# DEVELOPMENT OF VALIDATED HPTLC FOR SIMULTANEOUS QUANTIFICATION OF GYMNEMIC ACID THROUGH GYMNEMAGENIN IN LEAF AND LEAF CALLUS OF *GYMNEMA SYLVESTRE* R. BR.

[1]Leena Koshti, [2]Darshana Patil, [3]Avinash Patil

[1]PhD Scholar, [2] Assistant Professor, [3] Principal

[1,3] Dept. of Biotechnology, B. K. Birla College of Arts, Science & Commerce (Autonomous), Kalyan (West), Maharashtra, India.

[2]Dept. of Botany, Smt. C.H.M. College, Ulhasnagar-03, Maharashtra, India.

## Abstract

*Gymnema sylvestre* R. Br. (Family: Asclepiadaceae), is a rare medicinal plant with significant medicinal attributes due to its principle phytoconstituent- "Gymnemic acid". The objective of the present study was to isolate and quantify Gymnemic Acid through Gymnemagenin in leaf and leaf calli of *Gymnema sylvestre* R. Br. as per ICH (International Conference on Harmonization) guidelines. The Extraction of Gymnemic acid involved simple solvent extraction, followed by successive basic and acidic hydrolysis process. A precise and rapid high-performance thin-layer chromatography (HPTLC) method was developed and validated for Gymnemic acid analysis. The method involved separation on TLC aluminium precoated silica gel 60 $F_{254}$ plate using ethyl acetate and methanol [5:6 (v/v)] as mobile phase. The purity and identity of peaks of leaf and callus cultures were confirmed by matching Rf values and UV-spectrum with authentic standard Gymnemic acid. The method showed good linear relationship in the range 0.3-0.7 µg/band with $r^2$ 0.99. LOD and LOQ for Gymnemic acid was recorded as 52.8 ng/band and 160 ng/band respectively. The proposed HPTLC method was found to be simple and accurate to use and could be successfully used for routine quality control analysis of herbal material as well as formulations containing Gymnemic acid.

**Keywords:** *Gymnema sylvestre* R. Br.; Gymnemic acid; Gymnemagenin; Callus; high-performance thin-layer chromatography.

## I. Introduction

*Gymnema sylvestre* R. Br., commonly known as 'Gudmar' (sugar killer) in Hindi and 'Periploca of the woods' in English, is a highly valued medicinal plant (Climber). It is an endangered medicinal woody climber native to the tropical forests of southern and central parts of India [1, 2]. Traditionally the plant is believed to be good neutralizer of excess sugar present in the human body and hence it is one of the most important botanicals being used for treating diabetes [3]. Apart from these, the plant is found to be effective against a number of diseases because of its anti-hypercholesterolemia [4], anti-hyperlipidemia [5], antiviral and anti-inflammatory [6] activities.

The properties of this indigenous medicinal plant are believed to be due to the presence of Gymnemic acids predominantly found in its leaves. Gymnemic acids (Fig. 1A) are complex mixture of pentacyclic triterpenic saponins with Gymnemagenin as a common aglycone [7]. Gymnemagenin (Fig. 1B) are produced after successive acidic and basic hydrolysis process and are known to possess anti-saccharine properties that inhibit glucose absorption [8]. Also, they are commonly used as an analytical biomarker to determine the quality of plant materials from *G. sylvestre* R. Br. [9, 10].

Increased demand and over exploitation of the drug due to its immense pharmaceutical importance has led the plant species to become extinct. Lack of awareness about the scientific harvesting practices is the main cause for several medicinal species getting rare from their natural ecosystem [11]. Early attempts had been made for the isolation and extraction of Gymnemic acid from the *G. sylvestre* R. Br. using several methods

*viz*. solvent extraction followed by column chromatography, separation on the basis of acidic and neutral fractions, etc. [12]. However the methods used were found to be time consuming, tedious with low yield and hence could not be applied on a commercial scale.

Moreover, determination of Gymnemic acids in plant materials is ambiguous as these occur as mixture of glycosides and their pure reference standards are not available. Thus, the objective of the present study was to isolate Gymnemic acid through Gymnemagenin on a commercial scale. Also, an alternate HPTLC densitometric method for the isolation and quantification of this biomarker compound was developed and validated as per ICH guidelines.

High performance thin layer chromatography (HPTLC) is the most versatile and routinely applied chromatographic technique due to its simplicity, lesser analysis time and cost effectiveness with greater accuracy [13, 14, 15]. Also, the method is applicable for the quality evaluation and chemoprofile development of complex herbal compounds in extract or formulations.

A thorough literature survey revealed that very few reports are available for simultaneous isolation and quantification of Gymnemic acid from leaf and callus cultures of *G. sylvestre* R. Br. using HPTLC or any other analytical technique [16]. Thus, the HPTLC method developed in the present research work can be used to identify, quantify and standardize the raw materials used in industries as well as *in vivo* and *in vitro* cell or tissue extracts containing Gymnemic acid.

