HPTLC METHOD FOR ESTIMATION AND QUANTIFICATION OF β-SITOSTEROL FROM IN-VIVO AND IN-VITRO SAMPLES OF MERREMA AEGYPTIA AND MERREMA DISSECTA

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ABSTRACT

Objective: A normal-phase high-performance thin-layer chromatography (HPTLC) method has been developed and validated for estimation and quantitation of beta-sitosterol from the methanolic fraction of different plant parts of two medicinally important plants viz. Merremia aegyptia and Merremia dissecta. These plants have been reported to possess antimicrobial, antioxidant, and anti-inflammatory activities.

Methods: Chromatographic separation of beta-sitosterol from the methanolic extracts of plant parts of M. aegyptia and M. dissecta was performed on TLC aluminum plates pre-coated with silica gel 60F 254 using a suitable mobile phase. The densitometric scanning was done after derivatization at λ 254 nm for β-sitosterol.

Result: Only M. dissecta leaf sample was reported to contain β-sitosterol (4.6 ng/μl), whereas other samples such as seed, stem, and callus extracts of M. aegyptia and M. dissecta did not show its presence.

Conclusion: The developed HPTLC method is simple, rapid, and precise and can be used for routine analysis and quantification of β-sitosterol and other useful plant bioactives that are phytopharmaceutically important.

Keywords: High-performance thin-layer chromatography, Derivatization, Anti-inflammatory, Merremia aegyptia, Merremia dissecta.

INTRODUCTION

High-performance thin-layer chromatography (HPTLC) employs the principle of adsorption for separation. The mobile phase solvent moves according to capillary action. Different components of the solution separate according to their affinities toward the adsorbent. The component with more affinity toward the stationary phase travels slower and vice versa. However, it has certain limitations such as having limited developing distance and low plate efficiency as compared to high-performance liquid chromatography (HPLC) and gas chromatography still it is widely used for quality evaluation due to its simplicity, low cost, and requirements.

HPTLC is used to assess, compare, and quantify the reservoirs of secondary metabolites in different plants and also can be used in fingerprinting as conducted by Dawane and Fulekar [1] in which the results obtained by HPTLC fingerprinting method were found reliable, accurate, and economical for identification and authentication of Avicennia marina mangrove species from other similar mangroves, in a single TLC plate run.

Plant sterols (or phytosterols) are a naturally occurring part of all plants and are found in nuts, legumes, grains, cereals, wood pulp, and leaves. Phyto-sterols are similar in structure to cholesterol in the human body and therefore block cholesterol from being absorbed. β-sitosterol is one such phytosterol having properties such as reducing cholesterol levels, activities of cancer cells, promoting prostate gland health, and immunity enhancement in the human body (Fig. 1).

The structures of beta-sitosterol are quite similar to cholesterol, thus can inhibit the absorption of cholesterol in the body [2], reducing its levels in the plasma [3]. The liver function activity (GDP, GOP) can be improved with β-sitosterol [4], and it can also reduce prostate and colon cancer cell growth [5]. The presence of β-sitosterol in foods suppresses carcinogenesis. It can also be the factor in forming the lymph cells and Natural Killer cells in the immunity process circulation [6]. β-sitosterol in corn oil, rice bran oil, and other vegetable oil decreases the cholesterol level in the plasma [7]. It has antifungal, antibacterial, and anti-inflammatory activities and can be used to treat asthma, arthritis, allergies, and cancer [8]. It was isolated from tissue cultures of Balanites aegyptiaca [9]. The HPTLC analysis of Asparagus racemosus, Withania somnifera, Vitex negundo, Plumbago zeylanica, Butea monosperma, and Tephrosia purpurea extracts was investigated [10].

The present study was undertaken for identification and quantification of β-sitosterol from in vivo and in vitro plant parts of Merremia aegyptia and Merremia dissecta.

METHODS

Preparation of extracts

Plant parts such as leaves, stems, seeds, and callus of M. aegyptia and M. dissecta were dried in the dark at room temperature, powdered and methanol extracted by Soxhlet extractor. Afterward, the extracts were

Fig. 1: Chemical structures of β-sitosterol
filtered using Whatman filter paper no. 42 and the solvents obtained were dried in a rotary vacuum evaporator.

Reagents and other materials
β-sitosterol (Sigma Aldrich), toluene, ethyl acetate, and methanol (all reagents of analytical grade, E. Merck) and silica gel 60 F₂₅₄ pre-coated TLC aluminum plates (E-Merck) were used for the analysis.

Preparation of standard and sample solutions
Ten milligram β-sitosterol was dissolved in 10 ml methanol (1 mg/ml). The Soxhlet extracted and dried methanolic extracts were accurately weighed (100 mg) and dissolved in methanol and then solution was filtered through Whatman filter paper no. 42 and the filtrate was made up to the mark with methanol.

