

Simultaneous Quantitative Estimation of Scopoletin And B-Sitosterol in *Girardinia Diversifolia* Plant Extracts by HPTLC and HPLC Methods

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ABSTRACT

Girardinia diversifolia is a traditionally important medicinal plant recognized for its therapeutic benefits, primarily attributed to bioactive phytoconstituents such as scopoletin and β -sitosterol. Despite their pharmacological significance, reliable and validated analytical methods for the simultaneous estimation of these compounds are limited. Hence, the present study was designed to develop and validate simple, rapid, precise, and accurate HPTLC and HPLC methods for the concurrent quantification of scopoletin and β -sitosterol in *Girardinia diversifolia* extracts. The whole plant material was collected, authenticated, shade-dried, and powdered, followed by successive extraction using petroleum ether, ethyl acetate, and ethanol. Preliminary phytochemical screening confirmed the presence of key secondary metabolites including flavonoids, tannins, and triterpenoids. Initial TLC profiling indicated that the ethyl acetate extract produced well-resolved spots when developed in a mobile phase consisting of toluene:ethyl acetate:acetic acid:formic acid (7:2:0.5:0.5 v/v/v/v). HPTLC analysis was performed using a CAMAG system with densitometric scanning at 366 nm and after derivatization using anisaldehyde–sulphuric acid reagent at 525 nm. For HPLC analysis, a Shimpack RP-C₁₈ column (50 mm $\times$ 4.6 mm, 5 μ m) was employed with an isocratic mobile phase of acetonitrile:water (85:15 v/v). The HPTLC method showed distinct and well-resolved peaks with R_f values of 0.30 ± 0.03 for scopoletin and 0.54 ± 0.03 for β -sitosterol, while HPLC analysis revealed retention times of 1.04 and 1.40 minutes, respectively. Both methods demonstrated satisfactory linearity, precision, and accuracy. Overall, the validated HPTLC and HPLC methods proved to be reliable, efficient, and suitable for routine quality control and standardization of *Girardinia diversifolia* extracts.

Keywords: *Girardinia diversifolia*, Scopoletin, β -Sitosterol, HPTLC, HPLC, validation.

INTRODUCTION

Herbal drugs or herbs were used in the prime source for the treatments of various disease since the ancient times. The herbs contain different phytochemical constituents that have therapeutic effects on the human individuals and animals. Phenolic substances, flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans, and lignins are abundantly available in medicinal plant parts. In India the Ayurvedic medical system primarily treats a variety of human illnesses with plant-based medications or formulations¹. The standardization of herbal drugs is important because, natural drugs are combinations of

numerous components and the active principle of a particular plant is unknown in the most cases. For the standardization of herbal drugs and herbal formulations different analytical techniques are used. TLC, HPTLC, HPLC, NMR, MS, etc. are some of the modern analytical tools that give precise and accurate analysis of the plants and plant products⁶.

In Nepal, China, the Himalayan regions of India, and the Nilgiris, *Girardinia diversifolia*, often referred to as Allo or Himalayan giant nettle, is widely distributed in open forests, riverbanks, and damp environments¹. Depending on soil conditions and rainfall, this erect annual plant can grow to a height of

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75 to 300 cm. Its leaves are big, lobed, alternating, coarsely dentate and up to 8 cm wide. The base of the stem can reach a diameter of 4 cm². The plain decoction prepared from the plant which is

traditionally used for the management of fever^{2,3}. The decoction of the roots and stems when mixed with wine is used in the treatment of malignant boils. The leaves of *Girardinia diversifolia* is traditionally used externally as an astringent properties and in the management of scrofula. The plant ashes are applied externally for the treatment of ringworm and eczema. A decoction prepared from the root and mixed with *Centella asiatica* is employed in the treatment of gastric disorders and the root juice is used for constipation. The fresh juice of the leaves is used externally in the management of headache and swollen joints and is also employed in the treatment of microbial infections².

Phytochemical investigations revealed the presence of proteins, saponins, flavonoids, tannins, tri-terpenoids and phenolic compounds in the roots and stems of *Girardinia diversifolia*. The plant also contain phyto-constituents like β -sitosterol, scopoletin, accholine, histamine and 5-OH tryptamine^{3,5}. So far, the methods such as HPTLC and HPLC have not been developed for the simultaneous estimation of scopoletin and β -sitosterol in *Girardinia diversifolia*. Therefore, the present study aims to develop a method for the estimation of scopoletin and β - sitosterol as marker compounds in *Girardinia diversifolia*.

