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Developing a new high performance thin layer chromatography method for quantitative estimation of Hyperforin in Hypericum perforatum

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ABSTRACT

The present paper deals with development and standardization of HPTLC method used for quantification of Hyperforin in *Hypericum perforatum* L. *Hypericum perforatum* L. commonly known as St. John's wort, is a plant of the family Hypericaceae, is today most widely known as a herbal treatment for depression. One of its constituents, Hyperforin, appears to be responsible for its pharmacological activity. Hence, a need to develop and standardize an analytical method for quantification of Hyperforin, which can be used for standardization of extract of *Hypericum perforatum*. An attempt has been made to quantify Hyperforin in *Hypericum perforatum* extracts by HPTLC method. The lowest detectable limit of Hyperforin was found upto 100 nanograms and provides good resolution and separation of Hyperforin from other constituents of *Hypericum perforatum*. Further, recovery values of Hyperforin were found to be about 96%, which shows the reliability and suitability of the method. This HPTLC method was found to be reproducible, accurate and precise. The structure of isolated Hyperforin was characterized and confirmed by various advanced spectroscopic methods.

KEY WORDS - HPTLC, St. John's wort, *Hypericum perforatum*, Hyperforin

INTRODUCTION

Hypericum perforatum L. (St. John's Wort) has been widely used as an anti-inflammatory and healing agent in traditional medicine. Some preparations containing the extract of this plant are used for their anti-depressive and antiviral properties[1]. Efficacy of *Hypericum perforatum* L. in mild to moderate depression was demonstrated in a number of clinical trials versus placebo and standard anti-depressants[2]. The relatively low rate of adverse effects and the good tolerability result in high patient acceptance. Hypericin, flavonoids and hyperforin have been suggested as the constituents contributing to the anti-depressant activity of St. John's wort [3-6]. Many chemical constituents such as anthrones, phloroglucinol derivatives, flavonoids, xanthones and phenolic acid derivatives have been isolated from this plant growing in Europe[7]. The detection of additional pharmacological activities in recent years further stimulated the interest in Hyperforin[8]. Therefore, it is important to standardize the extract of *Hypericum perforatum* for its content of Hyperforin.

There are few chromatographic methods, such as HPTLC[9-10] and HPLC[11-13], available for estimation of Hyperforin, but these methods require presence of acids, which are harmful as well as time consuming and not precise. Therefore an attempt has been made to develop a HPTLC method, which is fast, precise, sensitive, free of any acid and reproducible with good recoveries for standardization of extracts of *Hypericum perforatum*.

MATERIALS AND METHODS

The commercial extracts of *Hypericum perforatum* L. were procured from two manufacturers from India, which were coded as IE-1 and RE-1. These samples were used as test samples for the quantification of Hyperforin.

Pure Hyperforin was isolated from the enriched extract of *Hypericum perforatum* (RE-1) by preparative HPLC method using 0.2% formic acid in methanol and 0.2% formic acid in water in gradient mode. Purity (97.42%) and structure of isolated compound (Hyperforin) was confirmed by HPTLC, HPLC and spectral analysis such as Mass Spectroscopy[14]. This isolated Hyperforin was used as working standard for quantification of content in commercial extracts.

Equipment: A Camag HPTLC system equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner III and Reprostar, Wincats and integration software 4.02 (Switzerland).

Test sample preparation (IE-1): Accurately weighed 100 mg extract of *Hypericum perforatum* and extracted with methanol (10 ml x 3) by vortexing and allowed to stand for 5 min. at room temperature. The methanol extract was then filtered through Whatmann no.42 filter paper; extracts were pooled and concentrated to dryness under vacuum. Final volume was made to 10 ml with methanol in volumetric flask. Hyperforin content was then analyzed after subjecting to HPTLC.

Test sample preparation (RE-1): Accurately weighed 6 mg extract of *Hypericum perforatum* and extracted with methanol (10 ml x 3) by vortexing and allowed to stand for 5 min. at room temperature. The methanol extract was then filtered through Whatmann no.42 filter paper; extracts were pooled and concentrated to dryness under vacuum. Final volume was made to 10 ml with methanol in volumetric flask. Hyperforin content was then analyzed after subjecting to HPTLC.

