High Performance Thin Layer Chromatography Fingerprinting of Phytosterols from Hydroalcoholic Extract of *Holoptelea Integrifolia* (Roxb.) Leaves

Ravindra C. Sutar[1*] Gowtham M[2] , Girish A. Kashid[3]

*1Department of Pharmacology, Sanjivani College of Pharmaceutical Education and Research, Kopargaon. At-Sahajanandnagar, Post-Shinganapur (Pin code- 423603), Tal- Kopargaon, Dist-Ahmednagar, Maharashtra, India

2.Department of Pharmaceutics, Sanjivani College of Pharmaceutical Education and Research, Kopargaon. At-Sahajanandnagar, Post-Shinganapur (Pin code- 423603), Tal- Kopargaon, Dist-Ahmednagar, Maharashtra, India

3.Department of Pharmaceutical Chemistry, Sanjivani College of Pharmaceutical Education and Research , Kopargaon. At-Sahajanandnagar, Post-Shinganapur (Pin code- 423603), Tal- Kopargaon, Dist-Ahmednagar, Maharashtra, India

Correspondence To Author:
Dr. Ravindra C. Sutar
Asso. Prof & Head;
Department of Pharmacology, Sanjivani College of Pharmaceutical Education and Research, Kopargaon. At- Sahajanandnagar, Post- Shinganapur (Pin- 423603),Tal-Kopargaon, Dist-Ahmednagar, Maharashtra, India.
E-mail: ravisutarbpharm@sanjivani.org.in

Abstract

Plant Sterols (PS) (Phytosterols And Phytostanols) Are Bioactive Compounds Of All Vegetable Foods Where Can Be Found As Free Sterol Alcohols And As Conjugates. These Latter Forms Have Been Less Studied, Although They May Have Potential Beneficial Effects, Whereas Some PS Have Several Approved Health Claims. *Holoptelea Integrifolia* Belongs To The Family Ulmaceae Commonly Called As Indian Elm And Commonly Used In India By The Tribal People For It's Medicinal Properties. Plants And Plant-Based Products Are The Basis Of Many Of The Modern Pharmaceuticals We Use Today For Various Ailments. Authentication Of Natural Products Can Be Done With Precious Method Like High Performance Thin Layer Chromatography (HPTLC). High Performance Thin Layer Chromatography (HPTLC) Technique Was Utilised To Detect Phytosterols From Hydroalcohol Extract Of *Holoptelea Integrifolia*. Phytochemical Screening Was Performed And Later HPTLC Studies Were Done. Instrumentation For HPTLC With CAMAG System Having Linomat V Applicator (Switzerland). Camag TLC Scanner IV With Reflectance Absorbance Mode At 277nm, Equipped With Win CATS Software (1.4.6 Camag), Tungstant Lamp Were Used To Perform Densitometric Scanning. Steroids, Tannins, Alkaloids, Glycosides, Flavonoids,
Phytosterols Were Found In *Holoptelea Integrifolia* Hydroalcohol Extract Undergoing Preliminary Phytochemical Analysis. Our Aim Is To Do HPTLC Studies From Hydroalcohol Extract Of Leaves Of *Holoptelea Integrifolia.* Objective That Were Assessed Are Hydroalcohol Extract Of Leaves Of *Holoptelea Integrifolia*. HPTLC Finger Printing Of Phytosterols Of Hydroalcohol Extract Of Leaves Revealed Twelve Multivalent

Phytochemicals Having Increasing Order Of Rf Values 0.10- 0.93. Rf 0.40 Has 46.10% Concentration.Phytosterols In Hydroalcohol Extract Were Confimed By Preliminary Phytochemical And Rf Values.

