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HPTLC in Herbal Drugs

Gayatri Sanjay Karanjawane[1]*, Rutuja Prakash Dokh[2], Prathamesh Prakash Dorugade[3], Tejaswi Santosh Ubhe[4], Rutuja Pandurang Vedpathak[5], Vaishali Pardeshi[6]
[1,2,3,4,5]Research Students, [6]Guide
Abhinav Education Society's College of Pharmacy, B-Pharm, Narhe,
Pune, Maharashtra, India.
Corresponding Author
Email Id: gayusk2612@gmail.com

ABSTRACT

"Nature itself is the best medicine", as said by Hippocrates; the herbs play great importance in human life. Mother Earth's medicine chest is full of healing herbs of incomparable worth. They effect or help to rectify the three doshas in the body and restore homeostatic balance. Standardization and analysis of the chemical marker of the herbal drugs have always been a concern due to the complex nature. For Researchers, Standardization is the need of the hour for the present era to set standards for maintaining the quality and efficacy of the herbal products. HPTLC is an ideal screening tool for adulterations and for evaluation and monitoring of cultivation, extraction processes and testing of stability. The present study compiles the fingerprinting analysis in HPTLC for herbal products which is the increasing demand that scaffolds for future drugs. HPTLC is a modern analytical separation method with extensive versatility, although already much utilized, is still with great potential for future development in research and development. It is accepted as a time-saving and most economical machine practically with minimum trouble shootings. This review attempts to focus the key feature, applications of HPTLC with some examples of herbal drugs and HPTLC performed for an herbal drug.

Keywords: *HPTLC; Herbal drugs; Diosgenin; Quercetin; Fenugreek; Fingerprinting Profile.*

INTRODUCTION

Science and technology have been evolving over years and delivering so many opportunities to improve health and extend lives, but continued investments are being invested in both the public and private sectors, in spite of the current economic climate [1]. Pharmaceutical industry success rates are increasing and delivering more medicines is very challenging, but very few predictive scientific and analytical tools are available leading to develop more superior techniques for analytical purposes [1]. Analysis of pharmaceutical and natural compounds is commonly used in all the stages of the drug discovery and development process.

India is one of the main hubs for herbal extracts and exports these products to all over the world [3]. Ayurveda is a medical system primarily practiced in India which has been originated from nearly 5000 years. It includes diet and herbal remedies while emphasizing the body, mind, and spirit in the prevention and treatment of diseases. World Health Organization defines Traditional herbal medicines as naturally occurring, plant-derived substances with minimal or no industrial processing that have been used to treat illness. Herbal drugs and their formulations are extensively used since time immemorial in developing and well-developed countries [2]. The assessment of quality is necessary for research as well as in practice. Herbal medicine is also called

botanical medicine or phytomedicine. It refers to the use of plant's seeds, berries, roots, leaves, bark, or flowers for medicinal applications. Herbalism has a long tradition of use outside of conventional medicine. Nowadays it is becoming more mainstream due to the improvements in analysis and quality control along with advances in clinical research which show the value of herbal medicine in treating and preventing disease limiting the side effects of allopathic drugs used.

Chromatographic techniques are generally been carried out for the separation and purification of phytoconstituents based on their charge, shape, or size. Thin-layer chromatography (TLC) is considered an authentic and reproducible method for the analysis of a variety of drugs. This technique is extensively adopted for the rapid analysis of drugs and drug preparations which provides a chromatographic drug fingerprint in a short period. A chromatographic fingerprint of herbal medicine is a chromatographic pattern of the extract of some common chemical constituent of pharmacologically active or chemical characteristics.

Herbal drugs have a complex nature and inherent variability of the chemical constituents; it is difficult to establish a quality control parameter. Quality assurance of herbal medicine is an important factor and basic requirement for the herbal drug industry and other drug development organization. Various problems influence the quality of herbal medicines. Some of the problems are listed below:-

1) Variable sources of the raw material.
2) The chemical constituents of herbs and herbal products may vary depending on the stage of collection, parts of the plant collected, harvest seasons, plant origins (regional status), drying processes, and other factors.
3) Extracts are usually mixtures of many constituents.
4) The active principle(s) is (are), in most cases unknown.
5) Selective analytical methods or reference compounds may not be available commercially.

