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COMPARATIVE QUANTITATIVE ESTIMATION OF β SITOSTEROL IN SELECTED INDIAN MEDICINAL PLANTS BY HPTLC FINGERPRINTING

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ABSTRACT

Background: Herbal plants are abundant in phytochemicals and these constituents plays promising role in the treatment and management of different diseased conditions. Different scientific methods were used for qualitative and quantitative estimation of these phytoconstituents to use these medicinal plants as therapeutic agents. In this study, investigation of β sitosterol in all selected plants was done by HPTLC fingerprinting. **Objectives:** Present research was aimed to do comparative quantitative estimation of β sitosterol in all selected Indian medicinal plants by using reference biomarker with the help of HPTLC fingerprinting. **Materials and methods:** Based on significant use in traditional medicine, the four Indian medicinal plants were taken. All the dried plant part materials were subjected for extraction by using the Soxhlet with a mixture of ethanol and distilled water (70:30) at temperature 60-70 ^{o}C for 24 hours. The extracts were concentrated using Rota evaporator. Redissolved methanolic extracts of all selected plants and reference biomarker β sitosterol were used for HPTLC analysis in mobile phase Toluene: Ethyl acetate (8:2) at λ=540nm by using CAMAG Switzerland and densiometric estimation was done with CAMAG TLC scanner and Win cat software. **Results:** HPTLC chromatogram for β-sitosterol was analyzed and Rf value for standard β-Sitosterol was found 0.53, and the area of the peak of β- sitosterol was 7783.39 at a concentration (20µg/10µl). Maximum concentration and peak area are obtained for extract of stem of *Tinospora cordifolia* Miers with Rf value 0.52 and area of peak 2229.57 (% β- sitosterol-1.30). Extract of roots of *Boerhavia diffusa* L. with Rf value 0.53 and area of peak 1879.40 (% β- sitosterol: 0.92), extract of leaves of *Moringa oleifera* Lamk with Rf value 0.53 and area of peak 653.2 (% β- sitosterol: 0.38) whereas β- sitosterol is not detected in hydroalcoholic extract of *Ficus religiosa* L. **Conclusion:** Results of present research on HPTLC analysis of all selected plant parts provide a tool for authentication, standardization and quality control parameters of all selected species. Values obtained by present research may be helpful for other researchers in future.

KEYWORDS: β- sitosterol, HPTLC fingerprinting, *F. religiosa* L., *T. cordifolia* Miers, M. *oleifera* Lamk, *B. diffusa* L.

1. INTRODUCTION

Herbal plants and their different parts are used for cure and maintenance of various diseases from ancient times. These plant parts as such or their extracted forms can be utilized for the management of various diseased conditions. Different pharmacological actions of medicinal plants are due to occurrence of different secondary metabolites.[1] These medicinal herbs are rich in phytochemicals which are classified in primary metabolites (sugars, amino acids, colored pigments etc.) and secondary metabolites (triterpenoids, volatile oil, alkaloids, flavonoids, saponins etc.). Standardization of medicinal plants by quantitative and qualitative analysis is a unique method to establish purity, quality of plant materials, biochemical quality and quantity of various phytoconstituents present in raw plant materials. Pre-established and validated procedure of estimation of these natural phytoconstituents and profiling with the standard biomarkers.[2] Several analytical estimations are done for quantification of secondary metabolites in different plant parts. High-performance thin-layer chromatography (HPTLC) is an important quantitative analytical tool which is based on the principle of adsorption chromatography for separation.[3] The mobile phase constitutes different solvents which move due to capillary action on stationary phase. Analytes present in the solution differentiated according to their affinities toward the stationary phase. The analyte with more affinity travels slower while analyte with less affinity travels faster on the stationary phase. This technique is widely used due to low cost, minimum purification of extracts, high reproducibility and less time consuming.[4]

In the present research four Indian medicinal plants have been selected based on their huge medicinal importance in different traditional system and folk medicine for comparative estimation of β sitosterol. The selected plants are *Ficus religiosa* L. (*F. religiosa* L.), *Tinospora cordifolia* Miers (*T. cordifolia* Miers), *Moringa oleifera* Lamk (*M. oleifera* Lamk), *Boerhavia diffusa* L. (*B. diffusa* L.) and selected plant parts are stem bark, stem, leaves and roots respectively.
F. religiosa L. (Family- Moraceae) is commonly known as bodhi tree and this plant has enormous attention in Indian heritage due to its sacred, mythological, and medicinal importance.[5,6]