Keywords: *Holoptelea Integrifolia*, Hydroalcohol Extract, Phytochemical Screening, Phytosterols, HPTLC Fingerprinting

1. INTRODUCTION

Many medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system, (ayurveda) and proposed for their interesting multilevel activities. Among the medicinal plants used in ayurvedic preparations for their therapeutic action, some have been thoroughly investigated and some of are still to be explored. Standardization of plant materials is the need of the day. Several pharmacopoeia containing monographs of the plant materials describe only the physicochemical parameters. Hence, the modern methods describing the identification and quantification of active constituents in the plant material may be useful for proper standardization of herbals and its formulations. Furthermore, the WHO has emphasized the need to ensure the quality of medicinal plant products using modern controlled techniques and applying suitable standards[1,2]. Natural remedies from medicinal plants are found to be safe and effective. Many plant species have been used in folklore medicine to treat various ailments. Even today compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries[3] Plants are well-known for the primary and secondary metabolites like carbohydrates, proteins and amino acids and steroids, flavonoids, phenolics, glycosides, saponins, tannins, terpenoids, and coumarins etc. These secondary metabolites impart medicinal properties to the plants[4]. Therefore, it is mandatory to resolve the type of secondary metabolites, their nature and pharmacological, antimicrobial, and clinical research, to reveal their bioactivities, to identify the active components and their side effects, and to enhance the purity of the pharmacologically important active compounds[5]. These active secondary metabolites are qualitatively and quantitatively estimated by various techniques such as spectroscopy and chromatography. Chromatography techniques are the popular tools for the separation and identification of the bioactive compounds. Thin layer and high performance thin layer chromatography (HPTLC) can be applied for this identification. HPTLC fingerprint analysis helps in the identification of the biochemical constituents of the plant[6] Plants used in traditional medicine contain a wide range of bioactive compounds that can be used to treat contagious diseases[7-9]. They are a source of active secondary metabolites which prove to be invaluable for the management of such diseases. In much of the developing world, 70-95% of the population relies on these traditional medicines for primary care[10]. Recent approach is the utility of natural products as sources of novel structures of therapeutic value[11]. Plants have developed chemical defenses over millions of years against environmental threats such as ultraviolet radiation, reactive oxygen species, and microbial attacks. Therefore,

phytochemicals are less toxic and biologically active[12]. Knowledge of the chemical constituents of plants is desirable because such information will be valuable for synthesis of complex chemical substances[13]. In India tribal people use *Holoptelea integrifolia* for its therapeutic uses. *Holoptelea integrifolia* also called as Indian Elm comes under family ulmaceae. The mucilaginous bark is boiled and the juice squeezed out and applied to rheumatic swellings[14] In traditional system of medicine, bark and leaves of *Holoptelea integrifolia* are used as bitter, astringent, acrid, thermogenic, antiinflammatory, digestive, carminative, laxative, anthelmintic, depurative, repulsive, urinary astringent and in rheumatism[15,16]. The plant *Holoptelea integrifolia* is used traditionally for the treatment of inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound healing, leprosy, diabetes, hemorrhoids, dysmenorrhoea and rheumatism[17].Hydroalcohol extract of leaves of *Holoptelea integrifolia* was subjected to preliminary phytochemical analysis and HPTLC analysis to find markers for quality evaluation and standardization of the drug.

2. MATERIALS AND METHODS

2.1 Collecion of Plant material

Holoptelea integrifolia leaves were assembled in agricultural fields in Tirunelveli district of Tamilnadu. The plant was authenticated by Dr. V. Chelladurai, Research Officer of Botany, Central Council for Research in Ayurvedic Sciences (Retired), Govt. of India, 476 F First South Street Thiyagaraja nagar Tirunelveli, Tamilnadu, Voucher Number 41371 by compairing morphological features (leaf and stem arrangement, flower /inflorescence arrangement, fruit and seed morphology etc.). The plant material was subjected to further extraction process.

2.2 Preparation and Extraction of Plant material for Preparation of Hydroalcohol extract

The powder of *Holoptelea integrifoila* leaves was charged in to the thimble of a Soxhlet Apparatus and extracted using equal volumes of water and ethanol (1:1), when colourless solvent comes in siphon tube which indicates completion of extraction process. Hydroalcohol extract was obtained by evaporating the extract into organ bath maintained at 50º C The extract was finally air dried thoroughly to remove all traces of solvent and its percentage yield was calculated.Hydroalcohol extract of *Holoptel ea integrifolia* leaves was subjected to the following investigations,

1. Preliminary phytochemical screening.
2. HPTLC Fingerprinting of Phytosterols

2.3 Phytochemical screening

Standardized protocol was utilized for preliminary phytochemical screening of leaves extract of *Holoptelea integrifolia* [18].

