Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences
ISSN- 0975-1491 Vol 6, Issue 1, 2014

Research Article

DEVELOPMENT OF A VALIDATED HPTLC METHOD FOR QUANTIFICATION OF ESCULIN IN DIFFERENT FRACTIONS OF *CICHORIUM INTYBUS* LEAF EXTRACT

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Received: 20 Oct 2013, Revised and Accepted: 14 Nov 2013

ABSTRACT

Objective: To develop a simple, rapid, sensitive and validated High performance thin layer chromatography method (HPTLC) for identification and quantification of esculin (6, 7-dihydroxycoumarin 6-glucoside), a glycosidic coumarin in different fractions of *Cichorium intybus* leaves extract prepared using soxhlet extraction .

Method: The separation was achieved on silica gel 60 F_{254} HPTLC plates using ethyl acetate: methanol: water: glacial acetic acid (20:2:1:4, v/v/v/v) as the mobile phase. Densitometric analysis of esculin was carried out using reflectance/fluorescence mode at 343 nm.

Results: Densitometric analysis of different fractions of leaf extract gave compact spots for esculin (R_f 0.36). The linear regression analysis of data for the calibration plots showed a good linear relationship with r=0.9989. The average recovery of esculin was 98.34%, indicating the good reproducibility. The amount of esculin was found to be equal in both water (94.9 µg g^{-1} dw) and ethanol (93.4µg g^{-1}dw) fraction. Statistical analysis of the data showed that the method is reproducible.

Conclusion: The method was found to be simple and convenient for rapid screening and quantification of esculin in the biological samples of *C. intybus*.

Keywords: Esculin, Coumarin, *Cichorium intybus*, HPTLC.

INTRODUCTION

Esculin (6, 7-dihydroxycoumarin 6-glucoside) (Fig 1) is a glycosidic coumarin of plant origin, belonging to a group of phenolic compounds. Esculin has multiple medicinal, pharmaceutical and industrial applications. It is a well-known natural UV-B protective agent showing vitamin P activity and skin regenerating properties [1]. Esculin possesses various biological activities such as anti-inflammatory [2], cytostatic [3] antimutagenic [4], and antioxidant effects [5]. Esculin can inhibit oxidative DNA damage and formation of aberrant crypt foci and tumors. Furthermore, it is reported that esculin can reduce thrombin-induced hyper-permeability in endothelial cells [6]. It has also been proven that esculin has the anti-apoptotic effect on dopamine-induced cytotoxicity in the human neuroblastoma SH-SY5Y cell line [7].

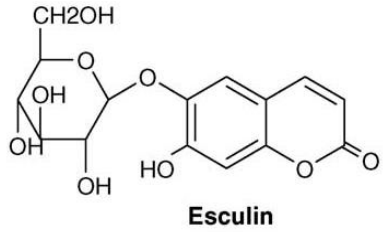

Fig. 1: Structure of esculin

Esculin is used in the manufacturing of pharmaceuticals with venotonic, capillary-strengthening and antiphlogistic action similar to that of Vitamin P. It is an important constituent of several anti-inflammatory drugs such as esqusan, esflazid and anavenol [8-9]. It is also present in several other drugs available in market such as proctosone, anustat and ariproct. Esculin has been reported in several plants such as *Cortex fraxini* [10], *Rhododendron tomentosum* [11] and *Cichorium intybus*. *Cichorium intybus*, known as "chicory", belongs to the family Asteraceae and is an important medicinal cultivated plant. Besides esculin, it also contains large number of other pharmaceutically important compounds, which include inulin, sesquiterpene lactones, coumarins, flavonoids and vitamins [12]. Because of the wide applications of esculin and its presence in *C. intybus* leaves, transgenic plants were developed using *in vitro* regeneration of leaf explants in our laboratory earlier which had enhanced quantity of esculin in the leaves [13-14]. The objective of the present study was to prepare different extracts from *C. intybus* leaves and develop a quick, simple, reproducible, standardized and validated HPTLC based method to quantify esculin.