T. cordifolia Miers (Family- Menispermaceae) is popular as "Rasayana" due to its immense use in traditional medicine.[7] Other synonyms for this are amrita, guduchi, shindilkodi, giloy etc.[8,9]

M. oleifera Lamk (Family- Moringaceae) is popularly called 'Miracle Tree'.[10] Every part of this plant has been utilized for treatment of different diseases in the indigenous system of medicine in South Asia.[11,12]

B. diffusa L. (Family- Nyctaginaceae) is popularly known as Punarnava in 'Sanskrit' which means that revitalizes the old body.[13,14]

1.1. β sitosterol

All plants are rich in naturally occurring phytosterols and are abundantly found plants like peanuts, legumes, rice, wheat, grains, cereals, wood pulp, and leaves. These sterols are structurally like cholesterol which is present in the human body.[15,16] Phytosterol (β-sitosterol) has amazing health properties like cholesterol levels reduction[17], fight to cancer cells, protection of hepatic cells, reduces the levels of free radicals, reduction in inflammation, regulating health of prostate gland and immunity booster activities in the human body.[18,19] Structure (Fig 1) and chemical profile given below:

Synonyms: Cupreol, Cinchol, α-phytosterol

Chemical class/group: Terpenes (Subclass: Triterpenes)

Molecular formula: $C_{29}H_{50}O$

Molecular weight: 414.71

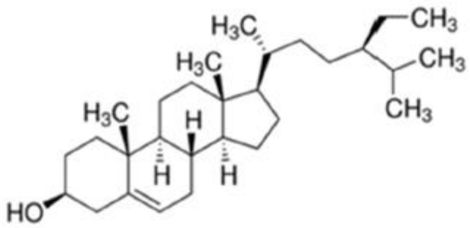

Fig. 1: Chemical structure of β-sitosterol.

1.2. Phytoconstituents and ethnomedicinal uses

F. religiosa L. bark is rich in phytosterols (lanosterol, β-sitosterol, stigmasterol) and β-sitosterol-d-glucoside. Other chemical constituents of bark are vitamin K1, n-octacosanol, methyl oleonate and lupen-3-one, bergapten and bergaptol (substituted furanocoumarins) and tannin content (8.7%).[20,21] The bark of *F. religiosa* L. has immense pharmacological importance in the treatment of gonorrhoea and other infections like bacterial, viral, protozoal infections, neurodegeneration, ulcer, hepatic disorders, kidney disorders, hepatic disorders used as antiulcer, astringent, anti-diarrhoeal, antineoplastic, nephroprotective.[22,23]

The chemical constituents have been isolated and identified from the stem of *T. cordifolia* Miers are cordifolioside A & B, berberine, 1,2-Substituted pyrrolidine, amritoside A, B, C and D, octacosanol etc.[24,25] Due to immense antioxidant effect stem of *T. cordifolia* Miers is used for the treatment of jaundice muscle contraction, pyrexia, neoplasm, urinary diseases, viral hepatitis, hyperlipidaemia, digestive disturbances diabetes, immunity disorders and liver diseases.[26,27]

Leaves of *M. oleifera* Lamk contain vitamins, carotenoids, flavone and polyphenolic compounds. It contains phytoconstituents like kaempferitrin, isoquercetin, rhamnetin, kaempferol and quercetin, zeatin, ascorbic acid, phenolic, flavonoids, vitamin E.[28,29] Leaves are used as antibacterial, antifungal, hepatoprotective, anti-inflammatory, anti-depressant, antineoplastic, in the traditional medicine system of South Asia.[30,31]

B. diffusa L. roots contain chemical constituents such as boeravinones A-1, B-1, C-2, D, E and F, beta sitosterol, boerhaavic acid, behenic acid, borhavine, campesterol, daucosterol, beta-ecdysone, hentriacontane N, hypoxanthine-9-l-arabinofuranoside, ursolic acid, and 5,7-dihydroxy-3,4-dimethoxy-6,8-dimethyl flavone.[32,33] This plant is used as bitter tonic, astringent, cooling, anthelmintic, diuretic, aphrodisiac, cardiotonic, and laxative.[34] It is also used in the treatment of inflammation, cough, bronchitis and general debility, leucorrhoea, ophthalmia, jaundice, anaemia, dyspepsia, constipation and immunomodulator.[35,36]