2.4 HPTLC Profile

Method of Harborne[19] and Wagner *et al* [20] was applied to do HPTLC Studies

2.4.1 Sample Preparation

Each extract residue was re-dissolved in 5 ml of chromatographic grade Hydroalcohol. Aluminium sheets were utilized to do sample

application on pre-coated silica gel

2.5 Developing Solvent System

A number of solvent systems were tried, for extracts, but the satisfactory resolution was obtained in the solvent chloroform-ethyl acetate in the volume ratio of 4:6 (v/v).

2.6 HPTLC instrumentation, Chromatographic conditions and detection of spots

The sample solutions were spotted in the form of bands of width 8.0 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F254 (20 cm × 10 cm with 250 μm thickness). The plates were activated at 120 °C for 20 min prior to chromatography. Slit dimension with 6.0mm×0.45 mm and 10 mm/s scanning speed and uniform application speed of 1.0 μl/s was applied. The space between 2 bands was 5mm. Chloroform-ethyl acetate having volume ratio of 4:6 (v/v) was utilized as mobile phase for fingerprinting of phytosterols, with anisaldehyde sulfuric acid for derivatization. 20 ml of mobile phase was used per chromatography. 20 cm x 10 cm twin trough glass chamber saturated with filter paper whatman no: 1 in the mobile phase was used for linear ascending development method. Length of chromatogram run was 8.0 cm. Subsequent to the scanning, TLC plates were dried in a current of air using an air dryer. Tungstant lamp was used to do densiomeric scanning with TLC Scanner IV in the reflectance absorbance mode at 540nm having WinCATS software. Subsequent to the development; TLC plate was dipped in Dragendorff reagent followed by drying in oven at 110^{o}C. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression[21-29] .

3. RESULTS AND DISCUSSION

Plant sterols (PS) are bioactive components of all vegetable foods.They are 28- or 29-carbon alcohols and resemble cholesterol in structure (steroid nucleus, 3-hydroxyl group, 5, 6 double bond). Phytosterols contain an extra methyl or ethyl group, or double bond, and most of their side chains contain 9–10 carbon atoms. Phytosterols have been classified as 4-desmethyl sterols of the cholestane series, all of which have double bonds at the C-5 position of the ring. More than 200 different types of phytosterols have been reported in plant species, being the most commonly encountered β-sitosterol, campesterol and stig-masterol. On the other hand, saturated PS, referred as phytostanols, have no double bond in the ring structure[30]. In foods, PS can be found as free sterols (FS) or as four types of conjugates in which the 3β-hydroxyl group is: esterified to (i) a fatty acid FASE), (ii) a hydroxycinnamic acid (HSE) (mainly ferulic or p-coumaric acid), (iii) glycosylated with a hexose (usually glucose) (SG) or glycosylated with 6-fatty acyl hexose (ASG)[31]. Preliminary phytochemical analysis of *Holopelea inegrifolia* hydroalcohol given the presence of phytosterols,flavonoids, steroids, tannins, alkaloids, glycosides. The chromatograms shown in fig.1 indicate that all sample constituents were clearly separated without

any tailing and diffuseness. Since in previous studies on Petroleum ether and Methanol extracts of *leaves of Holoptelea integrifolia* (Roxb.) were analysed for HPTLC Studies for fingerprinting of Flavonoids, alkaloids, steroids, phytosterols, phenols, glycosides[32-37]. Leaves of *Holoptelea integrifolia* (Roxb.) were not studied for HPTLC Studies till so far involving Hydroalcohol extract so Hydroalcohol extract of Leaves of *Holoptel integrifolia* (Roxb.) was analysed for phytochemical pro filing using high performance thin layer chr omatography (HPTLC).

3.1 Phytosterol Confirmation

3.2 Detection of Phytosterols in hydroalcohol extract at 277 nm

3D plot of Phytosterols of *Holoptelea integrifolia* leaf and the chromatograms in Fig. 1 shows separation of constituents.