MATERIAL AND METHODS

Chemicals and reference compounds

All chemicals and solvents used in this study were of analytical and HPLC grade (E.Merck, Mumbai, India). The standard esculin was purchased from Sigma Aldrich Chemicals. The HPTLC plates RP-18 F254S (20x20 cm) (E.Merck, Darmastadt, Germany) were used without any pre-treatment.

Plant material

Cichorium intybus leaves were collected in the month of January, 2012, at the bolting stage from Herbal Garden, Jamia Hamdard, New Delhi. The leaves were washed with water to remove soil particles, dried in shade and finely powdered. The powder was stored in air tight container at room temperature till extraction.

Preparation of stock and working standard solution

Standard stock solution (1mg/ml) of esculin was prepared by weighing accurately 1.0mg of pure compound and dissolving in 1.0ml of double distilled water. Further standard dilutions were prepared by diluting this stock solution with double distilled water.

Sample preparation

The dried powdered leaves (30g) were packed in Soxhlet extractor and extracted with ethanol (500 ml). The ethanolic extract (350ml) was filtered (1ml ethanolic extract was kept separately) and treated with charcoal (30mg/10 ml). It was filtered again after charcoal treatment. After addition of chloroform (1:1 v/v), the mixture was partitioned with water (1:1 v/v) twice. The water and the chloroform fractions were concentrated in vacuo. The concentrated water, chloroform and the ethanol fractions were used as sample solutions for esculin estimation.

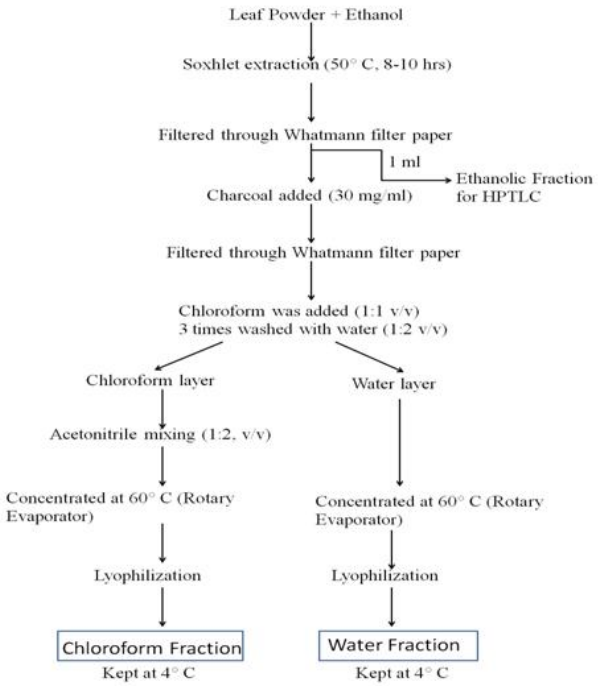

Fig. 2: Flow diagram showing steps involved in sample preparation.

Chromatography

The HPTLC was performed on 20x10 cm aluminium foil plate coated with a 200µm layer of silica gel $60F_{254}$ (E. Merck, Germany). A 5 µl of each sample solution was applied as 6mm bands by means of a Camag (Switzerland) Linomat 5 applicator fitted with a 100 µl syringe. A constant application rate of 150nl/s was used. Linear ascending development using the mobile phase (ethyl acetate: methanol: water: glacial acetic acid, 20:2:1:4 (v/v/v/v)) was performed in a glass twin-trough chamber (Camag) previously saturated with mobile phase for 20min (optimized saturation time) at room temperature. Reference marker compound was also applied on the TLC plate along with samples to confirm the presence of esculin in test samples. The development distance was 90mm. The densitometric analysis was performed at 343nm using Camag TLC Scanner 3 in fluorescence mode, winCATS software (v. 1.4.3.6335). Slit dimension was 6.00 x 0.45mm with scanning speed of 20mm/s.

Calibration plot of esculin

For calibration, a series of solutions of esculin (5 and 10ng/µl) were prepared in water by dilution of the stock solution with the same solvent, which were then applied to TLC plates to prepare nine point linear calibration curve. After, a number of experiments, optimal application volumes for esculin were chosen so as to provide 2.5, 5, 10, 15, 20, 25, 30 and 35ng esculin/spot, respectively. Peak area and the corresponding amounts were treated with linear least square analysis.

