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HPTLC FINGERPRINT PROFILING OF PHYTOSTEROL (β-SITOSTEROL) OF THE EXTRACTS OF LEAVES OF PLANTS *NERIUM INDICUM* MILL.AND *CASCABELLA THEVETIA* (L.) LIPPOLD

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ABSTRACT:
For the present study medicinally important two plant species of family Apocynaceae viz. *Nerium indicum* Mill. And *Cascabella thevetia* (L.)Lippold were selected. These important plant species are found in some region of India and traditionally used in indigenous system of medicine. An attempt has been made to give a HPTLC profile in leaves for the confirmation of presence of phytosterol - β-Sitosterol. Hexane extracts were prepared using soxhlet apparatus and unsaponified extract was done with petroleum ether. The mobile phase of Benzene: Ethyl acetate (5:1) was used for good separation. The fingerprint showed better resolution after derivatization in Antimony Trichloride in Chloroform reagent. The fingerprint has revealed a distinct pattern of bands of specific phytosterol, β-Sitosterol. Thus HPTLC profile of the plants was done for the identification of phytosterol - β-Sitosterol in the crude and unsaponified leaf extract.

Keywords: HPTLC, *Nerium indicum* Mill. and *Cascabella thevetia* (L.) Lippold, Derivetization, Identification.

INTRODUCTION:

Herbal medicine is one of the oldest forms of medical treatment in human history, and could be considered one of the forerunners of the modern pharmaceutical trade. Plants provide chemicals of unknown and unusual chemical structures. These compounds provide new pharmacological properties and may serve as a starting material for more complex biologically active compounds. Plants thus contain an enormous number of biologically active compounds with various chemical structures. These phytochemicals, often secondary metabolites present in smaller quantities in higher plants include the alkaloids, flavonoids, tannins, terpenoids, sterols, and many others. Out of all these, Sterols present in many plants as an imperative phytoconstituent. These phytohemicals can be detected by different standardized preliminary tests. These tests are principal analysis of the crucial compounds affiliation from the plant (Williams, *et al.*, 2005).

Nerium indicum Mill. And *Cascabella thevetia* (L.) Lippold which belongs to family Apocynaceae, are such plants which is famed for its therapeutic efficiency in different diseases globally. The leaves and the flowers of *Nerium indicum* Mill. Are cardiotonic, diaphoretic, diuretic, anticancer, antibacterial, antifungal and expectorant. A decoction of the leaves has been applied externally in the treatment of scabies and to reduce swellings. This is a very poisonous plant, containing a powerful cardiac toxin and should only be used with extreme caution (Patel, *et al.*, 2010). Oleander (*Thevetia peruviana* or *Thevetia neriifolia*) is a potent cardiotoxic plant. Preparations from the plant are used in native medicines for cardiac diseases, myalgia, parasitic infestations, wound healing, abortifacient, and so on (Rastogi, *et al.*, 1993).

Nowadays well developed quality standards can be achieved only through systematic evaluation of the plant material using some modern analytical techniques including chromatographic analysis. In the present scenario, TLC/HPTLC is very important, essential and viable tool for qualitative and quantitative analysis of herbal products. High Performance Thin layer Chromatography (HPTLC) is an enhanced form of Thin Layer Chromatography (TLC). HPTLC allows fast, inexpensive method of

analysis in the laboratory. HPTLC is a valuable tool for reliable identification because it can provide chromatographic fingerprints that can be visualized and stored as electronic images (Srivastava, 2011). Hence, an attempt has been made to identify specific phytosterols, β-sitosterol with HPTLC profile.

METHOD AND MATERIAL:

Plant Materials-

Some plants were analysed by field test for phytosterols (Anasane, P. 2014). Thus selected plants of family Apocynaceae, which had given positive field test for phytosterols were - *Neriumindicum* Mill. And *Cascabella thevetia* (L.) Lippold. Fresh plant parts (leaves) were collected from the different region of Nagpur, then identified and authenticated from Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Campus, Nagpur and Flora of Maharashtra State - Vol. I and II (Singh & Kartikeyan, 2000; Singh & Kartikeyan, 2001), Flora of Nagpur District (Ugemuge, 1986).