2. MATERIALS AND METHODS

In the present study, a HPTLC method is used for quantitative estimation of *β*- sitosterol in all selected parts of Indian medicinal plants.[37] The External Standard Method is generally used for quantification analysis in TLC studies as assures accuracy and precision in quantitative analysis.[38]

2.1. Plant material

The selected plant parts of *F. religiosa*, stem of *T. cordifolia*, root of *B. diffusa and* leaves of *M. olifera* were procured from nearby areas of Hyderabad and all were authenticated (Accession no. 1092,1094,1096,1098) by Dr. Mohammed Mustafa, Department of Botany, Kakatiya University, Telangana.

2.2. Hydroalcoholic extracts preparation

All the plant parts were grinded separately and all powders were passed through sieve 44. The powdered sample was treated with petroleum ether for defatting and 100 gm of each dried powdered sample was extracted by using the Soxhlet Apparatus with a mixture of ethanol and distilled water (70:30) at temperature 60-70 ^{o}C for 24 hours. The solvent was removed to get the solid extract.[39]

2.4. HPTLC fingerprinting and quantification of β-sitosterol

2.4.1. Preparation of standard and test solution for HPTLC

The stock solution of β-sitosterol was made by solubilizing 20 mg of the reference standard was transferred in 10 ml volumetric flask and volume was adjusted up to with methanol (2 mg/ml). Sample solution (50 mg/ml) was prepared by reconstituting in methanol of each hydroalcoholic extract.

2.4.2. Development of chromatogram and detection of β- sitosterol

The development of chromatogram was performed in a glass chamber of size 20×10 cm, which was saturated with mobile phase for twenty minutes before putting the plates. The standard and methanolic extracts of all hydroalcoholic plant extracts were spotted at the appropriate intervals form of bands with the help of CAMAG LINOMAT 5 applicator on precoated silica gel 60F_{254} pre-coated on aluminium sheet at the 10 mm distance from bottom edge. The volume applied 2, 4, 6, 8, 10, 12 µl for standard stock solution of β- sitosterol (2mg/ml) respectively in six bands while the volume of sample is applied 5, 10, 15µl sample stock solution of each plant extract respectively in three bands. The developed TLC plates sprayed with anisaldehyde sulphuric acid reagent for derivatization of. Detection and densiometric quantification of *β*-sitosterol in all hydroalcoholic extracts were done at λ=540nm using tungsten lamp by CAMAG TLC Scanner 3 and Win CAT software.[40,41] Estimation was done with the help of calibration curve. analysis was developed by using the software. Chromatographic specification illustrated (Table 1).

Table 1: Chromatographic conditions for HPTLC fingerprinting of β- sitosterol.

Instrument	CAMAG Switzerland
Stationary Phase	Silica gel 60F254 pre-coated on aluminium sheet
Mobile phase for β- sitosterol	Toluene: Ethyl acetate (8:2)
Prewashing of TLC Plate	Methanol, activated at 110 oC
HP- TLC Plate Development	Pre-saturated CAMAG Twin trough Chamber
Chamber saturation	20 min
Applicator	CAMAG LINOMAT 5 automated application
Band length and development distance	8 mm and 80mm respectively
Scanner	CAMAG TLC Scanner 3
Photo Documentation Chamber	CAMAG REPROSTAR 3
Drying of plate	At 110 oC for 5 min
Chromatographic evaluation	CAMAG TLC scanner and Win cat software
Scan Wavelength	540 (white R line)

3. RESULTS AND DISCUSSION

3.1 Extraction and phytochemical screening

All the selected plant parts were extracted with a mixture of ethanol and distilled water (70:30) and percentage yield of extracts for *F. religiosa* L. (4.75%), *T. cordifolia* Miers (5.67%), *B. diffusa* L. (4.62%) and *M. olifera* Lamk (7.54%). All the hydroalcoholic extracts showed the presence of phytosterols (β- sitosterol) by qualitative chemical tests like Salkowski's test and Liebermann Burchard's test. Further this is quantitatively estimated by HPTLC fingerprinting.

3.2. HPTLC fingerprinting and quantification of β-sitosterol

All the hydroalcoholic extracts were evaluated by HPTLC fingerprinting for biomarker β-sitosterol by using toluene: ethyl acetate (8:2) as mobile phase. The image of TLC plate fingerprinting (Fig. 2) at 540 nm.