Method validation

The method was validated for repeatability, precision and accuracy. Repeatability of the method was affirmed by multiple measurements (n=8) of esculin after application on the TLC plate (10ng/spot) under the same analytical and laboratory conditions. Intermediate precision of the method was studied by analyzing aliquots of standard solution of esculin (5, 15 and 25ng/spot) on the same day (intra-day precision) and on different days (inter-day precision). The results were expressed as relative standard deviation (%RSD) between different days.

Accuracy of the method was tested by performing the recovery studies at three levels (50, 100 and 150%). To the pre-quantified *C. intybus* leaf extract, known amount of esculin was added in ethanol (46.7, 93.4 and 140.10µg/g) and water fraction (47.5, 94.9 and 142.35µg/g) and estimated as described above.

RESULTS AND DISCUSSION

The objective of this work was to quantify esculin in different fractions of extract prepared from *C. intybus* leaves. Esculin is among the common coumarins found in leaves. For this purpose, the extraction procedure described above was followed.

Optimization of chromatographic conditions

Detailed TLC studies revealed that the mobile phase comprising of ethyl acetate: methanol: water: glacial acetic acid in the ratio of 20:2:1:4 (v/v/v/v) gave well resolved bands for all samples, with the marker compound, esculin, at R_f 0.36. The three dimensional patterns obtained from standard and test samples (water and ethanol fraction) revealed that the peaks at R_f 0.36 for both the samples were super-imposable as shown in Fig 3.

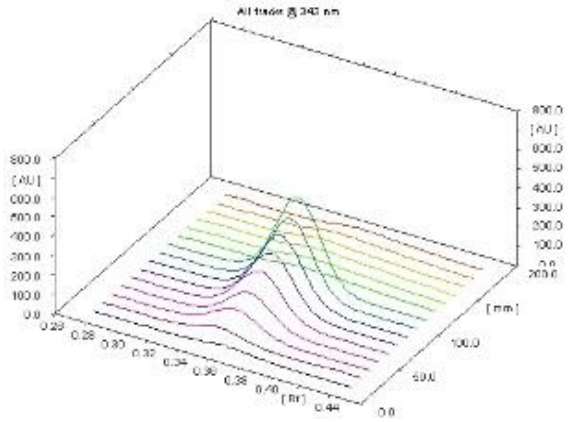

Fig. 3: 3-D chromatogram of standard esculin scanned at 343nm along with ethanol fraction and water fraction.

Initial HPTLC fingerprinting study was performed on the pure marker compound, esculin. The marker compound band was scanned and its spectrum was recorded at 343 nm. Thus, for better

quantitative analysis all the plates were scanned at 343 nm. Fig 4 shows the chromatogram obtained at 343 nm from the standard marker compound. Fingerprint patterns obtained from the test samples under identical conditions showed that, during extraction procedure the esculin present in the leaves of *C. intybus* did not enter in the chloroform fraction when partitioned thrice with distilled water as shown from the absence of peak in the HPTLC chromatogram obtained from this fraction, as shown in Fig 5.