Extraction and Saponification of Plant Material (Wong, *et al.*, 1999) -

The leaves of selected plants were separated, washed thoroughly in distilled water shade dried at room temperature. Dried leaves were then uniformly grinded separately using mechanical grinder to make fine powder. The powdered form stored for future use. Extraction of all samples then done by Soxhlet method with the selected solvent Hexane. 10g of powder was weighed and it was taken in a thimble. Thimble was kept in a Soxhlet extractor and run for 12 hrs until the last cycle when the colour of solvent hexane (250ml) becomes almost colourless. The crude extracts of *Nerium indicum* and *Cascabella thevetia*, were kept in cool environment for further exercise. 10ml of the extracted samples (crude extract-CE) were hydrolyzed with 50ml Potassium Hydroxide (2M) (KOH) by refluxing at 40℃ for 90min. The saponified sample then allowed for cooling for 1 hr. The cooled saponified sample then washed with 60ml distilled water. The saponified sample then extracted with 40ml petroleum ether (PET) to get unsaponified sample. The immiscible liquids were separated in separating funnel. The unsaponified material obtained by evaporating the petroleum ether. The crude extract and unsaponified extracts were then used for HPTLC fingerprinting.

Preliminary Phytochemical Analysis-

Two standard methods were done to determine the presence of sterols for the comparative confirmation with field tests (Krishnaiah, *et al.*, 2007). Salkowski test was done with the maceration of selected plants. 2ml extract taken in a test tube. 2ml Chloroform and 2ml conc. Sulphuric acid was added in it. Brown or red colored ring on the sulphuric acid layer given the confirmatory test. Liebermann and Burchurd's test was done after the extraction and reflux of the plant material. 2ml extract taken in a test tube. 2ml Chloroform, 2ml Acetic Anhydride and 2ml conc. Sulphuric acid was added in it. Translucent green color given the confirmatory test.

High Performance Thin Layer Chromatography (HPTLC) (Verma, *et al.* 2014) -

HPTLC is an enhanced form of TLC. It is a simple, sensitive and accurate method use to separate the components of sample.

i. Instrument details:

Applicator: CAMAG LINOMAT 5 (Made in Switzerland), UV Chamber: CAMAG (Made in Switzerland), Scanner: CAMAG TLC Scanner 3 (Made in Switzerland), Software: WinCATS, version – 7.4.2.

ii. Sample and Standard Preparation:

Samples were taken in different concentrations (on the basis of its percent extractable matter) as in Table 1.

iii. Selection of Solvent System and Chamber saturation:

Satisfactory resolutions was obtained by using solvent system Benzene : Ethyl Acetate (5:1). A rectangular Twin trough glass chamber was used in the experiment. To avoid insufficient chamber saturation and the undesirable edge effect, a smooth filter paper in a 'U' shape was placed in the glass chamber and was allowed to soak in the Methanol. The moistened paper was pressed against the walls of the chamber so that it adheres to the walls. The chamber was allowed to saturate for 20min. before use. The experiment was carried out in an AC room (temperature 20-25^{o}C).

iv. Application of Sample and Standard:

All the samples and standards were applied on the readymade precoated silica gel G plate (10 × 10) with CAMAG LINOMAT 5 applicator. Samples were run in a specially designed needle which was placed in the applicator. Amount of each sample was taken as in Table 2.

v. Chromatographic Development:

The applied plates were kept in a properly saturated twin trough glass chamber in a selected solvent system, Benzene: Ethyl Acetate (5:1) for 10-15 min. Developed plates were then removed from the glass chamber and kept to dry for 5min.

vi. Derivatization:

The developed plates derivatize with spraying reagent (20% Antimony Trichloride in Chloroform) with sprayer for the visualization of phytosterol spots.

vii. Scanning and Documentation of Chromatographic Plate:

Derivatize plates kept inside the scanner and Photographs were taken with Digital Camera. All the densitometeric fingerprint readings were documented on the computer having WinCATS software.