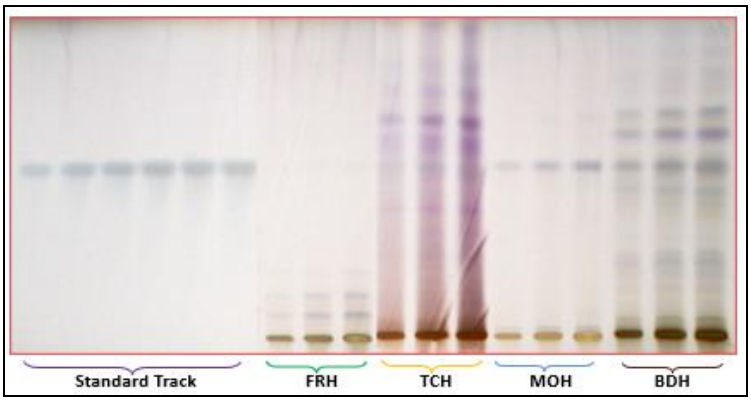

Fig. 2: HPTLC fingerprinting profile of standard compound (β-Sitosterol) and hydroalcoholic extract samples: FRH (*F. religiosa* L.), TCH (*T. cordifolia* Miers), MOH (*M. olifera* Lamk), BDH (*B. diffusa L.*)

Identification of β-Sitosterol in all the hydroalcoholic plant extract of selected Indian medicinal plants were done by comparing the Rf value of reference standard β-Sitosterol solution. Chromatogram of reference standard β-sitosterol solution have given better results with volume ranging from 4 µl to 12 µl and 10 µl standard reference solution was used for quantification of β-sitosterol. In the similar way, chromatogram was developed for all hydroalcoholic extracts and maximum concentration was found in 15 µl concentration track for each plant extract and this one was used for quantification of β-sitosterol. Calibration curve for reference standard β-sitosterol was plotted in between concentration (in mg) and peak area which was used for densiometric quantification of β-sitosterol in all plant extracts (Fig. 3 A and B).

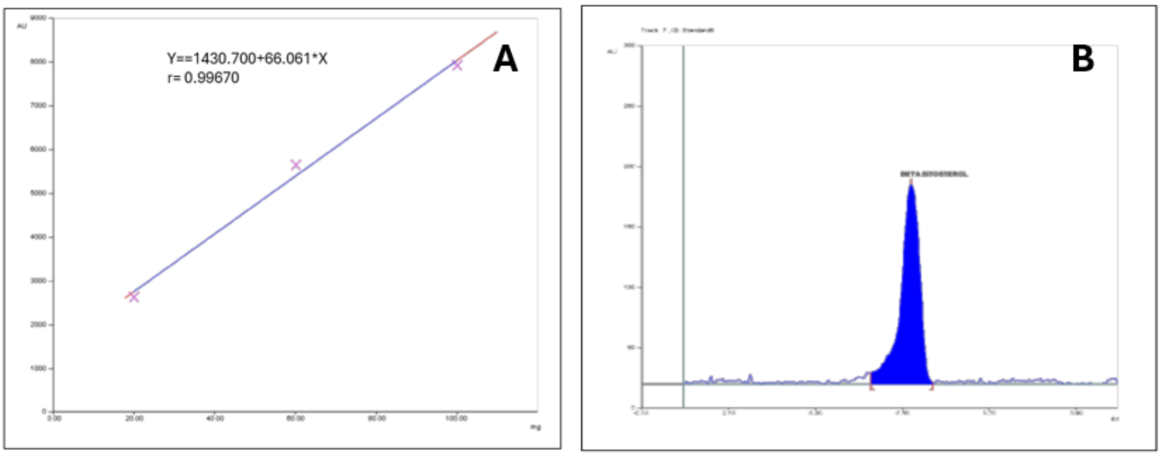

Fig. 3: Calibration curve and HPTLC densitometric chromatogram of β-Sitosterol.

Densiometric chromatogram for quantification of β-sitosterol are given (Fig. 4) for all the hydroalcoholic extracts of selected Indian medicinal plants and 3D densiometric chromatogram of β-sitosterol and hydroalcoholic extract samples at 540 nm (Fig. 5).