Procedure
The samples were spotted in the form of bands with CAMAG microliter syringe on a TLC A1 pre-coated silica gel plates 60 F₂₅₄ (10 cm×10 cm with 0.2 mm thickness, E. Merck) using CAMAG Linomat V applicator. 10 µl of test solution and standard solution was applied on pre-coated layer maintaining a distance of 10 mm from the bottom edge, band length 6 mm, distance between tracks 10 mm and distance from the side 15 mm. Application position 10 mm with solvent position 70 mm. Measurement mode was ultraviolet (UV) absorbance/reflectance with scanning wavelength 580 nm, tank saturation 10 min with filter paper. Total no. of tracks was 8 with the position of first track X-axis 15.0 mm, distance between tracks 1.0 mm, scan start position Y-axis 5.0 mm, scan end position Y 75.0 mm, slit dimensions 4.00×0.30 mm, micro with scanning speed 20 mm/s, and data resolution 100 µm/step. Detector was used as an automatic mode with sensitivity 33%, peak threshold minimum slope-5, peak threshold minimum height-10AU, peak threshold minimum area-50, and peak threshold maximum height-990AU.

The plates were developed in a solvent system in CAMAG glass twin trough chamber (20×10 cm) with S.S. Lid. Of the various solvent systems tried that containing toluene-ethyl acetate (8:2v/v) gave the best resolution of β-sitosterol (retention factor-0.91). It was developed up to 80 mm in the twin trough chamber using mobile phase, dried with the help of air drier, and subjected for TLC scanning using TLC scanner 3 (CAMAG Switzerland) and spots were visualized under UV Light at 254 nm, 310 nm, and under visible light at 580 nm. Deuterium and tungsten lamps were the source of radiation utilized.

A 10 µl suitably diluted sample volume was applied in triplicate on a TLC plate. The standard zones were quantified by linear scanning at 580 nm by use of a TLC Scanner III CAMAG. Data of peak height and peak area of each spot were recorded. The purity of β-sitosterol bands was confirmed by comparing the absorption spectra at start, middle, and end position of the bands. The calibration curve was prepared by plotting concentration (microgram/spot) versus peak area corresponding to each spot. Hence, the amount of β-sitosterol in different samples was calculated using the respective calibration curve. The plates were developed and scanned at 580 nm. The peak areas and absorption spectra were recorded. Concentration of the metabolite in sample was calculated by considering the sample initially taken and dilution factors. Quantitative evaluation of the plate was performed in the remission/absorption mode at 580 nm with the following conditions slit width 4.00×0.30 mm, micro scanning speed 20 mm/second and data resolution 100 µm/step.

RESULT AND DISCUSSION
HPTLC is a reliable analytical technique for quantification of analytes at micro and nanogram levels [11]. It is a useful technique reason being its low operating cost, less analysis time, high sample throughput, and need for minimum sample clean-up. HPTLC offers precise control over sample application, chromatographic development, derivatization, detection, and determination [12,13].

HPTLC studies of methanolic sample extract for identification of stigmasterol, quercetin, and kaempferol simultaneously in Indigofera tinctoria and quantification of kaempferol and quercetin in various medicinal plants has been performed [14,15]. Isolation and identification of stigmasterol and β-sitosterol through HPTLC from methanolic extract of root bark of Calotropis gigantea and quantification of stigmasterol and lupeol from Ficus religiosa [16,17] and lupeol from Andrographis echioides leaves has been done [18]. These types of quantification studies thus stand useful in suggesting a wide array of possible therapeutics from natural sources.

In the present study, β-sitosterol was estimated and quantified from four different plant parts viz. leaf, stem, seed, and callus (in vitro) samples of M. aegyptia and M. dissecta, respectively, as it is a medicinally important compound and has antifungal, antibacterial, and anti-inflammatory activities and is also used to treat asthma, arthritis, allergies, and cancer.

HPTLC studies revealed resolution of bands of β-Sitosterol at Rf-0.91 (Fig. 2). The identity of the bands of β-Sitosterol in the samples was confirmed by overlapping their UV absorption spectrum with that of standards. The calibration curve was plotted using concentration (µg/spot) and peak area of each spot. The amount of β-sitosterol in different samples was calculated using the calibration curve. The plates were developed at 580 nm. M. dissecta leaf sample reported to contain β-sitosterol in quantity 4.6 ng/µl. The presence of such a useful metabolite in vivo makes this plant phytopharmaceutical important.

Fig. 2: Chromatogram of standard solution of β-Sitosterol peak (Amount/Fraction =1200 ng)
**CONCLUSION**

Advantages of HPTLC include the capability to detect more compounds than HPLC simultaneously and it can identify compounds with no UV absorption by reagent spraying. It also provides semi-quantitative information on the major active constituents of a drug or drug preparation, thus enabling an assessment of drug quality. Furthermore, the colorful picture in HPTLC provides extra intuitive parameters of visible color and/or fluorescence [16]. Realizing the medicinal importance of \( \beta \)-sitosterol, the present study was performed and it was found that *M. dissecta* leaf sample contained \( \beta \)-sitosterol (4.6 nanogram/microliter, Fig. 3).

**CONFLICTS OF INTEREST**

The authors have no conflicts of interest.

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**AUTHORS’ CONTRIBUTIONS**

Dr. Ridhi Joshi carried out all the laboratory work as part of her Ph.D. and compiled the manuscript. Dr. Vidya Patni and Dr. Preeti Mishra analyzed the data and guided throughout the research work. Dr. Rishikesh Meena finalized the manuscript. All the authors read and approved the final manuscript.

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