Following are the chromatographic conditions required to get an effective resolution by selected mobile phase:

Stationary Phase	HPTLC precoated, Silica gel G (Merck)
Size	10 × 10
Developing Chamber	Twin Trough Glass Chamber
Solvent System	Benzene : Ethyl Acetate (5 : 1)
Mode of Application	Band
Band size	6mm
Separation Technique	Ascending
Temprature	20-25 ^{o}C
Saturation Time	15 min
Scanning Wavelength	540nm
Scanning mode	Absorbance/ Reflectance

RESULTS AND DISCUSSION

Preliminary Phytochemical Analysis-

Present investigation was completely about Phytosterols (a class of triterpenes). According to the reports of Tiwari, Kumar, Kaur, Kaur & Kaur (2011) and Venkata, Kantamreddi, Nagendra Lakshmi & Kasapu (2010), Salkowski's Test and Liebermann Burchard's Test are the two important tests for the detection of sterols from the plant material.

In the study of Shah & Chakraborthy (2010) on leaves of *Nerium indicum*, alcoholic extract of leaves contain steroids and saponins. The studies of Ravichandra & Paarakh (2011) on leaves of *Cascabella thevetia* also showed that the extracts of different solvents like petroleum ether, benzene, chloroform, acetone, methanol and aqueous extarct indicated the presence of sterols, triterpenoids, flavonoids, phenols and tannins in large amount. Likewise in the present research Hexane extract of leaves of selected plant material was investigated with Salkowski and Liebarman Burcahrd Tests for the confirmation of Phytosterols. Salkowski test had given Brown to red colored ring on the sulphuric acid layer while Lieberman Burchard Test given the translucent Green color, which confirmed the presence of phytosterols in the selected plant materials (Table 3).

High Performance Thin Layer Chromatography (HPTLC)-

High Performance Thin Layer Chromatography was performed on both the active crude (Hexane) and

unsaponified extracts selected plant materials. In HPTLC study, from Chromatograms and Densitograms (Dg), β-Sitosterol was observed in crude extracts NIE, CTE at Rf value 0.40 while in unsaponified extract NIUE and CTUE at 0.44. (Table 4 and Table 5, Fig. 1 and 2, Graph 1). (Only those graphs mentioned having same/nearly same Rf values that of the standards).

CONCLUSION:

HPTLC has confirmed the presence β–Sitosterol in the selected plant materials. The peaks of particular Rf value of the bands in standard track and that of the plant material was sensitively and precisely compared which confirms the presence of the particular type of β-Sitosterol in the selected plants. Unsaponified extracts showed less bands than crude extract which could make easy to detect particular type of sterol i.e. β–Sitosterol in the selected plant extracts. This has been showed that unsaponified extract is more isolated form for β–Sitosterol.

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REFERANCE :

Anasane, P., Chaturvedi, A. (2014). Field Test for the Detection of Phytosterols. International Research journal of Pharmacy, 5(9), 734-736.

Burchard. (1890). Dissertation, Rostock. *Chem. Zbl.*

De Filipps, R., Maina, S., &Crepin, J. (2006).Medicinal Plants of the Guianas. Smithsonian Institute .

Krishnaiah, D., Sarbatly, R., & Bono, A. (2007). Phytochemical antioxidants for health and medicine: A move towards nature. *BiotechnolMolBiolRev, 1*, 97-104.

Liebermann, C. (1803). *Ber.dtch. chem. Ge, 18*.

Patel, G., Nayak, S., &Shrivastava, S. (2010).Antiulcer Activity of Methanolic Leaves of NeriumIndicum Mill.*International journal of biomedical Research* , 55-61.

