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HPTLC Method Development and Validation for Standardization of Drynaria Quercifolia Rhizome Powder

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ABSTRACT
Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease affecting 0.5–1% of adults worldwide, with women exhibiting a 2–3 times higher prevalence than men. Current therapeutic options, including non-steroidal anti-inflammatory drugs, disease modifying anti-rheumatic drugs, glucocorticoids, immunosuppressants, and biological agents, often present limitations such as adverse effects and high cost. This has led to increased interest in plant-based alternatives. Drynaria quercifolia known as 'Mudavaatukkal' in Tamil and traditionally used in Indian ethnomedicine, has been reported to treat various ailments, including rheumatism. The rhizomes contain bioactive compounds such as phenols, tannins, alkaloids, flavonoids, β-amyrin, β-sitosterol, and naringin. β-Sitosterol, in particular, exhibits anti-inflammatory, immunomodulatory, antioxidant, and anti-arthritic properties. Secondary metabolites, crucial for the efficacy of herbal medicines, are highly susceptible to seasonal variations, making quality control and standardization challenging. These fluctuations, coupled with diminishing plant sources, increase the risk of adulteration. In this study, a rapid, simple, precise, and selective high-performance thin-layer chromatography (HPTLC) method was developed for the quantification of β-sitosterol in marketed preparations of D. quercifolia. Separation was achieved on TLC aluminum plates precoated with silica gel 60 F_{254} using toluene:ethyl acetate:formic acid (5:5:1, v/v/v) as the mobile phase. Densitometric detection was performed at 248 nm, yielding a Rf value of 0.74 for β-sitosterol. The method was validated in accordance with ICH guidelines, confirming its suitability for quality control and standardization of herbal formulations containing D. quercifolia.
KEYWORDS: *Drynaria quercifolia, Standardization, HPTLC, β-sitosterol*

I. INTRODUCTION

A chronic systemic autoimmune inflammatory illness, rheumatoid arthritis (RA) affects 0.5 to 1% of adults globally each year.[1] Women have a 2-3 times higher prevalence of RA than males have. Research indicates that RA primarily affects people between the ages of 40 and 70, and numerous evidences point to RA as a significant social, medical, and financial burden.[2]

They days, a variety of synthetic pharmaceuticals are used to treat RA; they include glucocorticoids, immunosuppressants, biological agents, non-steroidal anti-inflammatory drugs, and anti-rheumatic therapies.[3] But due to their high cost and many negative effects have prompted many to look for plant-based treatments for arthritis.[4] Throughout history, humans have used natural products especially plants to heal a wide range of ailments.[5] Many medicines made from plants are invaluable for treating arthritis because they are affordable, have no negative side effects, and work well for long-term ailments.[6] The World Health Organization has noted the utility of medicinal plants to public health care in developing nations and come up with guidelines to help member states formulate national policy on traditional medicine and conduct research on its potential benefits, which include determining its efficacy, safety, and quality. Standardization is the process of determining the quality and purity of crude pharmaceuticals through morphological, microscopical, physical, chemical, and biological observations, among other criteria.[7]

Medications made from natural substances found on Earth are referred to as traditional medicine. Nowadays, herbal medicines are frequently found in pharmacies, grocery stores, pharmacies, and health stores under the heading of "health products." Because traditional medicine is accessible, affordable, and culturally acceptable, most people in developing nations still use it for their fundamental medical requirements.[8]

Common names for the plant Drynaria quercifolia (L.) J. Sm. include oakleaf fern and oakleaf basket fern. Other frequent names include Aattukal kilangu or Mudavaattukal kilangu in Tamil, Pankhiraj in Bangla, Pannakizhangu in Malayalam, Aswakarti in Sanskrit, Asvakatri in Hindi, and Pakpak lawin in the Philippines. It is extensively distributed in India's evergreen forests and is a member of the Polypodiaceae family of Pteridophyta.[9] At its youngest, the fleshy rhizome can grow up to 18 cm long and 8 cm wide. The dried rough rhizome is up to 12 cm x 6 cm x 2 cm in size, irregularly shaped, and almost flat in color. It is covered in copper-colored scale leaves that are flexible and velvety. The inner surface has a light reddish-brown color and noticeable longitudinal wrinkles; it is splintery and has no particular bitter flavor or smell.[10]

Tribal groups in Tamil Nadu and Kerala are said to have employed the rhizome to treat a number of illnesses, and this custom is still followed today. The Kolli Hills locals by eating it as a soup for its health advantages.