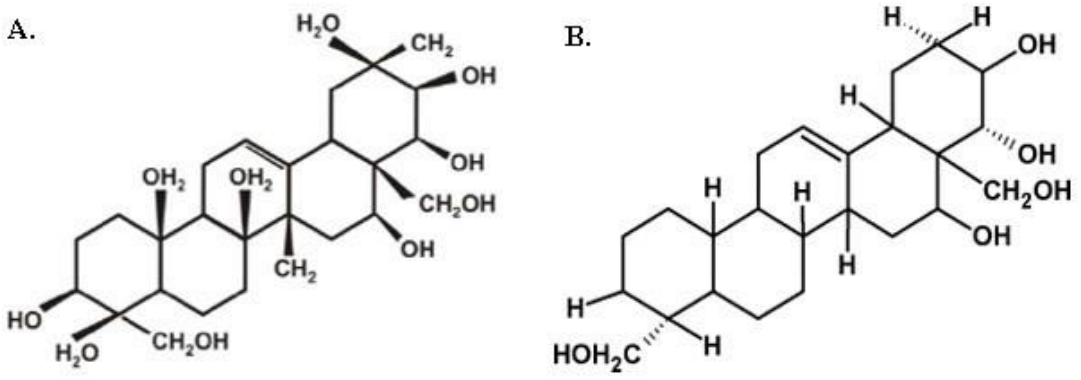



**Figure 1: A. Structure of Gymnemic acid [17] and B. Gymnemagenin [10]**

## II. Materials and methods

### 2.1. Reference Standard and Reagents

Ethyl acetate, chloroform, glacial acetic acid and methanol were of laboratory grade and purchased from S. D. Fine Chemicals, Mumbai, India. Standard Gymnemic acid as Gymnemagenin (≥ 94% purity) was purchased from Yucca enterprises, Mumbai, India.

### 2.2. Plant Material

*Gymnema sylvestre* R. Br. plants were collected from Alibaug, Raigad district, Maharashtra, India. A Herbarium of the plant was prepared and authenticated from Blatter Herbarium, St. Xavier's College, Mumbai. The fresh and healthy leaves of *Gymnema sylvestre* R. Br. were selected as explants for in *vitro* callus culture study and some leaves were air dried, ground into fine powder and used for extraction and estimation of Gymnemic Acid.

### 2.3. Establishment of Callus culture

The leaf explants were thoroughly washed under tap water and finally washed using distilled water. The explants were then treated with 70% ethanol for 15 seconds under aseptic conditions with frequent swirling so that the surface of plant material come thoroughly in contact with sterilant. The explants were further treated

with 3% Sodium hypochlorite (NaOCl, v/v) for 2 minutes and then rinsed thrice with autoclaved distilled water to remove all traces of sterilant. Later, they were treated with 0.1% Mercuric chloride ($HgCl_2$, w/v) for 1 minute and rinsed thoroughly with sterile distilled water thrice to remove traces of sterilant. Each leaf was then cut into four regions (apical, middle, basal and petiole), trimmed from edges and separated. Further, these explants were inoculated on Murashige and Skoog (MS) medium [18] and Woody Plant Medium (WPM) [19] supplemented with various concentrations and combinations of auxins *viz*. 2,4-dichlorophenoxyacetic acid (2, 4-D), indole 3 acetic acid; (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA) and cytokinins *viz*. 6-benzylaminopurine (BAP) and Kinetin(KIN) singly and in combination. After inoculation, the culture tubes were first placed in dark for one week and then transferred to illumination with 16 hours photoperiod and 8 hours dark period at 25 ± 20 $^0$C. Callus cultures were observed periodically in terms of growth and color and the results were recorded and analyzed. The calli were sub cultured at regular intervals in respective medium for further growth. Further, the calli showing maximum growth were selected on the basis of their fresh weight and subjected to drying in hot air oven at 50 $^0$C for 3- 4 days. After drying, calli were powdered using mortar and pestle and stored in air-tight bottles at room temperature.

### 2.4. Standard Preparation for HPTLC analysis

A stock solution of reference standard (1mg/ml) was prepared by accurately weighing 10 mg Gymnemic acid as Gymnemagenin in 5 ml of methanol, followed by vortexing and making up the total volume of solution to 10 ml with methanol. The above stock solution was further diluted with methanol to give a working standard solution of appropriate concentrations ranging from 0.3 - 0.7 µg/band.