MATERIALS AND METHODS

Collection of plant material:

The whole plant material was collected from Anmapuram, close to Pykara falls in Ooty, identified and authenticated by Dr. C. Kunhikannan, Scientist,

Biodiversity division, IFGTB, Coimbatore, Tamil Nadu.

Chemicals and reagents:

Scopoletin and β -sitosterol were obtained from the Synergy Scientific Services Pvt Ltd, located in Chennai. AR/HPLC grade methanol, water, acetonitrile, petroleum ether, ethyl acetate, ethanol, formic acid, toluene and glacial acetic acid were purchased from S.D. Fine chemicals Ltd., Mumbai.

Instruments:

Camag HPTLC System with WinCATS software, TLC Scanner III, and Linomat V sample. Jasco V-630 UV-Visible Spectrophotometer; Shimadzu HPLC system with LC AT10 VP Pump, SPD M 10 AT VP, Detector, and CLASS M 10A Software. Soxhlet Borosil extractor. Leela Sonic Ultrasonicator with SHIMADZU electronic balance L-220H. pH meter Elico Li 127.

TLC study of *Girardinia diversifolia* plant extract and marker compounds:

An aqueous slurry of the finely powdered substance (Silica gel G) was spread over the clean surface of glass plates to form thin layer chromatography plates. The plates were then heated in an oven to 110°C to 120°C for 30 minutes before the sample spotting.

Based on its solubility, methanol was chosen as the solvent for markers. The mobile system selected was toluene: ethyl acetate: formic acid: acetic acid (7:2:0.5:0.5 %v/v/v/v) by optimization of different solvent systems (Table 1) and its R_f value (Table 2) are tabulated as follows

Table 1: Selection of mobile phase

Systems for solvent tried	Observation
Toluene: ethyl acetate: formic acid (6:3:1 %v/v/v)	Separation was not good
Toluene: methanol (9:1 %v/v)	Separation was not good
Toluene: chloroform: methanol (6:3:2 %v/v/v)	Separation was not good
Toluene: ethyl acetate: formic acid: acetic acid (7:2:0.5:0.5 %v/v/v/v)	Good Separation



Table 2: R_f value of biomarkers

Compound	R _f Value
Scopoletin	0.35
β-sitosterol	0.54
Marker mixture	
Scopoletin (Spot 1)	0.34
β-sitosterol (Spot 2)	0.55

HPTLC analysis of plant extract and marker compounds of *Girardinia diversifolia* HPTLC conditions:

The sample was applied on a pre-coated silica gel 60 F₂₅₄ plates (10 x 10 cm with 250 μm thickness) using a Camag Linomat V sample applicator and a 100 μL syringe. The sample was spotted in band width of 6 mm and the migration distance was 80 mm from the sample application. Plates were developed using a mobile phase consisting of toluene: ethyl acetate: formic acid: acetic acid in the ratio of 7:2:0.5:0.5 %v/v/v/v. The development of the plate was carried out in 10x10 cm twin trough glass chamber previously saturated with mobile phase. The chamber saturation time for mobile phase was 15 minutes at room temperature. The slit dimension was set to 5 x 0.45 mm and scanning rate of 20 mm/second. The scanning of developed plate was performed on Camag TLC scanner III in absorbance mode at 366 nm and 525 nm operated by winCATS software.

Post chromatographic derivatization

Anisaldehyde-Sulphuric acid reagent:

The reagent was prepared by mixing 0.5 ml of anisaldehyde with 10 ml of glacial acetic acid, followed by the addition of 85 ml methanol and 5 ml of concentrated sulphuric acid. After the development, the plates were derivatized using anisaldehyde-sulphuric acid reagent and heated to 105°C until purple colour spots appeared. Then the derivatized spots were scanned at 525 nm.

Preparation of standard solutions Scopoletin:

To obtain a concentration of 100 μg/ml, 5 mg of scopoletin was added to a 50 ml standard flask,

dissolved in a small amount of methanol, and the volume was made up to 50 ml with methanol.

β-sitosterol:

A quantity of 5 mg of β-sitosterol was transferred into 50 ml volumetric flask and dissolved in a small quantity of methanol, then the volume was adjusted to 50 ml with methanol to achieve a concentration of 100 μg/ml.