The rhizome of the plant DQR was traditionally used to treat a variety of ailments, including fever, headache, cholera, typhoid, chronic jaundice, joint pain, cholera, body pain, diarrhea, and skin conditions, according to ethnomedical data.[7,11] A traditional healer demonstrates the ethnomedical applications of Drynaria quercifolia in the Thiruvolakkampaarai Hills of Tamil Nadu. In the Sathuragiri Hills of Tamil Nadu, a traditional healer sells soup made from rhizomes that have been harvested for rheumatic ailments.[12] This plant has been shown to have antimicrobial, antidermatophytic, neuropharmacological, antipyretic, anti-inflammatory, and analgesic properties. Additionally, it was stated that using this plant as a treatment promoted bone growth and prevented bone resorption.[13]

The compounds found in Drynaria quercifolia include proteins, xanthoproteins, phenols, tannins, alkaloids, carboxylic acid, coumarins, and saponins.

There are also other phytochemicals such as triterpenes, flavonoids, steroids, and catechins. Dried rhizomes include β-sitosterol, β-amyrin, 3-β-D-glucopyranoside 3, 4 dihydroxyl benzoic acid, flavone glycosides, friedelin, acetyl lupeol, naringin and naringinin, aglycone.[14]

A subclass of steroids, phytosterols are a significant family of bioorganic molecules found in a wide variety of plants, animals, marine life, and fungus. Their structures are similar to those of cholesterol.[15] Although their side chains differ, all sterols share the same sterol ring. Known by many as phytosterols, plant sterols are a crucial component of the lipid bilayer that keeps cell membranes stable. Plants are known to have about 40 different types of sterols. The three phytosterols that are most common and abundant are βsitosterol, compesterol, and stigmasterol. An estimated 500 different plants contain β-sitosterol.

Several studies have demonstrated that higher plants use mevalonic acid to convert acetate into C29 sterols. Additionally, phytosterols can be found as conjugates, especially fatty acyl sterol esters, which are abundant in seeds and lipid bodies of the cytoplasm. It is thought that this plant sterol acylation activity contributes to the preservation of the cell membrane's free sterol content at physiological levels.[16]

Numerous dietary and non-dietary plants have been shown to have BS. Revealed about its ability to stabilize cell membranes. It has been demonstrated that BS has numerous potential advantages and is usually regarded as a safe, natural, and efficient dietary supplement. It has been discovered that giving rats BS does not result in cytotoxicity or genotoxicity. BS is present in certain nutraceutical formulations that are sold on the market.[17]

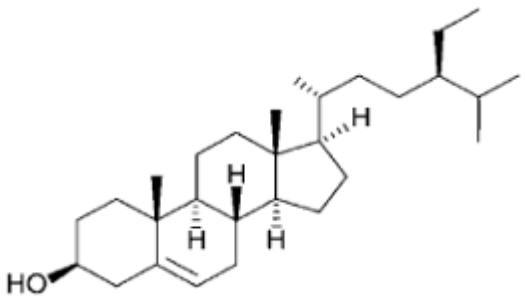

Figure 1.1: chemical structure of beta sitosterol

Chemical Properties:[3,18,19]
Molecular formula: $C_{29}H_{50}O$

IUPAC name: 17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17 dodecahydro-1 H-cyclopenta[a]phenanthren-3-ol
Solubility: It is soluble in some organic solvents like ethanol, dimethyl sulfoxide, chloroform, and ethyl acetate but insoluble in water.
Melting point: 130°C–140°C
Molecular weight: 414.7g/mol
Beta sitosterol possesses antibacterial, angiogenic, immunomodulatory, antidiabetic, anti-inflammatory, anticancer, antinociceptive, and antioxidant properties. Additionally, it can be safely employed to treat lower urinary tract infections, hypercholesteremia, immunosuppression, rheumatoid arthritis, and androgenetic alopecia.[14]

II. MATERIALS:

2.1. Chemicals and reagents:

β-sitosterol were purchased from Yucca Enterprise at Mumbai. The organic solvents and chemicals of analytical grade were procured from Technico chemicals, Coimbatore, India. HPTLC plates (10×10 cm) pre-coated with silica gel.