HPTLC method: Silica gel 60 F_{254} precoated plates (20 x 10 cm) were used with Petroleum ether : Ethyl Acetate (90:10) as solvent system. 1-10 µl of working standard of Hyperforin as well as test samples were spotted on pre-coated HPTLC plates. The band width applied on plate was 6 mm and ascending mode was used for development of thin layer chromatography. Saturation time was 20 mins along with humidity level – 65% ± 5% RH and room temperature - 25°C ± 2°C. TLC plates were developed upto 8 cms. The TLC plates were scanned at 290 nm for quantification purpose and then were sprayed with 10% methanolic-sulphuric acid reagent (10 ml concentrated sulphuric acid added in 90 ml of methanol with cooling and the reagent should be freshly prepared), heated at 110^0C for 2-3 min and brought to room temperature.

Procedure: -1 (Calibration curve of standard Hyperforin)

2 milligram of working standard Hyperforin was dissolved in 10 ml of methanol to yield stock solution of 0.2 mg/ml concentration. Calibration curve from 0.2 µg to 2 µg was prepared and checked for reproducibility, linearity and validating the proposed method. The correlation coefficient, coefficient of variance and the linearity of results were calculated.

Procedure: -2 (Calibration curve using extract spiked with Hyperforin)

The content of Hyperforin in two extracts (IE-1 & RE-1) were determined by comparing with the calibration curve of the working standard of Hyperforin. The extract-IE-1, which showed lowest content of Hyperforin, was then used as blank. This blank was then used to spike with the working standard of Hyperforin. Different samples with varying amount of standard Hyperforin in range of 0.5 mg to 1.3 mg were spiked separately in 100 mg of blank extract in which the content of Hyperforin had already been estimated. Procedure for sample preparation was followed as mentioned above. In each sample preparation, 10 µl of spiked solution were then subjected to HPTLC with 10 µl of blank solution for comparison. The percent recovery of Hyperforin standard was calculated. Reproducibility, precision and validation of the method were achieved by analyzing six replicate of spike sample solutions. Correlation coefficient, coefficient of variance was calculated.

Linearity along with limit of detection and limit of quantitation: For a long-term use of the analytical method a rigorous validation is indicated and requires the following procedures. For the preparation of calibration curve the stock solution was diluted freshly with methanol to obtain a set of 9 calibration standards. These standards were measured and the integrated peak areas were plotted against the corresponding concentrations of the standards. The complete procedure was repeated on three consecutive days. The so obtained three calibration curves were used to calculate a mean calibration graph. The limit of detection was obtained by analyzing signal to noise ratio and limit of quantification was defined as the lowest concentration of linear range.

Intraday and interday analysis using Hyperforin: Three different concentrations using a different stock solution of hyperforin were prepared (0.3; 0.9 and 1.9 µg/mL). For the determination of the intraday precision and accuracy three replicates of the standard solutions were analysed at the same day in triplicate. The precision and the accuracy of the interday analysis were determined by analysing the standard solution on 3 different days in triplicate.

Results & Discussion: Working standard Hyperforin showed single peak in HPTLC chromatogram (Figure I). The calibration curve of working standard Hyperforin was obtained by spotting standard Hyperforin on HPTLC plate after scanning at 290 nm. Brown yellowish

Table I: Percentage of Hyperforin in different samples of Hypericum perforatum commercial extracts by measuring area in HPTLC method

Sr.	Sample name	Concentration of test sample	Hyperforin content (% w/w)
1	IE-1	10 mg/ml	2.06
2	RE-1	0.6mg/ml	27.04

Table II: Precision & accuracy of the method applied to spiked Hyperforin samples

Amount added (μg / spot)	Amount found (μg /spot) (Mean ± S.D., n=6)	Precision / Reproducibility (C.V.)	Mean Recovery (%)
0.5	0.48 ± 0.014	3.12	97. 09
0.9	0.86 ± 0.034	4.78	96.70
1.3	1.24 ± 0.048	3.91	96.31

Table III: Intraday and interday precision & accuracy of the method applied to Hyperforin

Amount added (μg / spot)	Intraday			Interday		
	Conc. found (μg / spot)	Precision (R.S.D., %)	Accuracy (%)	Conc. found (μg / spot)	Precision (R.S.D., %)	Accuracy (%)
0.3	0.2903 ± 0.010	3.950	96.75	0.2871 ± 0.008	2.808	95.70
0.9	0.8629 ± 0.021	2.451	95.88	0.8676 ± 0.026	2.991	96.4 1
1.9	1.8247 ± 0.053	2.784	96.04	1.8115 ± 0.055	3.017	95.34

spot of Hyperforin with Rf ≈ 0.32 – 0.35 was visible after spraying with 10% methanolic sulphuric acid reagent. Various extracts (IE-1 & RE-2) of *Hypericum perforatum* were analysed by the proposed method and the data are recorded in table I.