### 2.5. Sample Preparation

500 mg of leaf and callus samples were extracted using 10 ml of methanol: water (1:1) and 2 ml of 11 % potassium hydroxide (KOH) solution by refluxing for 1 hour. 1.8 ml of HCl was added to extract and refluxed again for an hour. The mixture obtained was then cooled to room temperature and the pH was adjusted in between 7.5 to 8.5 with 1 % NaOH solution. Further, the mixture was diluted to 50 ml with 50 % of methanol in volumetric flask and filtered through 0.45 µm nylon filter (Millipore). The volume was made up to 50 ml with methanol and the clear supernatant was used for HPTLC analysis [1, 20].

### 2.6. HPTLC analysis

2.6.1. Chromatographic conditions

Chromatographic separation were performed on TLC aluminium precoated silica gel 60 $F_{254}$ Plate (Merck). 10 µl of extracts (leaf and calli) and standards were applied in triplicate to the plate as 8 mm band length using CAMAG Linomat 5 TLC applicator equipped with 100 µL syringe (Hamilton, Bonaduz, Switzerland). After the application, the linear ascending development of plate was carried out in a glass twin trough chamber (CAMAG, Switzerland) pre-saturated using ethyl acetate and methanol as mobile phase (10 ml) in the ratio of 5:6 (v/v) for 20 minutes at room temperature. The length of chromatogram run was 8 cm from the lower edge of the plate. After development, the plate was dried with the aid of hair dryer for complete removal of mobile phase. The plate was further derivatized by dipping in Anisaldehyde-sulphuric acid reagent for 2 sec. and dried at room temperature. After drying, the plate was heated on HPTLC Plate heater at 110 $^0$C for 10 sec. and photo documented using photo-documentation chamber (CAMAG REPROSTAR 3).

Quantitative estimation was performed using CAMAG Scanner 3 in conjunction with winCATS software version 1.4.6. The plate was scanned under UV 366 nm and visible light after derivatization. The slit dimension used was 6.0×0.45 mm with scanning speed of 20 mm/sec., throughout the analysis. Precision of retention factor (Rf) values was based on 10 successive measurements. The developed and derivatized HPTLC plate and HPTLC densitogram (at 540 nm) for standard and sample tracks of leaf and callus extracts are shown in Plate 1 A and B and Fig.2 A, B and C respectively.

## 2.7. Method Validation

The HPTLC method was validated as per the International Conference on Harmonization (ICH) guidelines for linearity, detection limit (LOD), quantification limit (LOQ), precision, accuracy, specificity and ruggedness [21].

2.7.1. Linearity

Five serial dilutions *viz*. 0.3 µg/band, 0.4 µg/band, 0.5 µg/band, 0.6 µg/band and 0.7 µg/band of stock solution (1mg/ml) of Gymnemic acid were analyzed to construct the calibration curve. The calibration curves was determined by plotting peak area against concentrations of standard applied.

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

The limit of detection (LOD) and limit of quantification (LOQ) were determined on the basis of signal to noise ratio by using the following formula, (LOD = 3.3 σ /S) and (LOQ = 10 σ /S).Where, 'σ' is the residual standard deviation of the intercept and 'S' is the slope of the calibration curve.

2.7.3. Precision

Precision was assessed on the basis of two parameters *viz*. repeatability and intermediate precision. In repeatability, the spiked samples were examined under the same operating conditions over a short time and inter assay precision. Whereas, Intermediate precision involves intraday and interday precision studies. Intraday precision studies deals with analysis performed on the same day (intra-day) with an interval of 1 hour while in intraday precision studies, the analysis was carried out on three different days (inter-day). The complete precision study was carried out in triplicate and the results were expressed as % CV.

2.7.4. Accuracy

The accuracy was calculated by performing recovery studies using a method of standard addition. A known amount of Gymnemagenin with three different levels (80, 100 and 120 %) was spiked to the known concentration of sample and each sample mixture was performed in triplicate to get the percent recovery.