2.2. Collection:

Raw materials of *DRYNARIA QUERCIFOLIA* were procured from Pooja store at sulur and stored in air tight containers at room temperature. Market product of DQ were procured from Kottakkal Ayurveda at Sulur. The stationary phase used was TLC plates precoated with silica gel 60 F254 (20×20 cm) of 0.2 mm thickness obtained from E. Merck Ltd. Mumbai, India

2.3. Preparation of standard solutions:

Stock solutions of β-Sitosterol (1000µg/ml) were prepared separately by dissolving 0.01g of accurately weighed standard in 10 ml of chloroform. From this stock solution, 100µg/ml solution was prepared by transferring 1 ml stock solution to 10 ml volumetric flask then volume adjusted with chloroform. The standard stock solution containing 100µg/ml of β-Sitosterol was further dilute with chloroform to make the series of working standard solutions (200,400,600,800µg/mL) for chromatographic analysis.

2.4. Preparation of In-house formulation:

The rhizome was thoroughly washed with running tap water. The fur portion was removed from the rhizome. Then it was grinded and dry in the shadow, passed through 100 # sieve. The powder mixture was packed in air tight containers for further analysis.

2.5. Maceration and standardization of the extract:

The required quantity of *D. quercifolia* powder was soaked in chloroform and kept for 7 days following the standard maceration procedure. In this method, the powdered plant material is placed in a stoppered container with the solvent (chloroform) and allowed to stand at room temperature for a period of 7 days with occasional shaking or stirring to facilitate the extraction. After the maceration period, the extract is filtered to separate the solvent containing the dissolved constituents from the marc (residue). The chloroform extract thus obtained was concentrated and used for further analysis

2.6. Preparation of post derivatization reagent:

The post-derivatization reagent (Vanillin sulfuric acid) was freshly prepared and used for derivatization of the developed plate. The reagent was prepared by mixing of 2mL of Sulfuric acid in 100mL of 95% ethanol and dissolve 1g of vanillin, to the above solution. Prepared reagent mixed thoroughly and used for the analysis as per requirement.

2.7. High-performance thin-layer chromatographic instrumentation and conditions

2.7.1. Instrumentation:

Camag Linomat 5 semiautomatic sample applicator equipped with a 100µl Hamilton syringe (Camag, Switzerland) and visionCATS software (Ver.3.2.23095.1), Camag TLC Scanner 4, Twin trough chamber.

2.7.2. Chromatographic conditions:

Chromatographic separation was achieved on HPTLC plates (10×10 cm) pre-coated with silica gel 60 F254 of 0.2 mm thickness with aluminium sheet support. Standard solutions of markers and extracts were applied to the plates as bands 6.0 mm wide, 10.0 mm from the bottom edge of the same chromatographic plate by using of a Camag (Muttenz, Switzerland) Linomat 5 sample applicator equipped with a 100µl Hamilton syringe. Ascending

development to a distance of 70 mm was performed at room temperature (24 ± 2°C) with mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried and then scanned at 360 nm with a Camag TLC Scanner 4 using the deuterium lamp with win CATS software.

2.7.3 Selection of mobile phase

Selection mobile phase was done by trial of TLC plate on different mobile phases. Based on Rf value of the drugs, the best mobile phase was selected. Mobile phase tried were as follows:

Toluene: Chloroform: Methanol (4:4:1)
Toluene: Ethyl acetate: Formic acid (5:5:1)
Hexane: Ethyl acetate (7:3)

Fixed mobile phase

Toluene: Ethyl acetate: Formic acid (5:5:1)

2.7.4. Optimization of Mobile phase:

The standard stock solution containing 100µg/ml of β-Sitosterol was spotted on to TLC plate and developed in different solvent systems. Many preliminary trials were carried out for selection of mobile phase. Mobile phase composition was optimized to provide accurate, precise and reproducible results for the determination of β-Sitosterol.