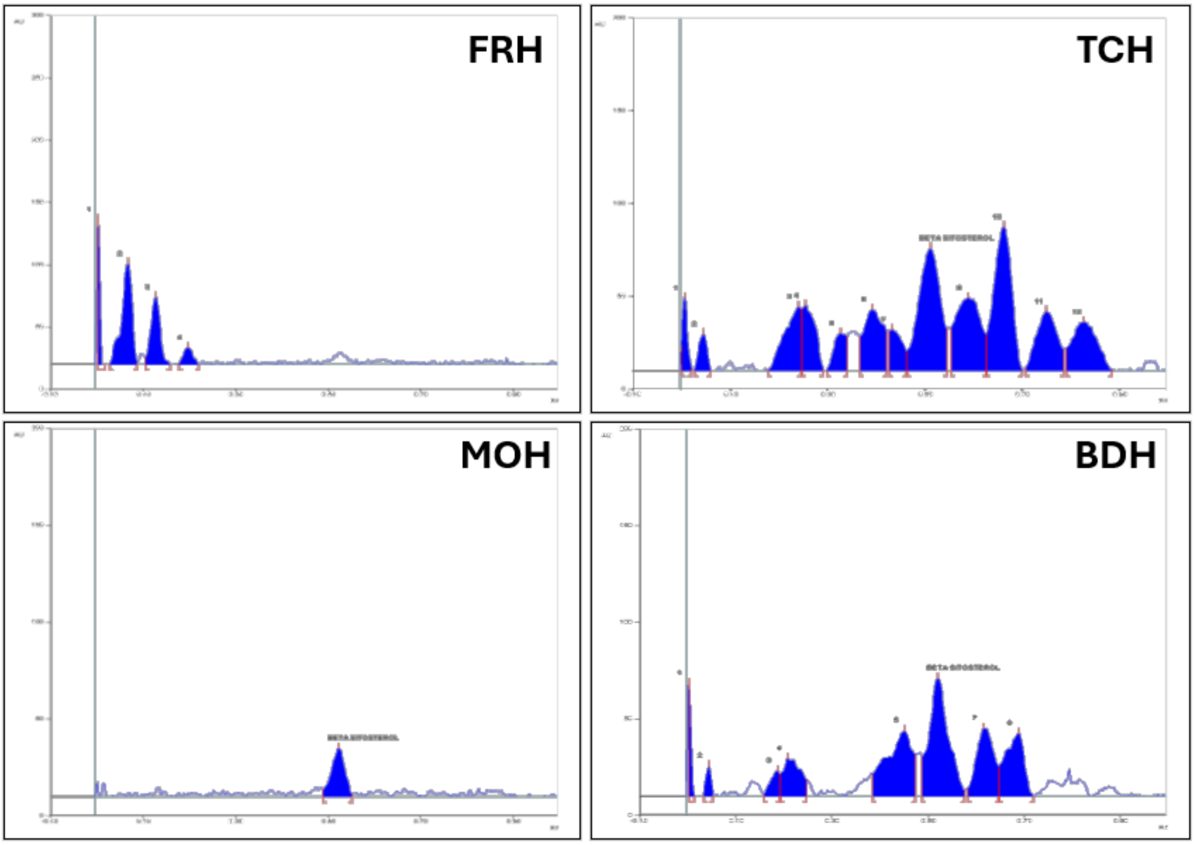

Fig. 4: HPTLC chromatogram of hydroalcoholic extract samples: FRH (*F. religiosa* L.), TCH (*T. cordifolia* Miers), MOH (*M. olifera* Lamk), BDH (*B. diffusa L.*).