Chromatographic precision and recoveries from spike sample solution

Specificity: It was observed that the other phytoconstituents present in the extracts did not interfere with the peak of Hyperforin. Therefore the method was specific and help in separation of Hyperforin from other constituents of herb and hence, help to get the exact content of Hyperforin. Test sample of *Hypericum perforatum* extract (IE-1) showed separated peak of Hyperforin along with other phytoconstituents as obtained in HPTLC chromatogram (Figure II).

Limit of Detection: By applying the proposed method, the minimum detectable limit was found to be 100 nanogram / spot at 290 nm.

Limit of Quantification: By applying the proposed method, the minimum quantification limit was found to be 200 nanogram / spot at 290 nm, the lowest concentration in linear range.

Linearity: The linearity of the method was checked with working standard Hyperforin with the calibration curve in the concentration range of 0.2 – 2.0 μg / spot based on a 1-10 μl sample volume. The regression equations (Y = 574.512 + 2.865 * X) and correlation coefficient were obtained with 6 replicate analysis for each concentration. Correlation coefficients were obtained in the range of 0.9950-0.9972 indicated excellent linearity of the procedure for working standard Hyperforin analyzed. Calibration curve of working standard Hyperforin is shown in figure. III.

The linearity of the method was checked with test sample of *Hypericum perforatum* extract (IE-1) with the calibration curve in the concentration range of 10 – 100 μg / spot based on a 1-10 μl sample volume. The regression equations (Y = 363.578 + 74.056 * X) and correlation coefficient were obtained with 6 replicate analysis for each concentration. Correlation coefficients were obtained in the range of 0.9991-0.9994 indicated excellent linearity of the procedure for Hyperforin in test sample analyzed. Calibration curve of Hyperforin in test sample (IE-1) is shown in figure. IV.

Accuracy and precision

The method was applied to determine concentration of spiked Hyperforin test sample in the range of 0.5 – 1.3 μg / spot for assessing the accuracy & precision of the procedure. Table II represents the mean values and Coefficient Variance (C.V.) results indicate the levels in

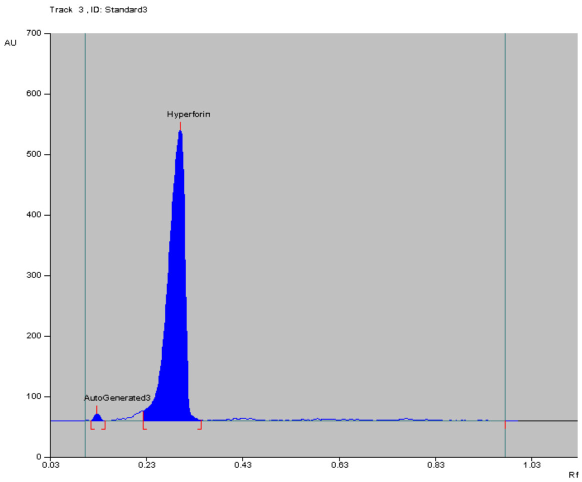
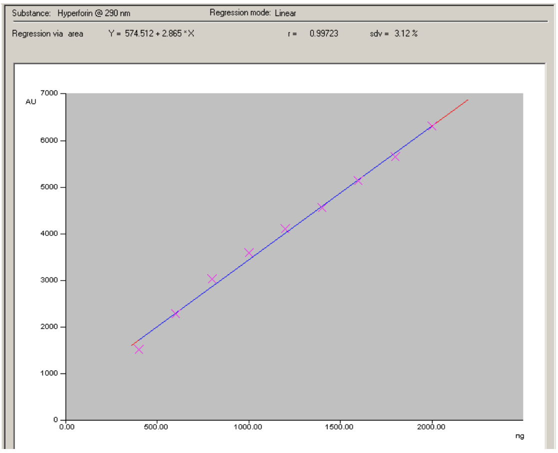