2.7.5. Assay:

For assay purpose standard and sample (extract) solutions were applied on TLC plate in triplicates. Standard solutions of β-Sitosterol 200-800ng/ml was applied. Calibration curves constructed from peak areas obtained from standard solutions of β-Sitosterol. Sample (extract) solution was used for quantification of markers. The amount of β-Sitosterol present per gram of formulation was calculated by comparison of the areas measured for the sample with the calibration curves.

2.7.6. Method validation:

In accordance with ICH guidelines Q2 (R1) the optimized HPTLC method was validated with respect to following parameters.

2.7.7. Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. It was determined by plotting a graph of peak area v/s concentration of standards to obtain correlation coefficient (r2) and equation of the line.

2.7.8. Specificity:

Specificity is the ability to assess the analyte in the presence of components that may be expected to be present in the sample matrix. The specificity of the method was ascertained by comparing the Rf value and the peak purity was assessed by comparing the spectrum of standard β-Sitosterol with sample.

2.7.9. Precision:

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (%RSD) for a statistically significant number of samples. As per the ICH guidelines precision should be performed at three different levels low quality control (LQC), medium quality control (MQC) and high-quality control (HQC). Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra-assay precision. It was assessed by using minimum of 9 determinations covering the specified range for the procedure. The intra-day assay precision was performed 3 times on same day, while inter-assay precision was performed on 3 different days.

2.7.10. Limit of Detection (LOD) and Limit of Quantification (LOQ):

Limit of detection (LOD) is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Limit of Quantification (LOQ) is the lowest amount analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. LOD and LOQ were determined by k x SD/s where k is a constant (3.3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal and s is the slope of the calibration curve.

2.7.11. Accuracy:

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations /3 replicates each of the total analytical procedure). The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% by adding known amount of standard mixture of β-Sitosterol. These samples were then analyzed and the results obtained were compared with expected results.

2.7.12. Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It was studied in triplicate by making small changes in mobile phase composition and the mobile phase saturation time. The final results were examined by calculation of %RSD of concentration.

III. RESULT & DISCUSSION

3.1. Optimization of Mobile phase:

The standard stock solution containing 100µg/ml of β-Sitosterol was spotted on to TLC plate and developed in different solvent systems. Many preliminary trials were carried out for selection of mobile phase. Mobile phase composition was optimized to provide accurate, precise and reproducible results for the determination of β-Sitosterol. The mobile phase of Toulene: Ethyl acetate: Formic acid: (5:5:1) the best resolution/band symmetry and retention factor with RF values at 0.74 ± 0.79.

3.2. HPTLC chromatogram:

Good resolution and sharp peaks with minimum tailing were obtained with mobile phase consist of Toulene: Ethyl acetate: Formic acid: (5:5:1). β-sitosterol was satisfactorily resolved with R_f value at 0.74 ± 0.79.

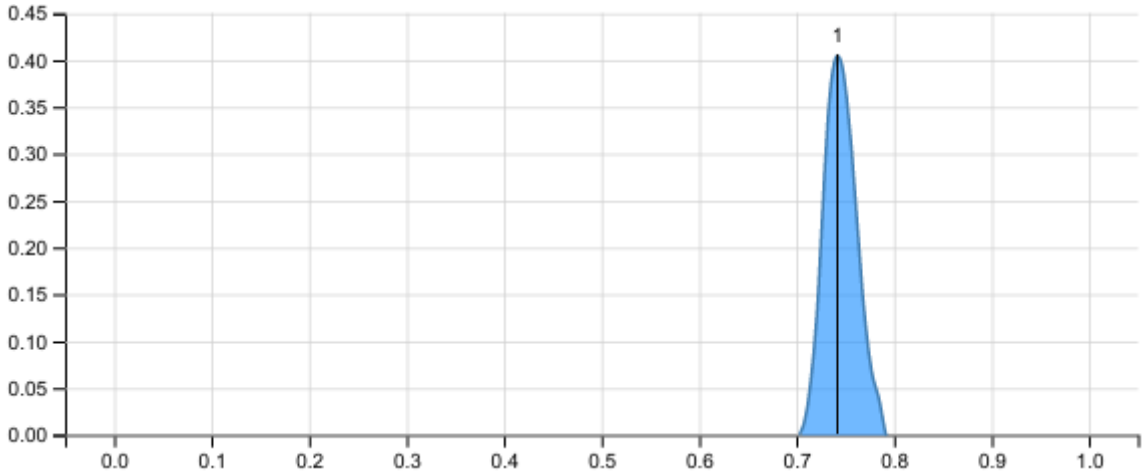

Figure 3.1.: HPTLC CHROMATOGRAM OF STANDARD β-SITOSTEROL [R_f 0.74 ± 0.79]