Rastogi, R., & Mehrotra, B. (1993).Compendium of Indian medicinal plants. Central Drug Research Institute. New Delhi: Publications and Information Directorate.

Salkowski, E. (1872). Berichte der DeutschenChemischenGesellschaft. 637.

Srivastava, M. (2011).*High Performance Thin Layer Chromatography (HPTLC)*.NewYork: Springer.

Ugemuge, N. (1986). *Flora of Nagpur District.*

Verma, G., Gupta, R., & Gupta, S. (2014). Development of HPTLC Densitometric Method for estimation of Quercetin in Bombaxceiba L. Leaves.*Journal of Advanced Scientific Research, 5* (2).

Williams, L., & Wilkins. (2005). Remington JP. Remington: The science and practice of pharmacy.

Wong, A., Norman, H., & MacMillan, A. (1999).Method for the preparation of phytosterols from tall-oil pitch.

Table 1: Concentrations of Samples for HPTLC

Sr. No.	Name of plant species	Crude Extract Conc. (mg/ml)	Unsaponified Conc. (mg/ml)
i.	*Nerium indicum*	3	12
ii.	*Cascabella thevetia*	6	4

Standards, β – Sitosterol (M P Biomeditech) was taken in the 1mg/ml concentration. Samples to apply were sonicate in a Sonicator for 15 mins.

Table 2: Amount of Samples loaded

Sr. No.	Name of plant species	Crude Extract Amount (µl)	Unsaponified Amount (µl)
i.	*Nerium indicum*	10	5
ii.	*Cascabella thevetia*	10	10

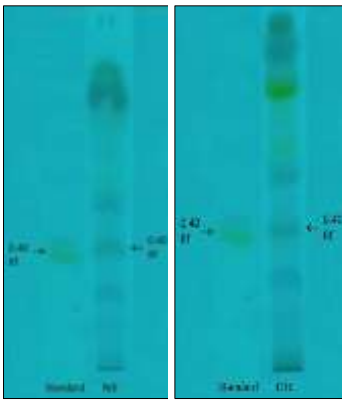
Table 3: Salkowski and Lieberman Burchard Test of all Plants

Sr. No.	Name of Plant species	Parts Used	Salkowski Test	Lieberman Burchard Test
1.	*Nerium indicum*	Leaves	+ +	+
2.	*Cascabella thevetia*	Leaves	+ +	+

'+' indicates the positive test for the presence of Phytosterols .Number of '+' indicates the intensity of color in the test

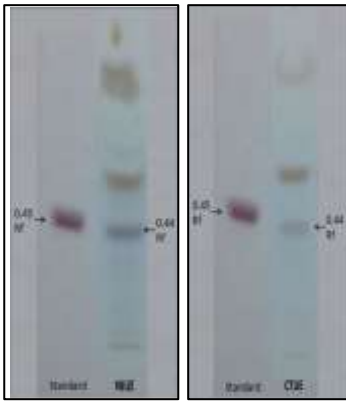
Table 4: HPTLC observations

Sr. No.	Name of Sample	Rf Values	
		Crude	**Unsaponified**
1.	β-Sitosterol	0.40	0.40
1.	*Nerium indicum*	0.40	0.44
2.	*Cascabella thevetia*	0.40	0.44



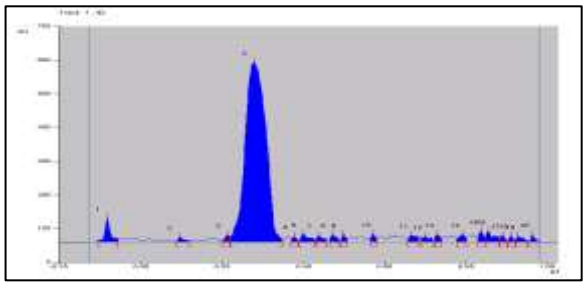
(A) (B)