Marker mixture:

From the prepared stock solution, 1 ml of Scopoletin and 1 ml of β-sitosterol solutions were individually transferred into 10 ml standard flasks, and the volume was adjusted to 10 ml with methanol to achieve each concentration of 10 μg/ml.

Preparation of sample solutions:

An amount of 10 mg of the extract was measured with diluted to 10 ml using methanol as the solvent, resulting in a concentration of 1000 μg/ml. The solution was then filtered through Whatmann filter paper. A volume of 10 μl/spot of the solution was placed onto the TLC plate.

Validation of the method Linearity and range:

The linearity of an analytical method refers to its ability (within a defined range) to produce test results that are directly proportional to the concentration of the analyte present in the sample. From the standard stock solution of marker mixture with scopoletin ranging from 50 to 1000 ng/spot and β-sitosterol from 200 to 1000 ng/spot was applied using a CAMAG semi applicator.

LOD and LOQ:



The LOD and LOQ of the standard were determined by the application on the plate of decreasing amounts of the drug in triplicate. The lowest concentration at which the peak is detected is referred to as the "Limit of Detection" and the lowest concentration at which the peak is quantified is referred to as the "Limit of Quantification".

Precision:

The precision of the analytical procedure expresses the closeness of the agreement between a series of multiple sampling measurements of the same homogeneous sample under the prescribed conditions.

- Intraday
- Interday
- Repeatability
 1. Repeatability of sample application
 2. Repeatability of sample measurement

Stability studies:

The analytes are liable to decompose when developed chromatographic plate is exposed to the atmosphere. Therefore, after development, the stability of the plates must be confirmed.

RESULTS

Preliminary phytochemical tests on the plant extracts were performed and they showed the presence of flavonoids, carbohydrates, tannins and tri-terpenoids. The preliminary TLC analyses revealed that the ethyl acetate extract spots were close to the standard scopoletin and β -sitosterol spots. Hence, the further analysis was carried out based on these results.

HPTLC Method:

For the HPTLC analysis of scopoletin and β -sitosterol, a mobile phase system consisting of toluene: ethyl acetate: acetic acid: formic acid (7:2:0.5:0.5 %v/v/v/v) was selected. For scopoletin, the plant extracts and markers were analyzed in the system at 366 nm (Fig. 1), and β -sitosterol was found after derivatization using anisaldehyde-sulphuric acid reagent at 525 nm wavelength (Fig. 2). The system yields symmetrical peaks for β -sitosterol and scopoletin with R_f values of 0.54 ± 0.03 and 0.30 ± 0.03 , respectively. Calibration graphs were plotted for both scopoletin and β -sitosterol (Fig. 3,4). The concentration ranges of 50-1000 ng/spot for scopoletin ($r = 0.9978$) and 200-1000 ng/spot for β -sitosterol ($r = 0.9987$) were shown to exhibit linearity. The amount of scopoletin and β -sitosterol present in ethyl acetate extract was quantified using linear regression equation method and was found to be 0.00516 mg/10 mg for scopoletin and 0.213 mg /10mg for β -sitosterol (Table No.4). The validation parameters for the marker mixture have been carried out and they are listed in the table (Table No. 3).