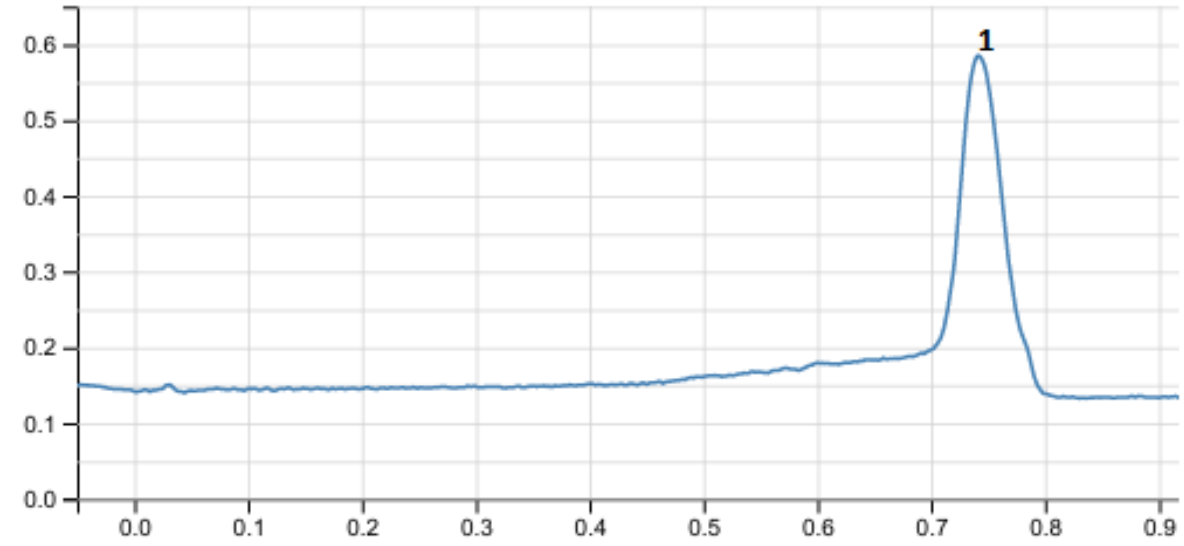

Figure. 3.2: Track S – β-SITOSTEROL standard scanning peak densitogram display

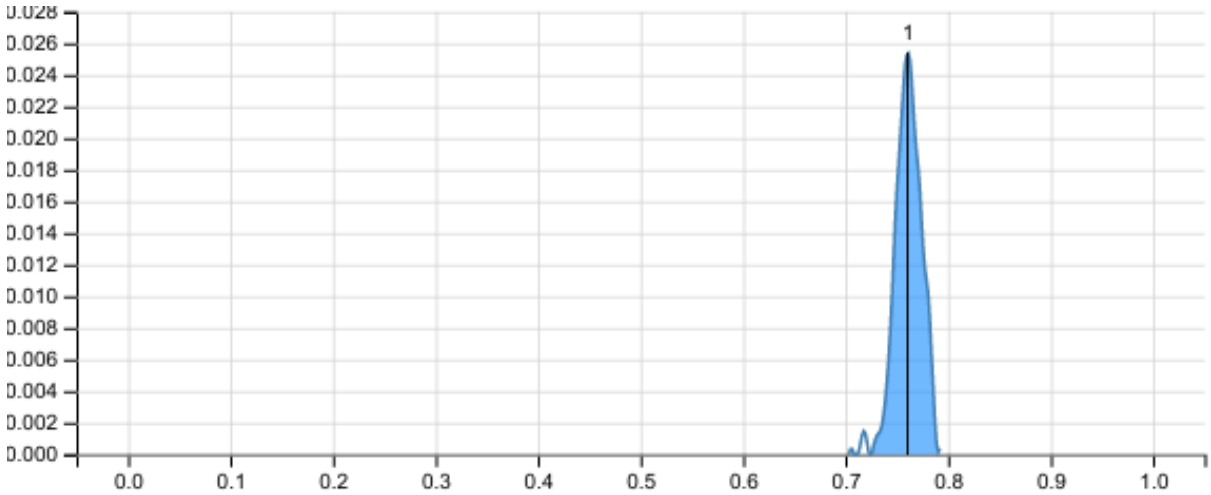

Figure 3.3: Track M – β-SITOSTEROL in marketed formulation scanning peak densitogram display

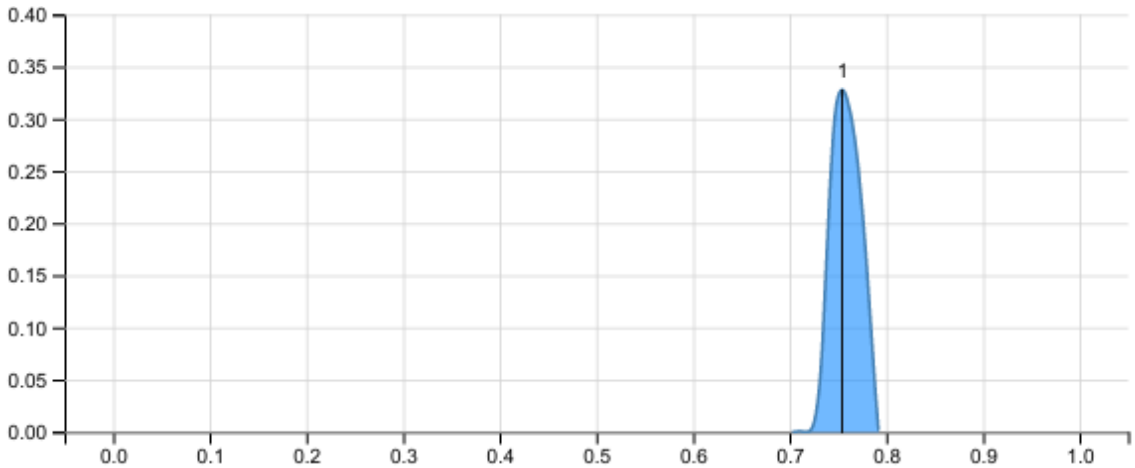

Figure 3.4: Track H – β-SITOSTEROL in Household formulation scanning peak densitogram display

3.3. Method validation:

3.3.1. Linearity:

To assess the linearity of the method, the calibration curve was plotted with four different concentration levels for each reference com pound in the range of 200-800ng/ml of β-Sitosterol. The linear regression equations were found to be y=2.225x+100 and the correlation coefficients (r2) was 0.9982.

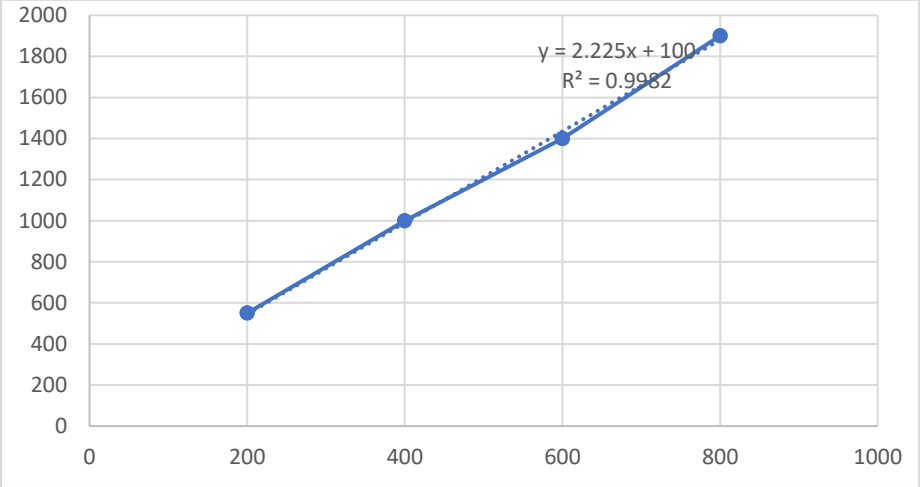

Figure:3.5 Linearity (200-800 ng/spot)