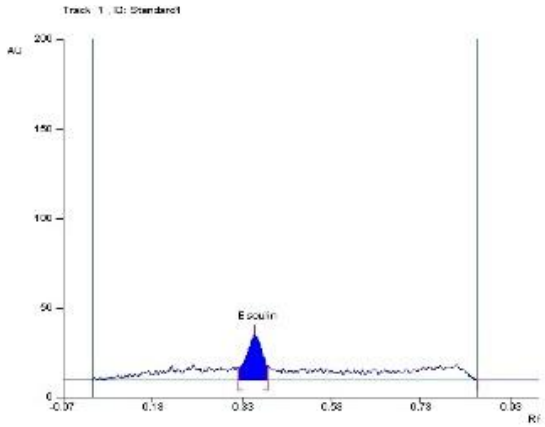

Fig. 4: HPTLC chromatogram of standard esculin at 343nm

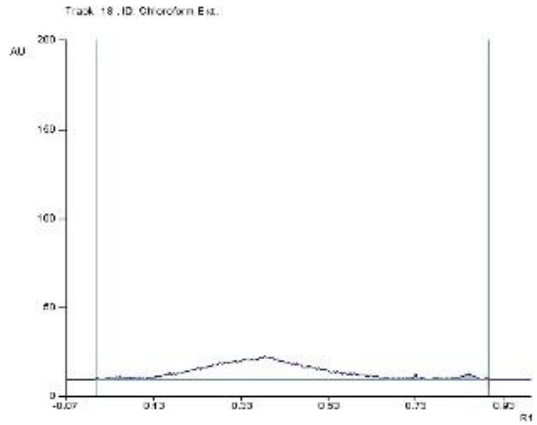

Fig. 5: HPTLC chromatogram of. chloroform fraction of *C. intybus* leaves extract at 343nm.

The HPTLC fingerprints obtained from ethanolic and water fractions as shown in Fig 6, 7 showed the peak corresponding to the peak of standard esculin marker. Further, the number of peaks present in the chromatogram obtained from water fraction as shown in Fig 7, was equal to that present in the chromatogram obtained from the ethanol fraction. Also, the quantity of esculin present in the ethanol fraction was found to be equal to that of water fraction. The data were shown in table1. These findings, hence suggest that the total number of compounds extracted in the ethanol along with esculin were also partitioned into the water and amount of esculin extracted from chicory leaves was partitioned equally into the ethanol and water fractions. One of the salient findings of our protocol is that those extracted compounds from *C. intybus* leaves which were soluble in ethanol were also soluble in water. Hence, their conversion into powder forms after lyophilisation of water fraction is an added advantage in making pharmaceutical formulations for administration to animals and humans.

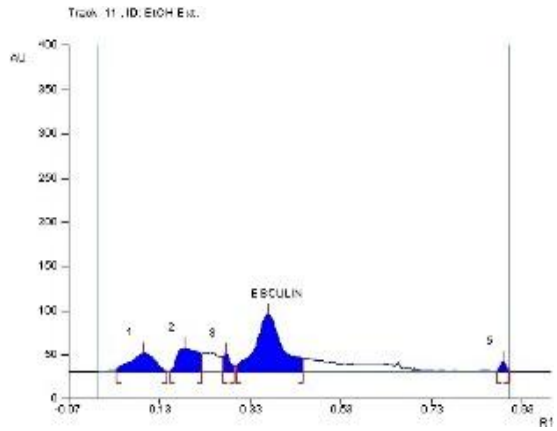

Fig. 6: HPTLC chromatogram of ethanol fraction of *C. intybus* leaves extract at 343nm.

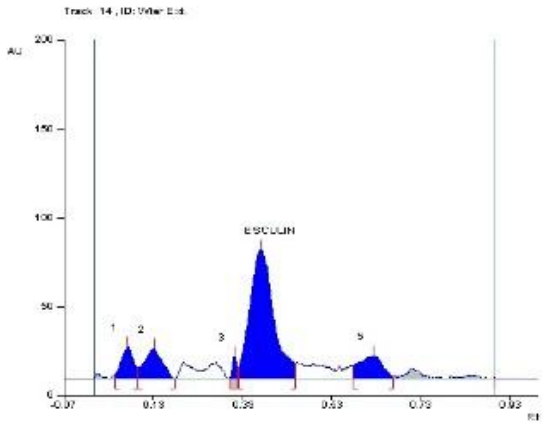

Fig. 7: HPTLC chromatogram of water fraction of *C. intybus* leaves extract at 343nm.

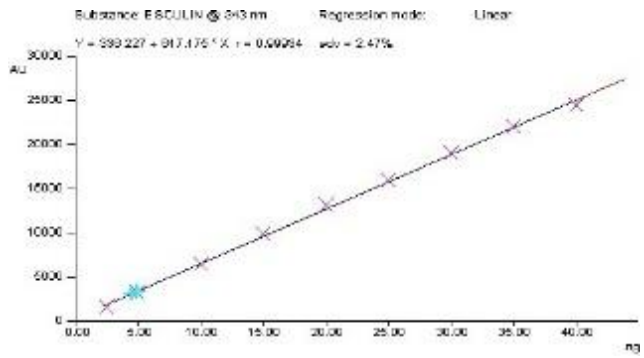

Fig. 8: Calibration curve of esculin.