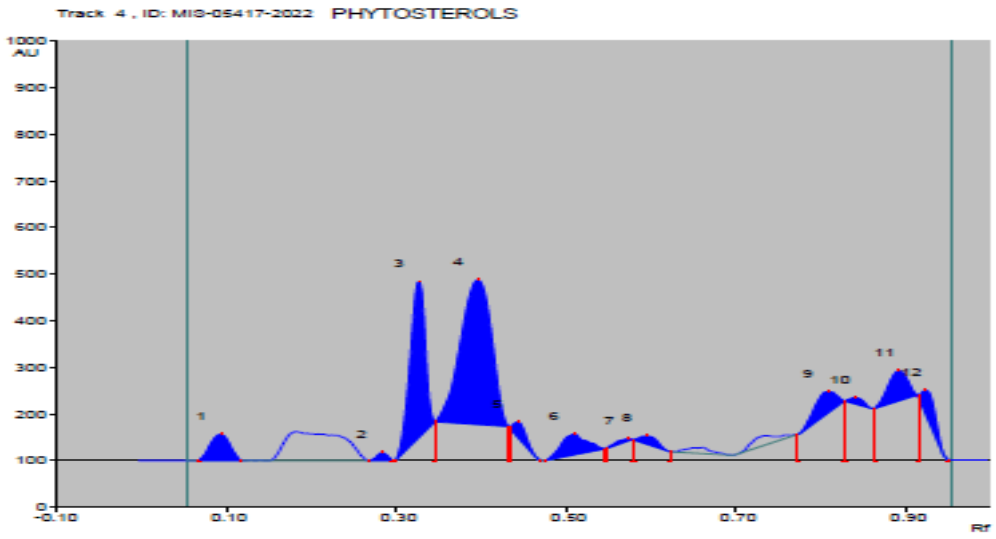

Fig 1: HPTLC chromatogram of extract at 277 nm, showing different peak (bands) unknown phytosterols of *Holoptelea integrifolia*

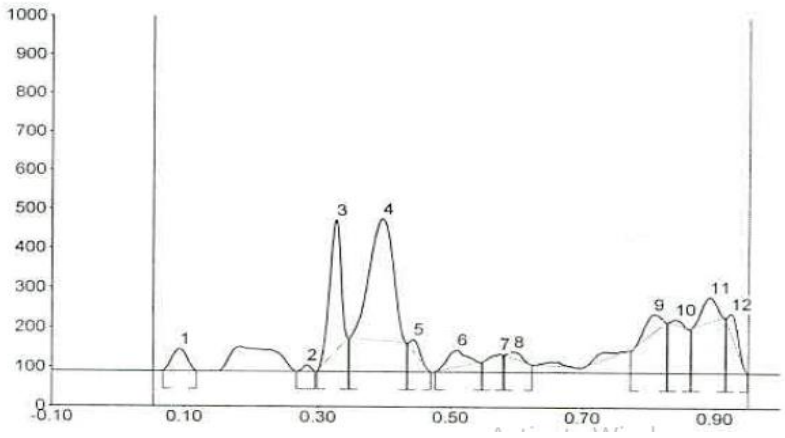

Fig 2: Peak table with Rf values, height and area of unknown phytosterols of *Holoptelea integrifolia* leaf

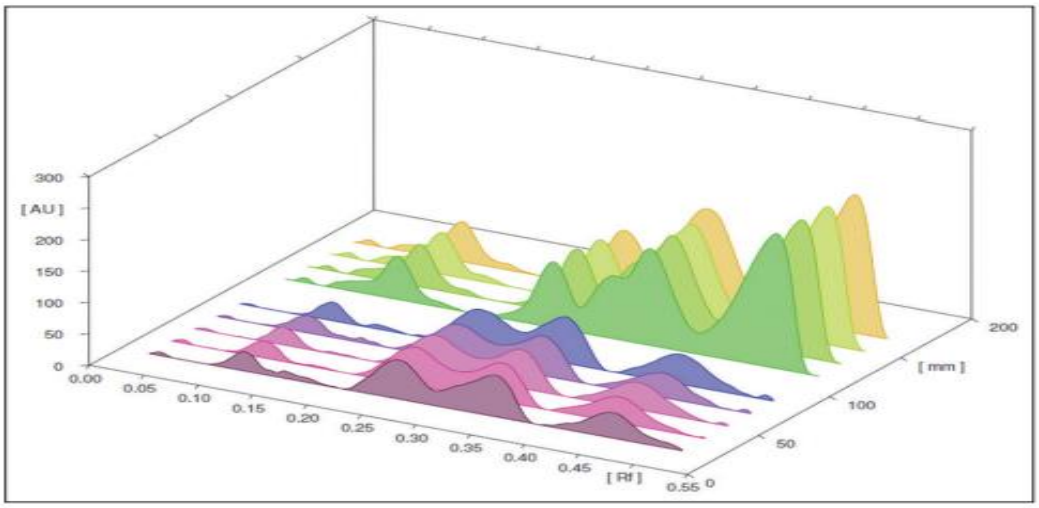

Fig 3: Three-dimensional plot of fingerprint of phytosterols of Holoptelea integrifolia leaf