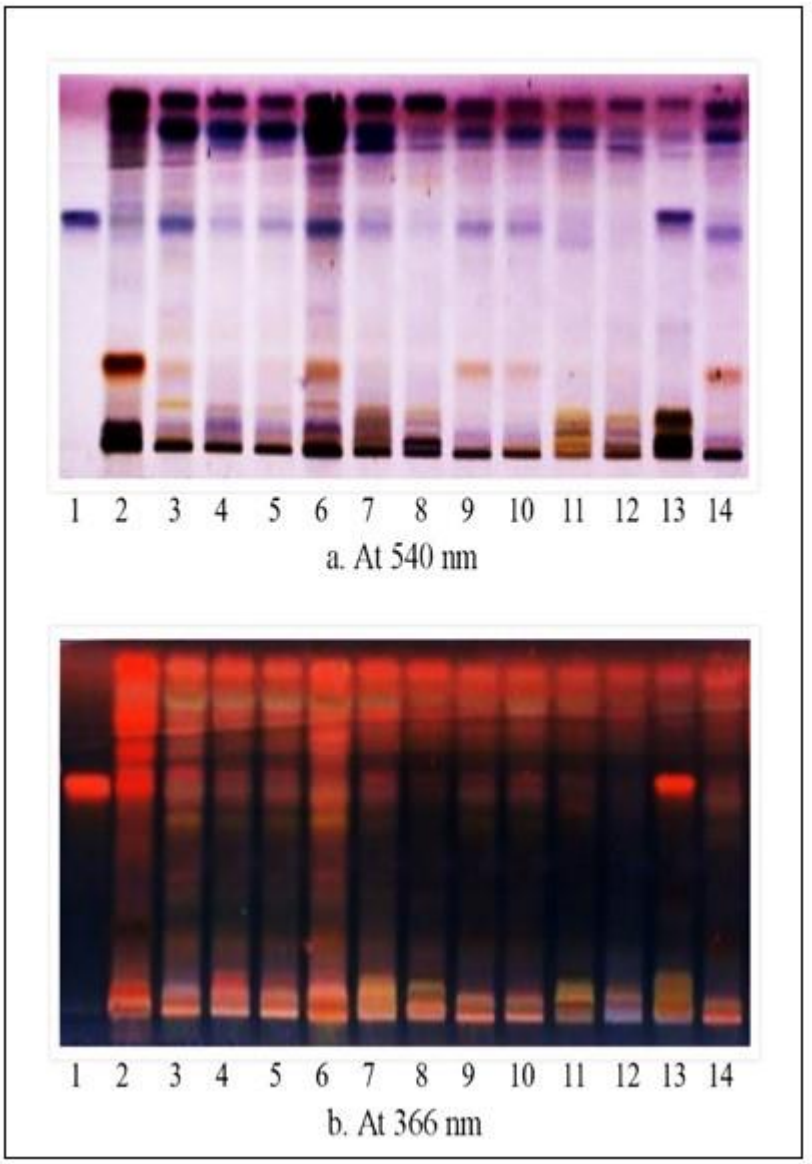
2.7.5. Specificity

The specificity was determined by analyzing the standard and samples. The bands for Gymnemic acid from leaf and calli extracts were identified by comparing the Rf values and UV spectra of the bands with those of standard Gymnemic acid. The peak purity of the standard was checked by comparing the peak area and position in the spectrum.

2.7.6. Ruggedness

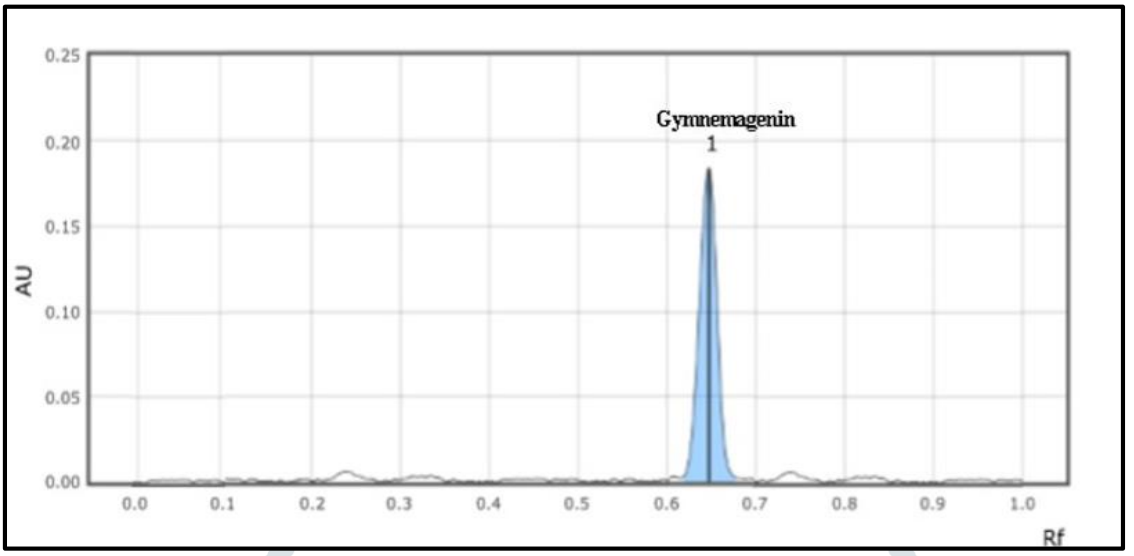
Ruggedness was checked by varying the selected parameters *viz*. mobile phase composition, duration of mobile phase saturation and spotting volume within certain limits to assess their effect on the retention factor and quantitative analysis.