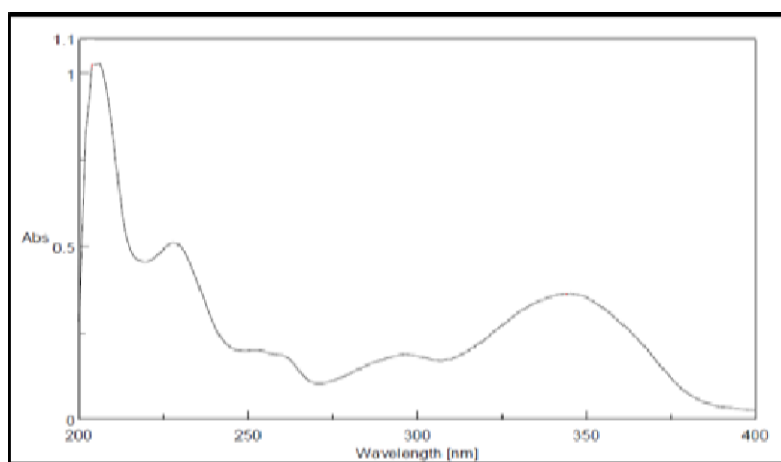


Figure 1: Spectrum of scopoletin

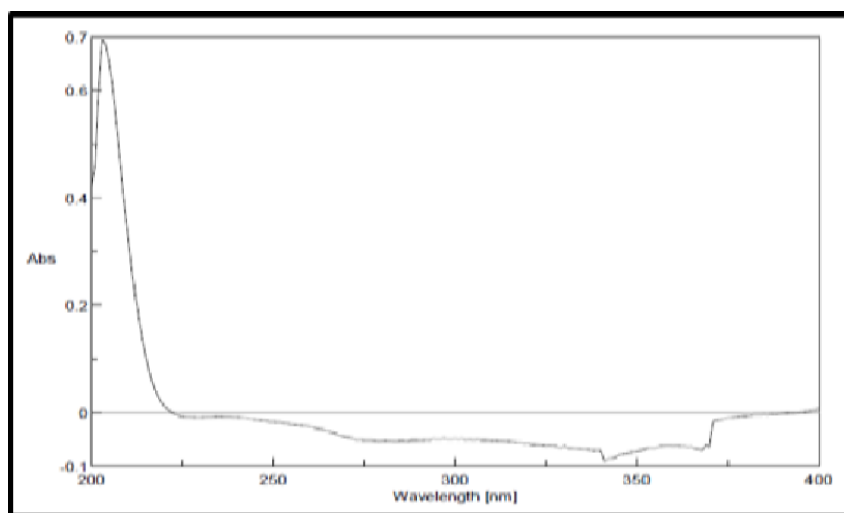


Figure 2: Spectrum of β -sitosterol

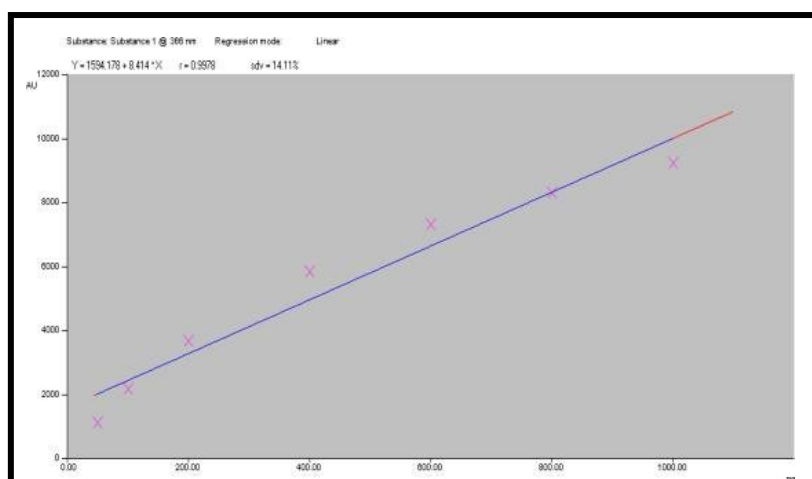


Figure 3: Calibration graph of Scopoletin (50- 1000 ng/spot)