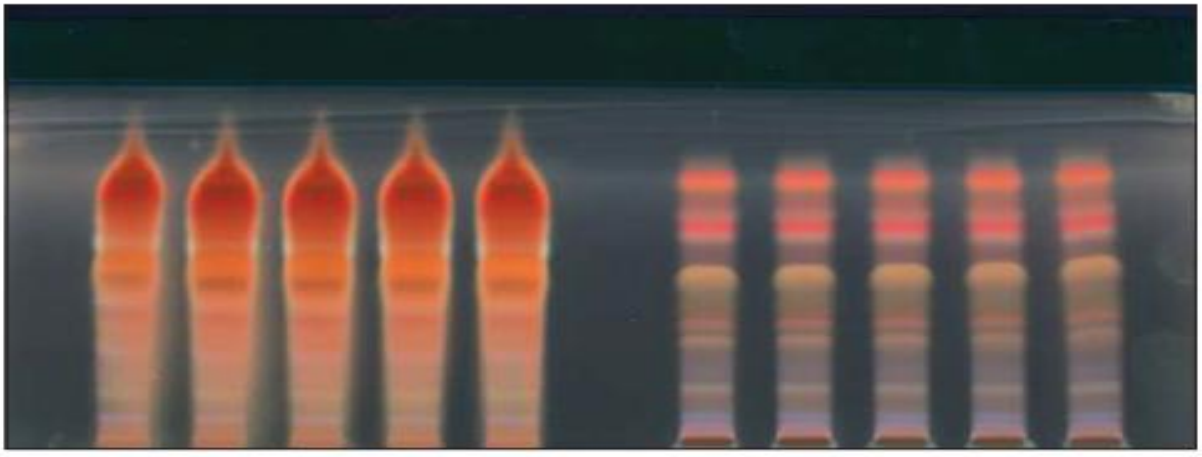

Fig 4: Fingerprint analysis of phytosterols of Holoptelea integrifolia (Roxb.) Planch leaves after derivatization with anisaldehyde sulfuric acid reagent

Table 1: Peak table with Rf values, height and area of Phytosterols

Peak	Start Rf	Start Height	MaxRf	Max Height	Max %	End Rf	End Height	Area	Area%	Assigned Substance
1	0.07	0.5	0.10	56.1	5.57	0.12	0.2	1208.7	5.32	Unknown
2	0.27	0.1	0.29	16.7	1.66	0.30	0.5	190.2	0.84	Unknown
3	0.30	0.1	0.33	333.5	33.10	0.35	83.5	5305.2	23.34	Unknown
4	0.35	84.6	0.40	309.4	30.71	0.44	72.2	10478.4	46.10	Unknown

5	0.44	72.3	0.45	34.4	3.41	0.47	0.6	433.9	1.91	Unknown
6	0.48	0.6	0.51	44.5	4.42	0.55	25.5	1227.1	5.40	Unknown
7	0.55	26.0	0.58	7.0	0.70	0.58	45.2	101.8	0.45	Unknown
8	0.58	45.2	0.60	18.7	1.86	0.63	19.7	333.9	1.47	Unknown
9	0.78	56.6	0.81	48.8	4.84	0.83	128.2	1095.8	4.82	Unknown
10	0.83	128.5	0.84	13.8	1.37	0.87	111.6	202.8	0.89	Unknown
11	0.87	112.3	0.90	64.1	6.36	0.92	140.7	1355.9	5.97	Unknown
12	0.92	140.9	0.93	60.4	6.00	0.95	2.2	795.5	3.50	Unknown

3.3 Fingerprinting study of Phytosterols of Hydroalcohol extract at 277 nm

Fingerprinting study of phytosterols shows twelve Rf Between the range of 0.10- 0.93. Rf 0.40 has 46.10% concentration in Figure 1, Table 1.