Other parameters describing the calibration data including correlation coefficient, slope, and intercept are given in Table1

3.3.2. Limit of detection (LOD) and Limit of quantification (LOQ):

LOD and LOQ results of β-Sitosterol were obtained as 53.73ng/spot and 162.8ng/spot respectively. (Table 1)

Table 1. SUMMARY OF VALIDATION DATA

Sl no	Parameters	β-sitosterol
1	Linearity (ng/spot)	200-800
2	Equation Correlation Coefficient (r^2±SD)	y=2.225x+100 0.9982 ± 29.6
3	Slope±SD Intercept±SD	2.225 ± 0.066 100 ± 36.23
4	LOD and LOQ (ng/spot)	53.73 and 162.8

3.3.3. Specificity:

When the spectra of standard β-sitosterol were overlayed or compared with extracts of *Drynaria quercifolia* it was observed that constituents present in the extract did not interfere with the peaks of β-sitosterol. Thus, the proposed method was proved to be Specific.It was shown in Figure 3.6

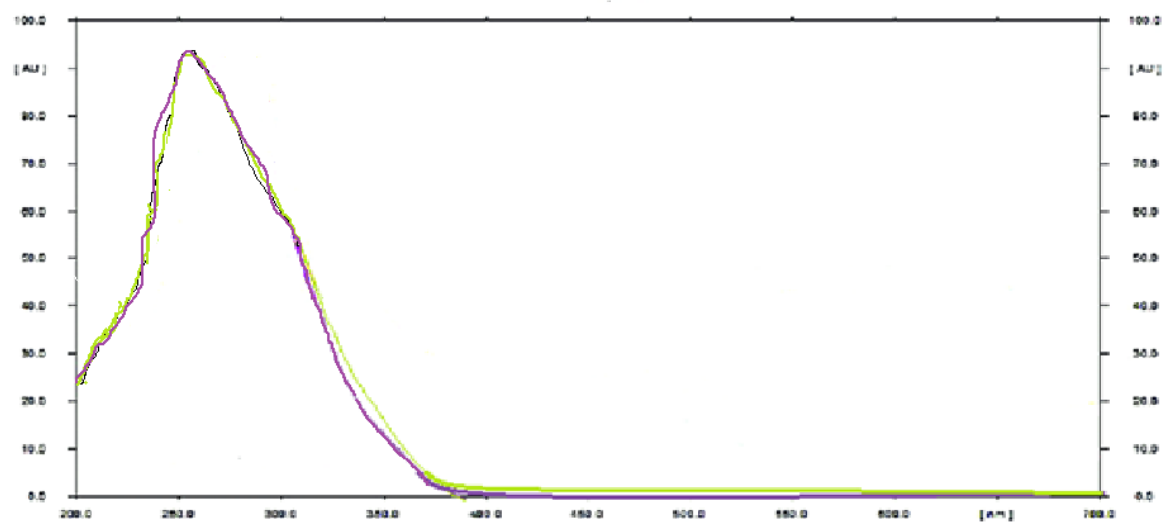

Figure 3.6.: OVERLAY SPECTRA OF STANDARD AND β-SITOSTEROL FROM EXTRACT.

3.3.4. Precision:

Intraday precision is used to describe the variation of the method, at three different concentration levels within the same day while intraday precision is for variation between different days. The % RSD values for both intraday and inter day precision were found within acceptable limit as shown in Table 2.

Table 2: INTRA-DAY AND INTER-DAY PRECISION RESULTS OF β-SITOSTEROL

β-SITOSTEROL	Concentration (ng/spot)	Interday			Intraday		
		Mean Area	S.D	%RSD	Mean Area	S.D	%RSD
	200	865.2	11.05	1.28	851.2	11.32	1.33
	400	1150.44	19.56	1.70	1189	23.4	1.97
	600	1864.48	32.37	1.74	1861.79	22.12	1.19

3.3.5. Accuracy:

Accuracy of the method is reported as percent recovery of known added amount of analyst in the sample. The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% by adding known amount of standard mixture of β-Sitosterol. Results obtained were given in Table.3.