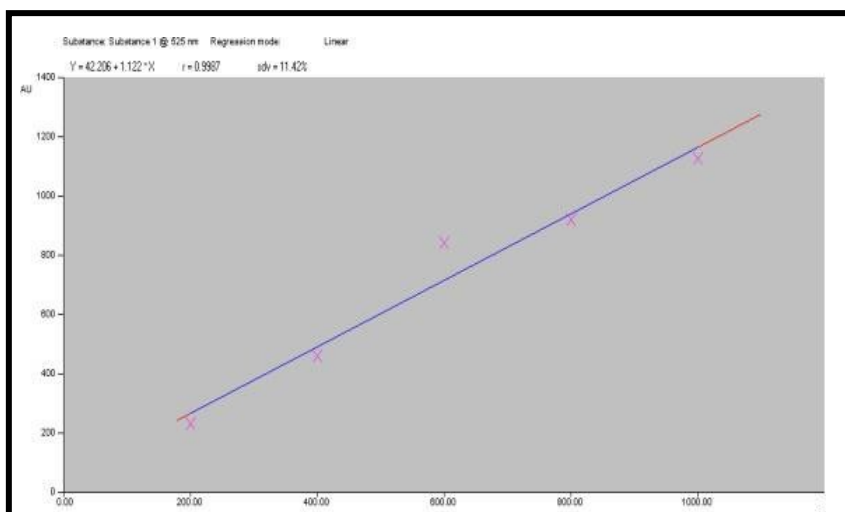
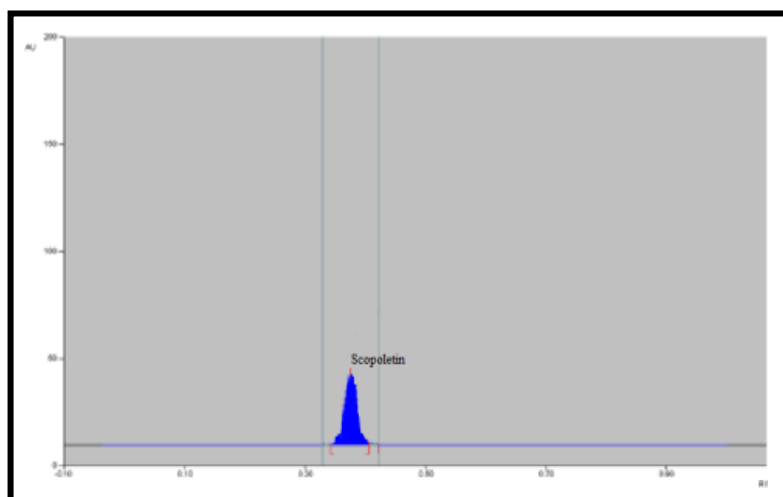
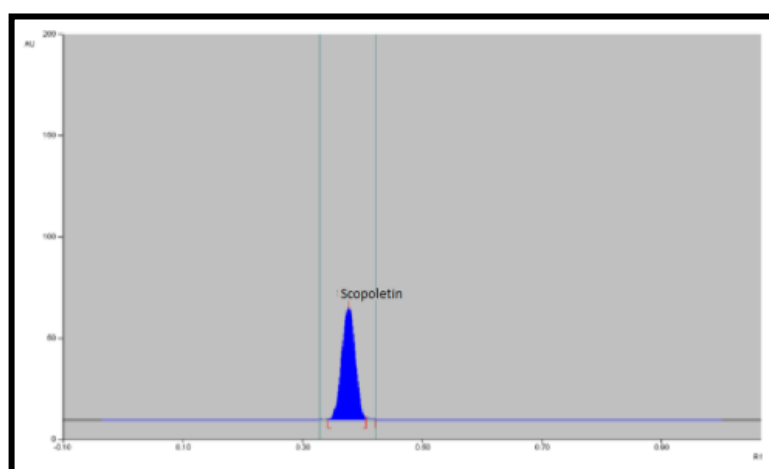
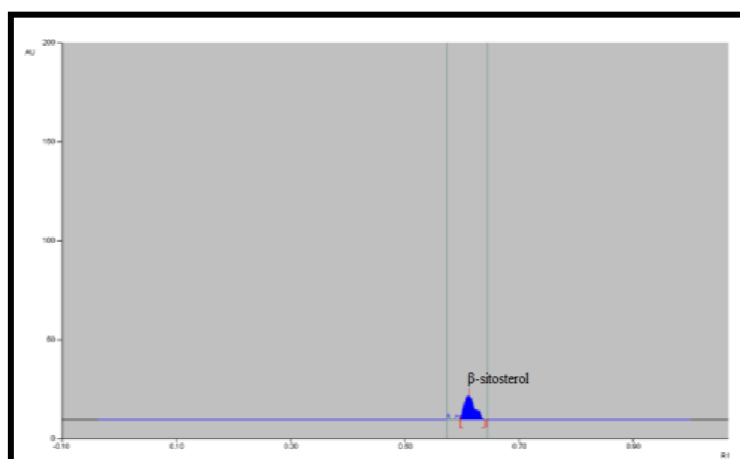


Figure 4: Calibration graph of β -sitosterol (200- 1000 ng/spot)

**Figure 5: LOD of scopoletin (5 ng/spot)****Figure 6: LOD of β -sitosterol (100 ng/spot)****Figure 7: LOQ of scopoletin (50 ng/spot)**