4. CONCLUSION

A novel method for HPTLC analysis of Hydroalcohol extract of *Holoptelea integrifoli* has been presented along with results show the presence of Phytosterols. The essences of these metabolites are beneficial for maintenance of human health and chronic degenerative diseases. The developed fingerprint analysis will help to isolate and identify new Phytosterols, which will offer a possibility to discover a lead molecule for drug development.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No human or animals were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data associated with this research paper is available in Dept.of Pharmacology Sanjivani College of Pharmaceutical Educati on and Research, Kopargaon, Maharashtra, I ndia The data will be shared upon request from the corresponding author.

CONFLICT OF INTEREST

The author declare no conflict of interest/competing interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

Dr. Ravindra C. Sutar conceptualized and designed the study, curated the data and prepared the original draft, discussed the methodology and analysed the data, prepared results Dr. Gowtham and Mr. Kashid contributed to the final manuscript.

FUNDING

We acknowledge the resource support for the

study was provided by the Hon'ble Management of Sanjivani Group of Institutes, Sanjivani College of Pharmaceutical Education and Research, Kopargaon. Maharashtra, India.

REFERENCES

1. Chaudhary RR. Herbal Medicine for Human Health. New Delhi: Regional Publication, SEARO, 2. No. 20, W.T.O; 1992. p. 1-80. 2.

2. World Health Organization. Quality Control Method for Medicinal Plant Materials. Geneva: WHO; 1989. p. 1-15.

3. Bobbarala V, Bramhachari PV, Ravichand J, Reddy YHK, Kotresha D, Chaitanya KV. Evaluation of hydroxyl radical scavenging activity and HPTLC fingerprint profiling of Aegle marmelos (L.) Correa extracts. J Pharm Res 2011; 4(1):252-255.

4. Prabavathy D, Valli Nachiyar C. Antimicrobial and antidiabetic activity of an endophytic fungi isolated from Adathoda beddomei. Int J Pharm Pharm Sci 2013;5(3):???. 2.

5. el-Mousallamy AM. Leaf flavonoids of Albizia lebbeck. Phytochemistry 1998;48(4):759-61. 3

6. Nazneen Bobby MD, Wesely EG, Johnson M. High performance thin layer chromatography profile studies on the alkaloids of Albizia lebbeck. Asian Pac J Trop Biomed 2012;2(1):S1-6.

7. Kiruba S, Mahesh M, Nisha SR, Miller Paul Z, Jeeva S. Phytochemical analysis of the flower extracts of Rhododendron arboretum Sm. Ssp. Nilagiricum (Zenker) Tagg. Asian Pac J Trop Biomed 2011;???:284-6

8. Tirupathi RG, Suresh BK, Ujwal KJ, Sujana P, Raoa AV, Sreedhar AS. Anti-microbial principles of selected remedial plants from Southern India. Asian Pac J Trop Biomed 2011;1(4):298-305

9. Raja RD, Jeeva S, Prakash JW, Antonisamy JM, Irudayaraj V. Antibacterial activity of selected ethnomedicinal plants from South India. Asian Pac J Trop Med 2011;4(5):375-8

10.Robonson MM, Zhang X. The World Medicine Situation, Traditional Medicine: Global Situation, Issues and Challenges. 3rd ed. Geneva: WHO; 2011

11.Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012;75(3):311-35. 6.

12.Osbourn AE, Lanzotti V. Plant Derived Natural Products. Synthesis Function and Application; New York: Springer Science; 2009. 7.

13. Parekh J, Chanda S. Phytochemical Screenig of some plants from western region of India. Plant Arch 2008;8(2):657-62

14. Nadkarni KM. Indian Materia Medica. Popular Prakashan Pvt Ltd. Mumbai, India. 1976, 651-652.

15. Kirtikar KR, Basu BD. Indian Medicinal Plants. Edn 3, Sri Satguru Publications, New Delhi, India, 2000; 3:2292- 2294.

16. Prajapati ND, Purohit SS, Sharma AK. A Handbook of Medicinal Plants a Complete Source Book. Agrobias. Jodhpur, India, 2003, 273.