Fig. 1: HPTLC Chromatograms of Standard β-Sitosterol, with
(A) *Neriumindicum*-NIE,
(B) *Cascabellathevetia*-CTE

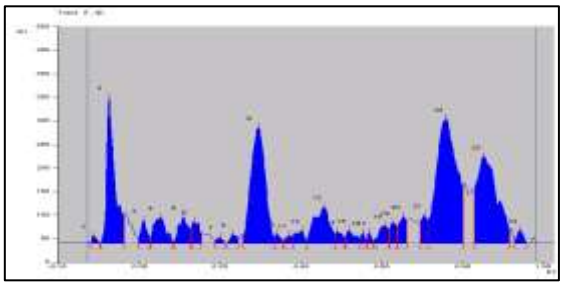


(C) (D)

Fig. 2: HPTLC Chromatograms of Standard β-Sitosterol, with
(C) *Neriumindicum*-NIUE,
(D) *Cascabellathevetia*-CTUE



(A)



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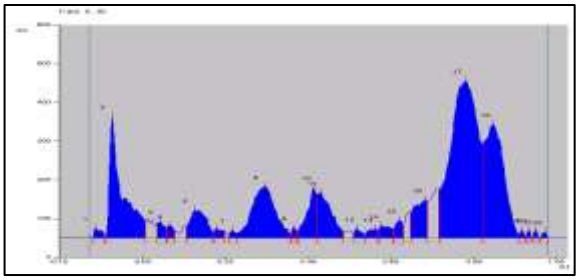
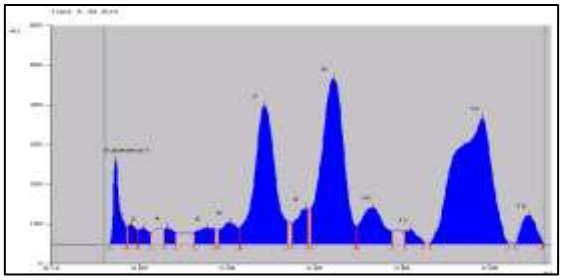
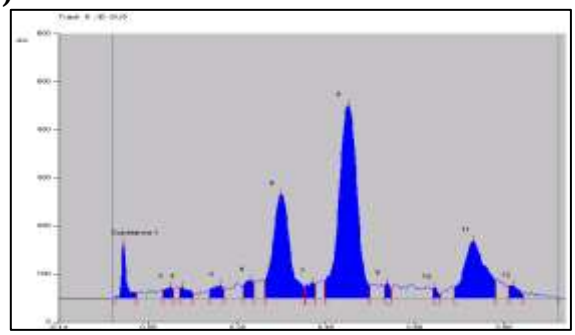


(C)



(D)



(E)

Graph.1 : HPTLC Densitograms (Dg) of (A) Standard β-Sitosterol, (B) *Neriumindicum*-NIE, (C)*Cascabellathevetia*-CTE, (D) *Neriumindicum*-NIUE, (E) *Cascabellathevetia*-CTUE

Dg Name	Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area%
A	4	0.27 Rf	8.4 AU	0.33 Rf	525.8 AU	100.00%	**0.40Rf**	1.9 AU	19630.3 AU	100.00%
B	10	0.38 Rf	13.3 AU	0.39 Rf	20.7 AU	1.20%	**0.40 Rf**	3.3 AU	201.5AU	0.52%
C	8	0.28 Rf	13.4 AU	0.34 Rf	133.5AU	6.90%	**0.40 Rf**	11.4 AU	5704.5AU	9.96%
D	8	0.41 Rf	49.3 AU	0.44 Rf	88.8 AU	4.90%	**0.44 Rf**	84.9 AU	1885.8 AU	2.82%
E	7	0.41 Rf	22.1 AU	0.43 Rf	33.2 AU	3.09%	**0.44 Rf**	28.3 AU	479.4 AU	1.69%