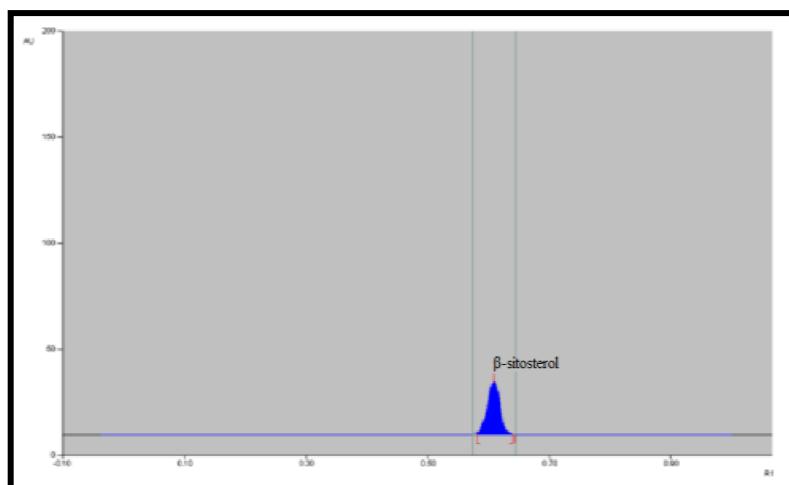
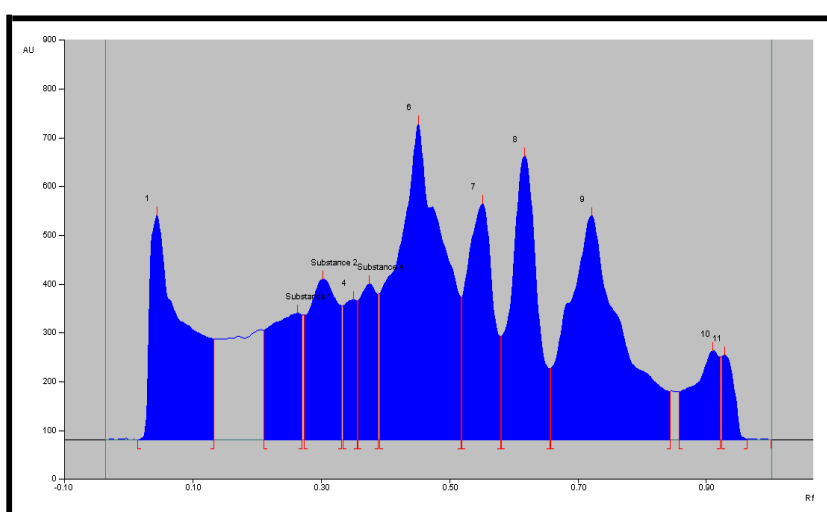
Figure 8: LOQ of β -sitosterol (200 ng/spot)

Table 3: Validation parameters of markers in HPTLC

Parameters	HPTLC	
	Scopoletin	β -sitosterol
Linearity	5-1000 ng/spot	200-1000 ng/spot
Correlation coefficient	0.9978	0.9987
LOD	5 ng/spot	100 ng/spot
LOQ	50 ng/spot	200 ng/spot
Precision		
1. Intraday	0.51	0.61
2. Interday	0.28	1.15
3. Repeatability	0.37	0.68
Plate stability	2 h	2 h

Table 4: Analysis of ethyl acetate extract of *Girardinia diversifolia*

Amount of scopoletin present in the ethyl acetate extract (mg/10 mg)	% RSD*	Amount of β -sitosterol present in the ethyl acetate extract (mg/10 mg)	%RSD*
0.005166	0.673	0.5069	0.213

*Mean RSD of 6 observation



HPLC Method:

In RP-HPLC method, a mobile phase comprising of acetonitrile: water (85:15 % v/v) as employed for the determination of scopoletin and β -sitosterol. This system provided symmetric peak shape and acceptable resolution with a retention time of 1.04 minutes for β -sitosterol and 1.4 minutes for scopoletin. The mixture of scopoletin and β -sitosterol was found to be linear over a concentration range of 0.3 to 1.5 $\mu\text{g/ml}$ for scopoletin and 200 to 1000 $\mu\text{g/ml}$ for β -sitosterol. The correlation coefficient values for

scopoletin and β -sitosterol were found to be 0.99242 and 0.99397 respectively (Fig. 11,12) indicating a strong relationship between peak area response and concentration. By using a linear regression equation, the amount of β -sitosterol in petroleum ether extract and ethanol extract was determined to be 0.237 mg/10 mg and 0.268 mg/10 mg, respectively (Table No. 6). The quantification of scopoletin was found to be difficult as the peak area of scopoletin was very low in the above extracts. The results of the validation parameters for marker mixtures were carried out and they are listed (Table No. 5).