Method validation

Method validation was performed on the parameters such as linearity, accuracy, specificity, limit of sensitivity and precision.

Linearity

The linearity was obtained by analyzing nine different solutions of esculin in the linear range of 1.25-35ng/spot and a representative linear calibration curve of esculin is shown in Fig 8. The regression data as shown in table 2 indicated a good linear relationship between the concentrations and peak areas over the concentration used.

Accuracy

The proposed method when used for extraction and subsequent estimation of esculin from ethanol and water extract after spiking with 50, 100 and 150% of excess esculin afforded recoveries of 98.43% and 98.64 %, respectively, as summarized in table 3.

Specificity

It was observed that the peak of standard esculin did not interfere with the peak of esculin in the extracts and therefore the method can be considered as specific. The chromatogram of standard esculin and esculin from the extracts were matched in a similar fashion.

LOD and LOQ

The LOD and LOQ were determined based on the lowest detectable levels of standard solution. The LOD and LOQ were found to be 1 and 2.5ng/spot, respectively. This indicated that the proposed method exhibits a good sensitivity for the quantification of esculin. The data is shown in table 1.

Intra and inter-day precision of HPTLC

The %RSD values for intraday precision determined at three different concentrations and interday precision determined three times at three different concentration of each compound on three different days over a period of one week (table 4) indicated that the method was precise and can be used for quantitative analysis under these conditions.

Table 1: Mean esculin content, standard deviation and total esculin content

S. No.	Sample	Mean of esculin content (ng spot $^{-1}$)	SD	Total esculin content (µg g^{-1} dw)
1.	Ethanol fraction	1.898	0.68	94.9
2.	Water Fraction	1.868	0.36	93.4

Table 2: Method validation parameters for the quantification of esculin by HPTLC

Parameters	Values
Linearity Range (ng/spot)	2.5-35ng
Standard Deviation	2.47
Correlation Coefficient	0.9993
Limit of detection (LOD)	1.0 ng
Limit of quantification (LOQ)	2.5 ng
Repeatability (% RSD , (n= 9)	2.42
Regression equation	Y= 336.227+617.175*X

Table 3: Recovery studies of esculin (n=3) in *C. intybus* leaves

Samples	Esculin added to extract (µg/g)	Amount of Esculin present (µg/g)	Amount of Esculin found (µg/g)	Recovery (%)	Average recovery (%)	% RSD	SD
Ethanolic extract	46.7		140.55	98.74			
	93.4	94.9	186.64	98.34	98.43	0.0027	0.27
	140.10		233.02	98.22			
Aqueous extract	47.45		138.53	98.88			
	94.9	93.4	184.07	98.54	98.64	0.0020	0.20
	142.35		230.04	98.52			

Table 4: Interday and Intraday precision of esculin (n=3)

Concentration	Intra-day Precision			Inter-day Precision		
(ng/spot)	Mean area	SD	% RSD	Mean area	SD	% RSD
5	3941.233	8.08	0.20	3948.067	9.22	0.23
15	11011.56	5.16	0.04	11029.23	5.89	0.05
25	17535.16	5.06	0.02	17578.59	10.23	0.05

CONCLUSION

Esculin, one of the simplest coumarins known to have multiple pharmacological and biochemical activities, is an established inhibitor of lipoxoygenase and cyclo-oxygenase (COX) enzymes and shows scavenging effects on ROS. Esculin have multiple biological activities including inhibition of xanthine oxidase activity, anti-oxidant activity, antitumor activity, and inhibitory effects on the growth of human breast cancer cells. Therefore, a simple, rapid, sensitive, effective and validated method for the quantitative analysis of this compound is urgently needed. The HPTLC method developed in the present study for the quantification of esculin in leaf extracts of *C. intybus* is simple, rapid, sensitive, reproducible and easily adaptable for screening and quantitative analysis.

ACKNOWLEDGMENT

The research facilities developed from UGC-SAP (DRS-1) grant sanctioned to the Department of Biotechnology used in this study are thankfully acknowledged. The Research Fellowship awarded to A. Ali is gratefully acknowledged.

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