**Plate 1: Developed and Derivatized HPTLC profile of leaf and leaf calli (MS medium) of *G. sylvestre* R. Br. with reference standard- Gymnemic acid under A. At 366 nm (UV light) and B. At 540 nm (Visible light)**



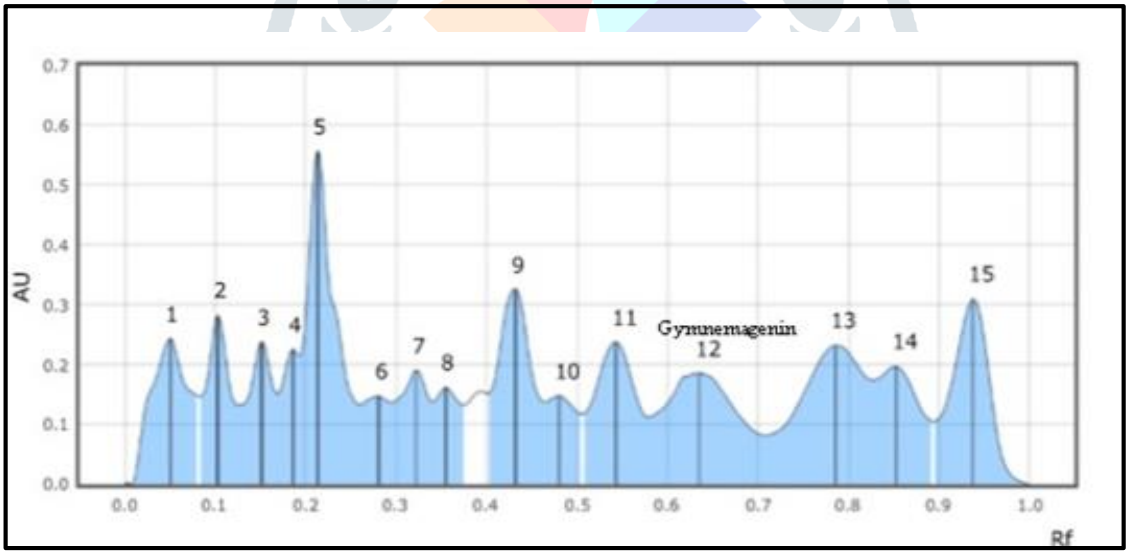

1. Standard Gymnemic acid as Gymnemagenin;
2. Leaf extract;
3. MS + 10mg/l 2, 4-D + 5 mg/l BAP leaf callus;
4. MS + 9 mg/l 2, 4-D + 4 mg/l BAP leaf callus;
5. MS + 8 mg/l 2, 4-D + 3 mg/l BAP leaf callus;
6. MS + 5 mg/l 2, 4-D + 5 mg/l BAP leaf callus;
7. MS + 5 mg/l 2, 4-D + 4 mg/l BAP leaf callus;
8. MS + 5 mg/l 2, 4-D + 3 mg/l BAP leaf callus;
9. MS + 5 mg/l 2, 4-D + 2 mg/l BAP leaf callus;
10. MS + 4 mg/l 2, 4-D + 3 mg/l BAP leaf callus;
11. MS + 3 mg/l 2, 4-D + 2 mg/l BAP leaf callus;
12. MS + 2 mg/l 2, 4-D + 2 mg/l BAP leaf callus;
13. MS + 2 mg/l 2, 4-D + 1 mg/l BAP leaf callus;
14. MS +1 mg/l 2, 4-D +1 mg/ml BAP leaf callus