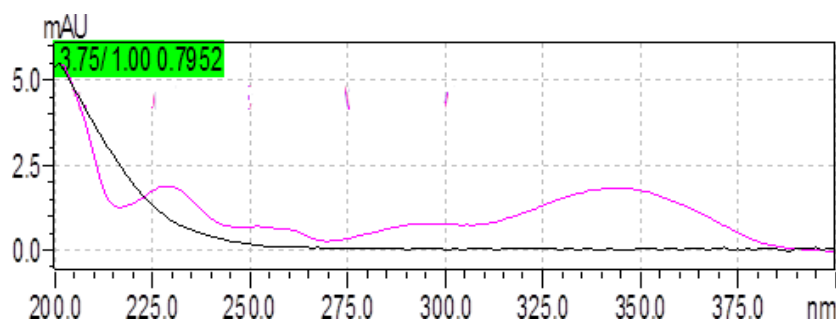


Figure 10: Overlay spectrum of scopoletin and β -sitosterol

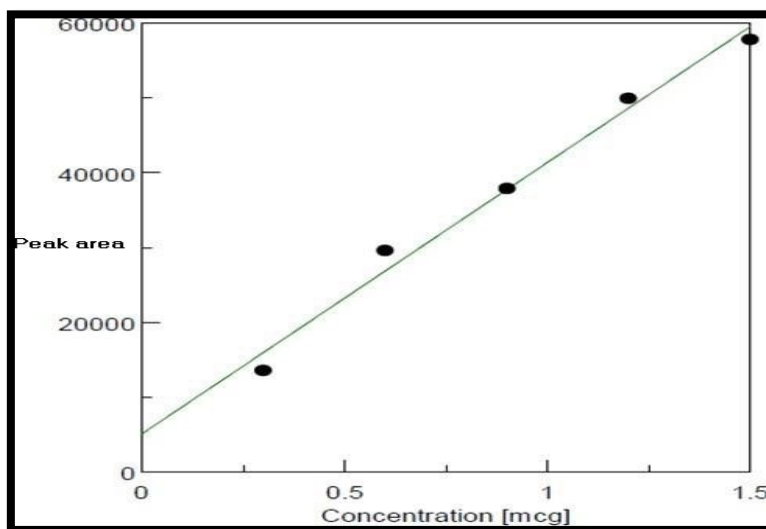


Figure 11: Calibration graph of scopoletin (0.3- 1.5 $\mu\text{g/ml}$)

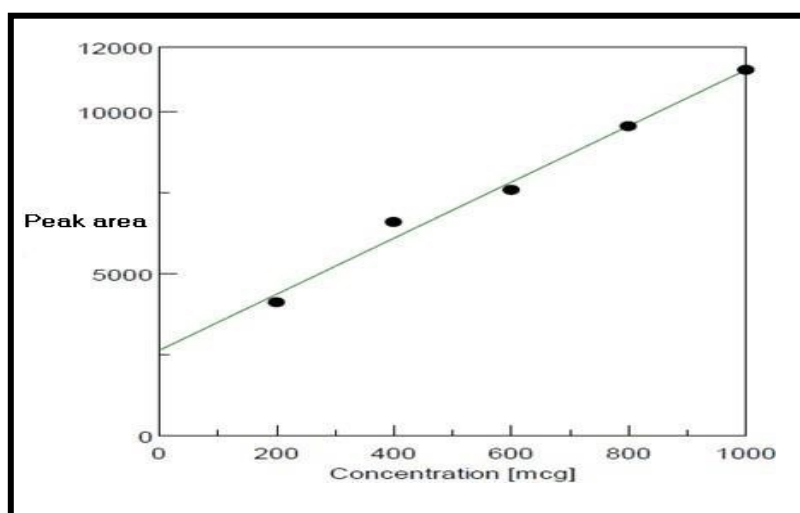


Figure 12: Calibration graph of β -sitosterol (200- 1000 $\mu\text{g/ml}$)