17. Warrier PK, Nambiar VPK, Ramakutty C. Indian Medicinal Plants a compendium of 500 species, Orient longman private Limited, 1995, 3:162.

18. Khandelwal K R. Practical

Pharmacognosy. Techniques and Experiments. 10th ed. Nirali Prakashan, Pune, India. 2006, 149-156.
19. Harborne JB. *Phytochemical methods;* 3rd edition, London: Chapman and Hall, 1998.
20. Wagner H, Baldt S. *Plant drug analysis;* Berlin: Springer; 1996. R.P.W. Scott, Encyclopedia of Chromatography, 10th edn, Marcel Dekker, USA, 2001, 252-254.
21. ICH/CPMP Guidelines Q2B, Validation of Analytical Procedures– Methodology, 1996.
22. Cazes J, Scott RPW. Chromatography Theory, Marcel Decker, NY, 2002, 443-454.
23. Reviewer Guidance, Validation of Chromatographic Methods, 1994.
24. Sethi PD. HPTLC: Quantitative Analysis of Pharmaceutical Formulations, CBS Publications, New Delhi, 1996, 162-165.
25. Heftman E. Chromatography Fundamentals and Applications of Chromatography and Related Differential Migration Methods. 6th edn, Elsevier, Amsterdam. 2004; 69A:253-291.
26. British Pharmacopoeia, International edn, HMSO, Cambridge, 2002; II, Appendix 112 (IB).
27. Sherma J. Encyclopedia of Pharmaceutical Technology, 2nd edn, Marcel Dekker, USA, 2001, 252-254.
28. ICH/CPMP guidelines Q2A, Text on Validation of Analytical Procedures, 1994.
29. USP 23, NF 19, Asian edn, United States Pharmacopeial Convention, Rockville, M.D., 982, 1225.
30. G. García-Llatas, M.T. Rodríguez-Estrada, Current and new insights on phytosterol oxides in plant sterol-enriched food, Chem. Phys. Lipids 164 (6) (2011) 607–624, https://doi.org/10.1016/j.chemphyslip.2011.06.005.
31. R.A. Moreau, L. Nystr¨om, B.D. Whitaker, J.K. Winkler-Moser, D.J. Baer, S. K. Gebauer, K.B. Hicks, Phytosterols and their derivatives: structural diversity, distribution, metabolism, analysis, and health-promoting uses, Prog. Lipid Res. 70 (2018) 35–61, https://doi.org/10.1016/j.plipres.2018.04.001
32. Sutar R C, Kasture S B, Kalaichelvan V K. Finger Printing Analysis of the Flavonoids from Holoptelea integrifolia (Roxb.) Planch Leaves using HPTLC Analysis. Journal of Pharmacognosy and Phytochemistry. 2014;3 (3):80-85
33. Sutar R C,Musmade D S , Ware A L . HPTLC Finger Printing Analysis of the Alkaloids from *Holoptelea integrifolia* (Roxb.) Planch leaves. Journal of Pharmacognosy and Phytochemistry. 2016; 5(4): 215-219.
34. Sutar R C, Kasture S B and Kalaichelvan V K . Phytochemical Profile Studies on the Steroids of Methanolic Leaf Extract of Medicinally Important Plant Holoptelea integrifolia (Roxb.)Planch using High PerformanceThin Layer Chromatography. Asian J Pharm Clin Res.2014;7(4):197-200.
35. Sutar R C , Kasture S B , Kalaichelvan V.K. Finger Printing Analysis of the Phytosterols from Holoptelea integrifolia (Roxb.) Planch Leaves using High Performance Thin Layer Chromatography Analysis. Asian J Pharm Clin Res.2014;7(4): 160-164
36. Sutar R. C. and Musmade D. S. Finger Printing Analysis of the Phenols fro

m *Holoptelea integrifolia* (Roxb.) Planch Leaves Using HPTLC. Indian Drugs. 2017 54(9):67-71.
37. Sutar R C ,Kasture S B , Kalaichelvan V K . Phytochemical Studies on the Glycosides of Leaf Extracts of Medicinally Important Plant Holoptelea integrifolia (Roxb.) Planch using High PerformanceThin Layer Chromatography. Asian J Pharm Clin Res. 2014;7(4): 192-196.