Table 3: ACCURACY DATA FOR β-SITOSTEROL

Compound	Level of % Recovery	Recovery (%)	RSD	Mean Recovery
Marketed Formulation	80 100 120	93.91 99.06 95.47	1.29 0.96 0.60	96.40
In house Formulation	80 100 120	92.15 94.63 95.04	0.61 0.55 0.70	93.84

3.3.6. Robustness:

The % RSD of the peak area was calculated in triplicate for changes in mobile phase composition, volume, and duration of saturation time. The values of % RSD were less than 2% which indicated that the developed method is robust as shown in Table 4.

Table 4: ROBUSTNESS RESULTS OF β-SITOSTEROL

Parameters	RSD
Mobile phase composition	1.025
Amount (volume) of mobile phase	1.033
Saturation time (±10min)	0.983

Estimation of β-sitosterol in marketed and In-house formulations:

The developed method was applied for the detection and quantification of β-sitosterol from marketed and in-house formulations of *Drynaria quercifolia*. The peaks for β-sitosterol are observed at R_f 0.74 ± 0.79. In the densitogram of extracts. The test samples of marketed formulations and in-house formulation were compared with the ingredients Figure 3.7.

S - STANDARD β-SITOSTEROL
M - MARKETED FORMULATION OF β-SITOSTEROL
H - INHOUSE FORMULATION OF β-SITOSTEROL

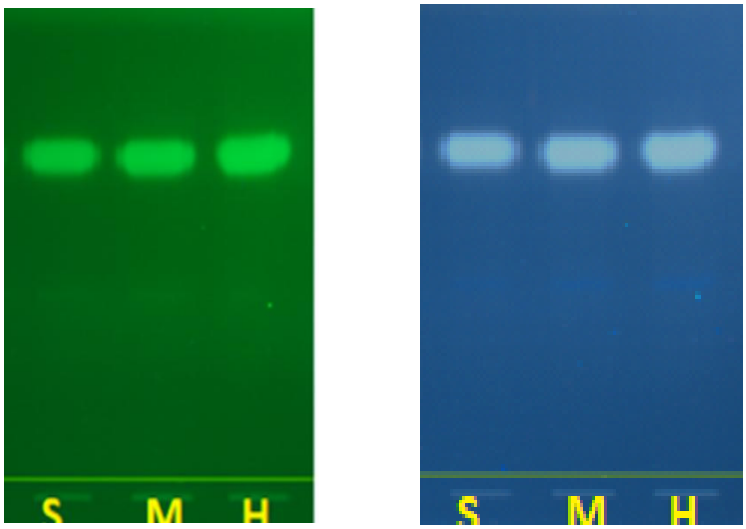

Figure 3.7: HPTLC FINGERPRINTING PROFILE OF EXTRACT OF *DRYNARIA QUERCIFOLIA* AT 248nm RESPECTIVELY.

There was no interference from other compounds present in the *Drynaria quercifolia*.
The total content of β-sitosterol in marketed formulations and in-house formulation is as shown in Table 5.

Table 5: β-SITOSTEROL CONTENT IN POLYHERBAL FORMULATIONS

Formulations	β-sitosterol content(%w/w)
Marketed formulation	0.20
In House Formulation	0.17

IV. CONCLUSION:

In the present work, attempt has been made for standardization of Drynaria quercifolia by developing chromatographic method. β-sitosterol from Drynaria quercifolia present in formulation were selected as marker compounds. A new, rapid, simple, precise, selective HPTLC method was developed for marketed preparation of Drynaria quercifolia. The separation was performed on TLC aluminum plates recoated with silica gel 60 F254, Toulene: Ethyl acetate: Formic acid: (5:5:1) v/v/v as mobile phase. The densitometric analysis was carried out at the detection wavelength of 248 nm. The Rf values of β-sitosterol was found to be 0.74. The developed method has been validated as per ICH guidelines.

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CONFLICT OF INTEREST:

Authors declare no conflict of interst.

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