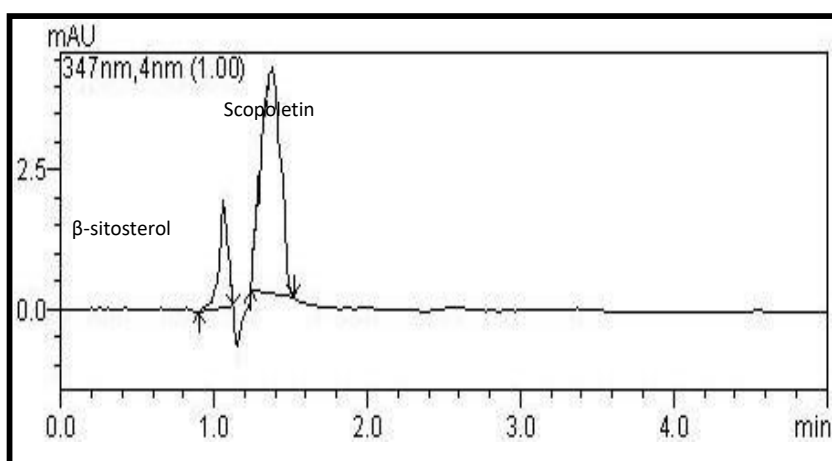


Figure 13: LOD (Scopoletin 0.01 $\mu\text{g/ml}$ and β -sitosterol 100 $\mu\text{g/ml}$)

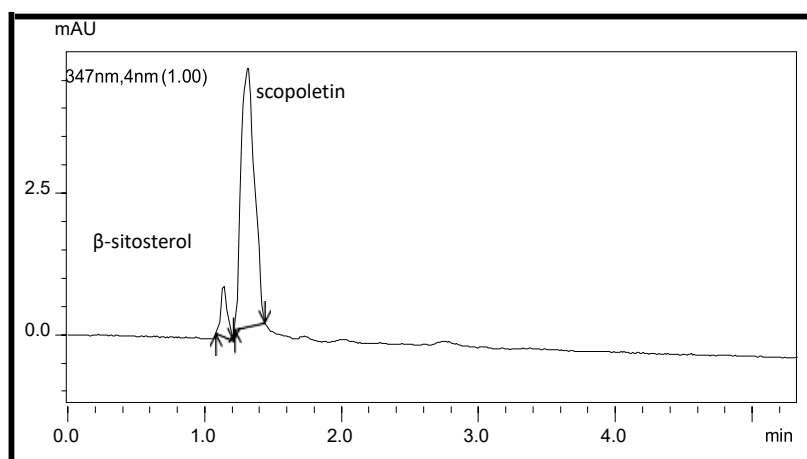


Figure 14: LOQ (Scopoletin 0.04 $\mu\text{g/ml}$ and β -Sitosterol 200 $\mu\text{g/ml}$)

Table 5: Validation parameters for marker mixture

Parameters	HPLC	
	Scopoletin	β -sitosterol
Linearity	0.3-1.5 $\mu\text{g/ml}$	200-1000 $\mu\text{g/ml}$
Correlation coefficient	0.99242	0.99397

LOD	0.01 µg/ml	100 µg/ml
LOQ	0.04 µg/ml	200 µg/ml
Precision		
1. Intraday	0.55	0.40
2. Interday	0.75	0.45
3. Repeatability	0.12	0.11
Stability in solution	2 days	2 days
Resolution		2
No. of theoretical plates	1216.42	2411.7
Tailing factor	1.6	0.984

Table 6: Analysis of extracts of *Girardinia diversifolia*

Extract	Amount of β -sitosterol present in 10 mg of extract (mg/10 mg)	% RSD
Petroleum ether	0.237	0.32
Ethanol	0.268	0.41

*Mean RSD of 6 observations

DISCUSSION

Scopoletin and β -sitosterol were simultaneously determined using HPTLC and HPLC in the whole plant extracts of *Girardinia diversifolia*. The suggested HPTLC and HPLC techniques can be used as an analytical tool to assess the quality of plants and formulations that contain phytochemical markers. This current research serves as a precursor for creating new herbal formulations with anti-inflammatory properties. These results may hold commercial value for pharmaceutical firms and research organizations in the creation and advancement of new medications.

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