**Figure 2A: HPTLC densitogram of reference standards- Gymnemic acid as Gymnemagenin**



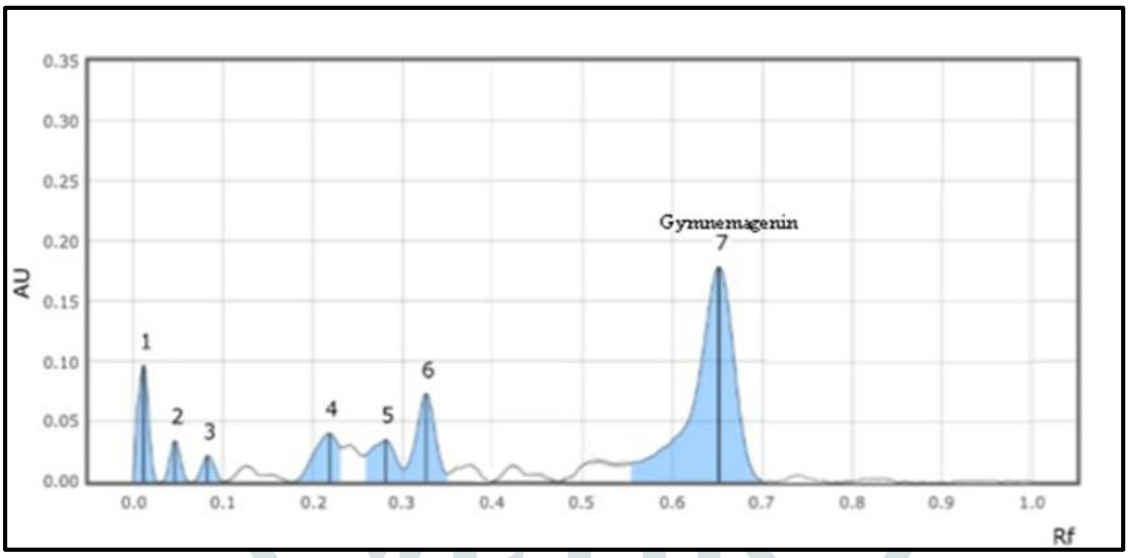

**A. Track 1, ID; Reference standard**

**Figure 2B: HPTLC densitogram of *G. sylvestre* R. Br. leaf extract**




**B. Track 12, ID; *G. sylvestre* R. Br. Leaf**

**Figure 2C: HPTLC densitogram of *G. sylvestre* R. Br. callus extract (MS + 2 mg/l 2, 4-D + 1 mg/l BAP)**




**C. Track 7, ID; *G. sylvestre* R. Br. Leaf Callus**

## III. Results and Discussion

The main objective of the present study was to achieve simultaneous quantification of gymnemic acid in *Gymnema sylvestre* R. Br. leaf and calli extracts by developing HPTLC method. Since, HPTLC is a valuable quality assessment tool for the evaluation of botanical materials, it allows single analysis of several samples efficiently and cost effectively in a single run. Also, with HPTLC, the same analysis can be viewed collectively under different wavelengths of light. Thus, making it a method of choice by providing a complete profile of the plant than is typically observed with other type of analytical technique [22].

### 3.1. Optimization of the chromatographic conditions

In order to obtain optimum chromatographic separation, several parameters like detection wavelength, flow rate, solvent type and strength were altered. The mobile phase conditions were optimized so that solvent and other eluents do not interfere with that of standard.

Preliminary trials were carried out using different solvent systems for separation of Gymnemic acid. Mobile phases composed of [Pet ether: ethyl acetate: formic acid], [Toluene: chloroform: ethanol], [Ethyl acetate: methanol], [Ethyl acetate] and [Chloroform: Glacial acetic acid: methanol] were used to separate Gymnemic acids. Out of these solvent systems tested, Ethyl acetate: methanol in the ratio of 5:6 v/v resulted in good separation of leaf and calli extracts under study and therefore finally selected for further method validation. Also, It was observed that chamber (CAMAG, Switzerland)) saturation time and solvent migration distance play vital role in chromatographic separation as chamber saturation time of less than 15 minutes and solvent migration distances greater than 8 cm resulted in diffusion of analyte spot. Therefore, Ethyl acetate: methanol solvent system in 5:6 (v/v) proportion with chamber saturation time of 20 minutes at 25 $^0$C and solvent migration distance of 8 cm was used in the present study. These chromatographic conditions produced a well defined compact band of Gymnemic acid with optimum migration at Rf = 0.64 ± 0.01. Gymnemic acid bands showed maximum absorbance at 540 nm after derivatization with anisaldehyde sulphuric acid reagent and thus chosen for densitometric analysis.

### 3.2. Validation of HPTLC method

3.2.1. Linearity

As shown in Table 1, Good linearity range was observed for each of the studied samples with a correlation coefficient close to unity ($r^2 > 0.98$). Method was found to be linear in a concentration range of 0.3-0.7 µg/band (n = 5), with respect to peak area.

3.2.2. LOD and LOQ

The results for LOD and LOQ values, determined using residual standard deviation (SD) and slope revealed that the method has very good sensitivity. The values for the same are depicted in Table 1.

3.2.3. Precision:

The intra- and inter-day precision data for the detection of studied standard are presented in Table 1.The precision analyses data both at inter and intra-day was expressed in terms of % RSD, which was found to be <3%, indicating the method is precise and reproducible (Table 1).

3.2.4. Accuracy

Accuracy of the developed method was calculated by recovery test method. Accordingly, it was clear that the method was accurate for the quantitative estimation of Gymnemic acid as the value for relative standard deviation (RSD) was found to be within the acceptance criteria (i.e. RSD, 3.0%). The percentage recovery of Gymnemic acid in leaf and calli extract was found in the range of 97.98 to 98.32, respectively (Table 2).

3.2.5. Specificity

Specificity was determined by analyzing standard and samples chromatogram. The spectra of standard Gymnemic acid, leaf and leaf calli of *Gymnema sylvestre* R. Br. were found to be comparable, indicating no interference by the other plant constituents and excipients so the method is found to be specific.

3.2.6. Ruggedness

The method was found to be rugged for the parameters like change in mobile phase composition, change in spotting volume and detection wavelength. No significant changes in Rf or response to Gymnemic acid was observed, indicating the ruggedness of the method.