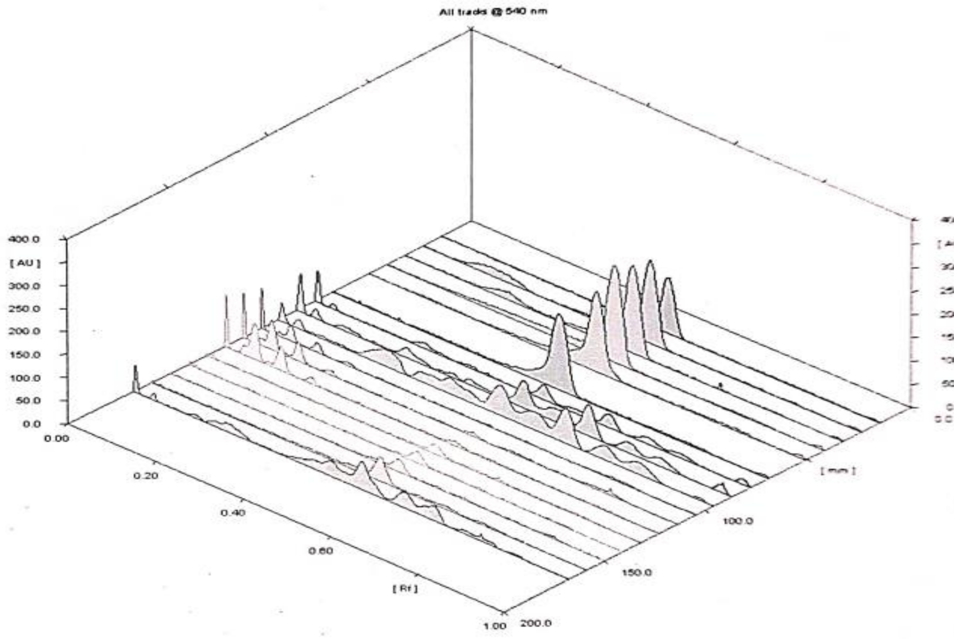

Fig. 5: 3D densiometric chromatogram of *β*-sitosterol and hydroalcoholic extract samples at 540 nm.

HPTLC chromatogram for β-sitosterol was analyzed for reference standard and hydroalcoholic extract of all selected plants by using the Win CATS software. Rf value for standard β-Sitosterol was found 0.53, and the area of the peak of β- sitosterol was 7783.39 at a concentration (20µg/10µl) and this was used as reference for calculation of the Rf value and area of the peak for β-sitosterol in all hydroalcoholic extract of selected plants. Comparative Rf value, area of peak and % β- sitosterol in hydroalcoholic extracts (Table 2).

Table 2: The amount of *β*-sitosterol in different hydroalcoholic plant extract.

S. No.	Extract name*	Applied Volume (µl)	Rf	Peak Area	Amount in 15 µl spot (%)
1	FRH	5,10,15	Not Detected	Not Detected	Not detected
2	TCH	5,10,15	0.52	2229.57	1.30
3	MOH	5,10,15	0.53	653.2	0.38
4	BDH	5,10,15	0.53	1879.40	0.92

***FRH (*F. religiosa* L.), TCH (*T. cordifolia* Miers), MOH (*M. olifera* Lamk), BDH (*B. diffusa L.*)**

4. DISCUSSION

HPTLC is a valuable technique for quantitative analysis due to its lower operating cost, high analyte throughput, very little sample requirement (nanogram to microgram) and less detection time. This technique requires minimum purification of samples and it provides explicit control over analyte application, development of chromatogram, derivatization of sample for detection and analysis.[42,43] Results of quantification for % β- sitosterol in hydroalcoholic extracts showed that maximum peak area and concentration is present in extract hydroalcoholic extract of stem of *T. cordifolia* Miers with Rf value 0.52 and area of peak 2229.57 (% β- sitosterol-1.30) followed by extract of roots of *B. diffusa* L. with Rf value 0.53 and area of peak 1879.40 (% β- sitosterol: 0.92), hydroalcoholic extract of leaves of *M. oleifera* Lamk with Rf value 0.53 and area of peak 653.2 (% β- sitosterol: 0.38) whereas β- sitosterol is not detected in hydroalcoholic extract of *F. religiosa* L.

5. CONCLUSION

HPTLC furnishes a semi-quantitative detail on the major phytoconstituents of different parts of medicinal plants, hence an assessment of drug purity. The quantification of β-sitosterol in extracts of all selected medicinal plant parts helps in the standardization of all these plants. β-Sitosterol is a valuable phytoconstituent due to its ability to treat diseased conditions such as diabetes, inflammation, pyrexia, cancer, liver disorders, bacterial and viral infections. In present comparative study, β-sitosterol was estimated maximum in the extract of stem of *T. cordifolia* Miers followed by the extract of roots of *B. diffusa* then in the extract of leaves of *M. oleifera* Lamk whereas β- sitosterol is not detected in the extract of *F. religiosa* L. The results of present research should be helpful for isolation of this phytoconstituent (β-sitosterol) and these standardized plants can be used for development of polyherbal formulations.

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7. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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