LOD:** Limit of detection; **LOQ:** Limit of quantitation**; s:** Second(s); **RSD:** Relative standard deviation; **SD:** Standard deviation; **w/w:** Weight by weight.

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