The presence of Gymnemic acid as Gymnemagenin was confirmed by comparing Retention factor (Rf) and the color of the sample extracts with that of standard. It was observed that, maximum concentration of Gymnemic acid i.e. 0.79 mg/g was detected in leaf callus obtained on MS medium fortified with 2 mg/l 2, 4-D and 1mg/l BAP as compared to leaf and other tested calli combinations (Table 3).

**Table 1: Quantification parameter for Gymnemic acid as Gymnemagenin**

| Sr. No. | Parameters | Gymnemic acid |
|---|---|---|
| 1. | Linearity range (µg/band) | 0.3 - 0.7 |
| 2. | Correlation Coefficient | 0.99 |
| 3. | LOD (ng/band) | 52.8 |
| 4. | LOQ (ng/band) | 160 |
| 5. | Instrument precision (n=12, %CV) | 1.2 |
| 6. | Intraday precision (n=3, %CV) | 1.462 |
| 7. | Interday precision (n=3, %CV) | 0.450 |
| 8. | Specificity | Specific |
| 9. | Robustness | Robust |

**Table 2: Recovery studies of Gymnemic acid (n=3)**

| Samples | Amount added (µg) | Amount present (mg/gm) | Recovery (%) | Average recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| Leaf | 0.4 | 0.65 | 97.45 | 97.98 ± 0.38 | 1.72 |
| | 0.5 | 0.65 | 98.23 | | 1.65 |
| | 0.6 | 0.65 | 98.28 | | 1.54 |
| Leaf callus [MS medium + 2,4- D (2mg/l) + BAP(1mg/l)] | 0.4 | 0.79 | 98.14 | 98. 32 ± 0.14 | 1.47 |
| | 0.5 | 0.79 | 98.46 | | 1.28 |
| | 0.6 | 0.79 | 98.38 | | 1.66 |

RSD = relative standard deviation.

**Table 3: Quantification of Gymnemic acid through gymnemagenin in leaf and callus cultures of *G. sylvestre* R. Br.**

| Sr. No. | Extracts | Content of Gymnemic acid (mg/gm) |
|---|---|---|
| 1 | *Gymnema sylvestre* R.Br. leaf extract | 0.65 ± 0.02 |
| 2 | MS + 10 mg/l 2,4-D + 5 mg/l BAP leaf callus | 0.67 ± 0.04 |
| 3 | MS + 9 mg/l 2,4-D + 4 mg/l BAP leaf callus | 0.59 ± 0.02 |
| 4 | MS + 8 mg/l 2,4-D + 3 mg/l BAP leaf callus | 0.65 ± 0.01 |
| 5 | MS + 5 mg/l 2,4-D + 5 mg/l BAP leaf callus | 0.24 ± 0.06 |
| 6 | MS + 5 mg/l 2,4-D + 4 mg/l BAP leaf callus | 0.35 ± 0.07 |
| 7 | MS + 5 mg/l 2,4-D + 3 mg/l BAP leaf callus | 0.28 ± 0.03 |
| 8 | MS + 5 mg/l 2,4-D + 2 mg/l BAP leaf callus | 0.29 ± 0.04 |
| 9 | MS + 4 mg/l 2,4-D + 3 mg/l BAP leaf callus | 0.31 ± 0.02 |
| 10 | MS + 3 mg/l 2,4-D + 2 mg/l BAP leaf callus | 0.43 ± 0.12 |
| 11 | MS + 2 mg/l 2,4-D + 2mg/l BAP leaf callus | 0.25 ± 0.12 |
| 12 | **MS + 2 mg/l 2,4-D + 1 mg/l BAP leaf callus** | **0.79 ± 0.02** |
| 13 | MS + 1 mg/l 2,4-D +1 mg/ml BAP leaf callus | 0.35 ± 0.11 |

## IV. Conclusion

HPTLC method has been developed for the identification and quantification of Gymnemic Acid as Gymnemagenin from leaf and *in vitro* cultured calli. Low cost, faster speed and satisfactory precision and accuracy are the main features of this method. Method was successfully validated as per ICH guidelines and statistical analysis proves that the method is sensitive, specific and repeatable. Thus, the developed HPTLC method can be conveniently employed for routine quality control analysis of the extracts and various formulations containing Gymnemic acid as a biomarker.

## Conflict of Interest

The authors report no conflict of interest.