Figure 1: TLC Chromatogram of working standard Hyperforin

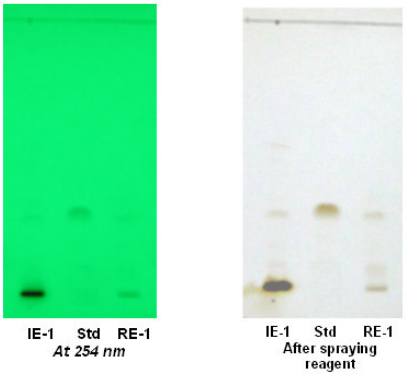


Substance: Hyperforin @ 290 nm   Regression mode: Linear

Regression via area   Y = 574.512 + 2.865 * X   r = 0.99723   sdv = 3.12 %

Figure 3: Calibration curve of working standard Hyperforin with respect to the area under curve at various concentration

Photograph 1: HPTLC Photograph of two commercial extracts of Hyperforin perforatum (RE-1 & IE-1) along with std. Hyperforin



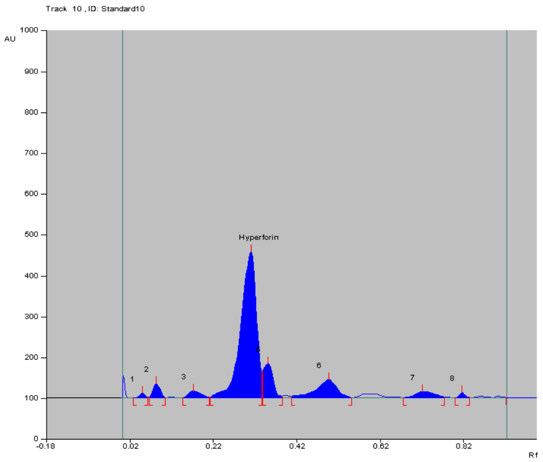
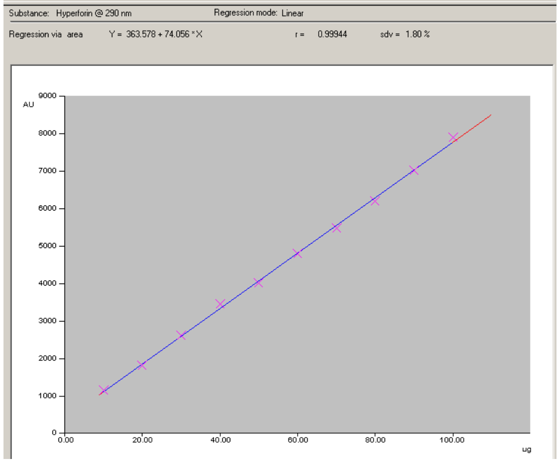


Figure 2: TLC Chromatogram of test sample (Hypericum perforatum extract –IE-1)



Substance: Hyperforin @ 290 nm   Regression mode: Linear

Regression via area   Y = 363.578 + 74.056 * X   r = 0.99944   sdv = 1.80 %

Figure 4: Calibration curve of test sample of Hypericum perforatum extract (IE-1) with respect to the area under curve at various concentration

the above range can be estimated with accuracy and precision. *Intraday and interday analysis using Hyperforin:* Furthermore the precision and accuracy of the intraday and interday analysis were investigated on the basis of a set of standard solution. The results given in Table III stands for a quite good trueness of the proposed method particularly considering interday and intraday analysis.

Robustness and Ruggedness studies

The method was found to be re-producible from one analyst to another. The low values of R.S.D. (2.897% - 3.214%) obtained after small deliberate changes of the conditions (mobile phase composition, mobile phase volume, saturation time, time from application to chromatography, and time from chromatography to scanning) used for the method indicated its robustness.

CONCLUSION

The lowest detectable limit of Hyperforin was found upto 100 ng / spot and provides good resolution and separation of Hyperforin from other constituents of *Hypericum perforatum*. Further, recovery values of Hyperforin were found to be about 96%, which shows the reliability and suitability of the method. The proposed HPTLC method is rapid, simple, free of acid in method and accurate for quantitative monitoring of Hyperforin in *Hypericum perforatum* L. samples.

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