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## References


1. Anonymous. The Ayurvedic Pharmacopoeia of India, Government of India, Ministry of Health and Family welfare, Department of Ayush 2016; 9: 128.
2. Padmapriya S, Kumanan K, Rajamani K. Studies on effect of organic amendments and bio-stimulants on morphology: yield and quality of *Gymnema sylvestre* R. Br. Afr. J. Agric. Res. (2015) 1655–1661. http://www.academicjournals.org/AJAR.
3. Reddy PS, Gopal GR, Sita GL. *In vitro* multiplication of *Gymnema sylvestre* R. Br. — An important medicinal plant. Curr. Sci. 75 (1998): 843–845. http://eprints.iisc.ac.in/1392/1/gymnema.pdf.
4. Bishayee A, Chatterjee M. Hypolipidaemic and antiatherosclerotic effects of oral *Gymnema sylvestre* R. Br. leaf extract in albino rats fed on a high fat diet. Phytother. Res 8 (1994)118–120. https://doi.org/10.1002/ptr.2650080216.
5. Baskaran K, Ahamath B, Shanmugasundaram R, Shanmugasundaram KER. Antidiabetic effect of a leaf extract from *Gymnema sylvestre* in non-insulin-dependent diabetes mellitus patients. J. Ethnopharmacol. 30 (1990) 295–305. https://doi.10.1016/0378-8741(90)90108-6
6. Satdive RK, Abhilash P, Fulzele DP. Antimicrobial activity of *Gymnema sylvestre* leaf extract. Fitoterapia. 74 (2003) 699–701.
7. Khramov VA, Spasov AA, Samokhina MP. Chemical composition of dry extracts of *Gymnema sylvestre* leaves. Pharm. Chem. J. 42 (2008):29–31. https://doi.10.1007/s11094-008-0051-8
8. Yoshikawa K, Arihara S, Matsuura K. A new type of antisweet principles occurring in *Gymnema sylvestre*. Tetrahedron Lett. 32 (1991) 789–792. https://doi.org/10.1016/S0040-4039(00)74887-8.
9. Trivedi PD, Pundarikakshudu K, Shah K. A validated reverse phase liquid chromatographic method for quantification of Gymnemagenin in the *Gymnema Sylvestre* R. Br. leaf samples, extract and market formulation. Int J App Sci Engg 9 (2011) 25-31.
10. Puratchimani V, Jha S. HPTLC standardization of *Gymnema sylvestre* R. Br. using Gymnemagenin as reference. Phytochem Anal. 15 (2004)164–166. https://doi.org 10.1002/pca.763
11. FRLHT. Medicinal plants of India. In: Guidelines for National Policy and Conservation Programmes. Foundation for Revitalization of Local Health Traditions, Bangalore 1997.
12. Chowdhary, F.; Rasool, M. H. Isolation and characterization of Gymnemic acid from indigenous *Gymnema sylvestre*, R.Br. J App Pharm. 3 (2010) 60-65.
13. Patel R, Patel M, Dubey N, Dubey N, Patel B. HPTLC Method Development and Validation: Strategy to Minimize Methodological Failures, Journal of Food and Drug Analysis 2 (2012). 794-804. https://doi.org 10.6227/jfda.2012200408.
14. Jain A, Parashar AK, Nema RK & Narsinghani T, High Performance Thin Layer Chromatography (HPTLC): A Modern Analytical Tool for Chemical Analysis. Current research in pharmaceutical science 4 (2010) 08-14.
15. Ram M, Abdin MZ, Khan MA, Jha P. HPTLC Fingerprint Analysis: A Quality Control for Authentication of Herbal Phytochemicals. Springer Verlag Berlin Heidelberg 2014; 105.http://doi.org.10.1007/978-3-642-14025-9_7
16. Ahmed, A. B. A.; Rao, A. S.; Rao, M. V. And Taha, R. M. (2012). HPTLC/HPLC and Gravimetric Methodology for the Identification and Quantification of Gymnemic Acid from *Gymnema sylvestre* Methanolic Extracts. *Acta Chromatographica*.
17. Kurihara Y. "Characteristics of antisweet substances, sweet proteins, and sweetness inducing proteins". Critical Reviews in Food Science and Nutrition. 32(1992) 231–52. https://doi.org 10.1080/10408399209527598
18. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture, Physiol. Plant. 15 (1962) 473- 497.
19. Lloyd G, McCown B. Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture, Proc. Intl. Plant Prop. Soc. 30 (1980) 421-427.
20. Manohar SH, Naik PM, Praveen N, Murthy HN. Distribution of Gymnemic acid in various organs of *Gymnema sylvestre*. Journal of Forestry Research 20 (2009) 268−270. https://doi.org 10.20959/wjpr 201710-9267
21. International Council for Harmonisation. Harmonised Tripartite Guideline. Validation of Analytical Procedures: text and methodology Q2 (R1). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use: Geneva; 2005, p.1-13.

22. Nafiu MO, Hamid AA, Muritala HF, Adeyemi SB. Preparation, Standardization, and Quality Control of Medicinal Plants in Africa. Medicinal Spices and Vegetables from Africa. (2017) 171–204. https://doi.org 10.1016/B978-0-12-809286-6.00007-8.