There is a high need for the analysis and separation of drugs for improving the quality of drugs. HPTLC is extremely versatile and sensitive for the identification of many herbal drugs and is especially useful for the detection of adulterations, often having a very high degree of sensitivity. High-performance thin-layer chromatography (HPTLC) is a modern, powerful analytical technique with separation power, performance, and reproducibility superior to classic TLC and advanced chromatographic technique. HPTLC features the use of modern apparatus; highly sensitive scanning densitometry, new chromatographic chambers, and more effective elution techniques, high-resolution sorbents with selected particle size or chemically modified surface, and video technology for rapid chromatogram evaluation and documentation. HPTLC has become one of the most flexible, reliable, and cost-efficient separation techniques ideally suitable for the analysis of botanicals and herbal drugs. Not only in any one field it has been the most widely applied method for the analysis in pharmaceutical industries, clinical chemistry, forensic chemistry, biochemistry, cosmetology, food and drug analysis, environmental analysis, and other areas as well.

Key Features of HPTLC are been listed below:

1) Visual evaluation and flexible use.
2) Reproducible analysis and reliable quantification.
3) Multiple detections of separated analytes.

4) Ability to analyze crude samples containing multi-components.
5) Several samples can be separated parallel to each other on the same plate resulting in high output, time-saving, and rapid low-cost analysis.
6) The choice of solvents for HPTLC development is wide as the mobile phases are fully evaporated before the detection step.
7) HPTLC can combine and consequently be used for different modes of evaluation, allowing identification of compounds having different light-absorption characteristics or different colors.
8) HPTLC method may help to minimize exposure risk of toxic organic effluents and significantly reduces its disposal problems, consequently, reducing the environmental pollution.
9) The requirements of a fingerprint analysis can be completely different from those for a quantitative determination of marker or key compounds, although the herbal preparation separated for fingerprints. It is necessary to fully separate those compounds from all others which are provided by HPTLC.
10) Constituents of herbals that belong to very different classes of chemical compounds can often create difficulties in detection. With this in mind, TLC and especially HPTLC can offer many advantages.

HPTLC Method for Estimation of Diosgenin and Quercetin in Fenugreek Seeds

Fenugreek one of the commonly used and well-known herb in India. The scientific name is *Trigonella foenum-graceum Linn.*, belonging to the Family Leguminosae which is the native herb of the Eastern region of India having a wide variety of pharmacological properties (Figure1). From the early decades, it has been extensively used in both Indian Ayurveda and Unani systems for the treatment of epilepsy, paralysis, gout, dropsy, chronic cough, diabetes, piles sinus, and lung congestion, inflammation, infection mitigation. Along with this, it has a variety of more applications also used in the treatment of hair, indigestion, and baldness as well. The biological and pharmacological actions of fenugreek seeds are mostly attributed to the variety of its bioactive chemical constituents that serve as raw materials for the manufacture of various hormonal and therapeutic drugs. It has the presence of specific bioactive compounds like steroidal diosgenin, alkaloid trigonelline, flavonoid quercetin, galactomannan, and unusual amino acid 4-hydroxy isoleucine. Among key bioactive constituents of fenugreek, diosgenin, and quercetin have been reported to be of vital importance and are well known to produce desirable therapeutic effects in patients.

Fig.1. Fenugreek

In Ayurvedic Pharmacopoeia, a couple of formulations such as Mustakarista and Mrusanivari Sura are reported to contain fenugreek seeds. Quercetin is a bioflavonoid that has been reported to have anti-inflammatory, antidiabetic, and anticancer uses and other properties which involve improvement in mental and physical performance [figure 2 a)]. It has also been reported to attenuate thermal hyperalgesia in a mouse model of diabetic neuropathic pain. Diosgenin (25R)-spirost-5-en-3 β-ol) is a plant-derived sapogenin which is known to suppress inflammation, inhibit proliferation, and induce apoptosis

in a variety of tumor cells. It also has other anti-rheumatic anti-viral properties. Diosgenin is often used as a raw precursor for the production of steroidal drugs and hormones such as testosterone, glucocorticoids, and progesterone.

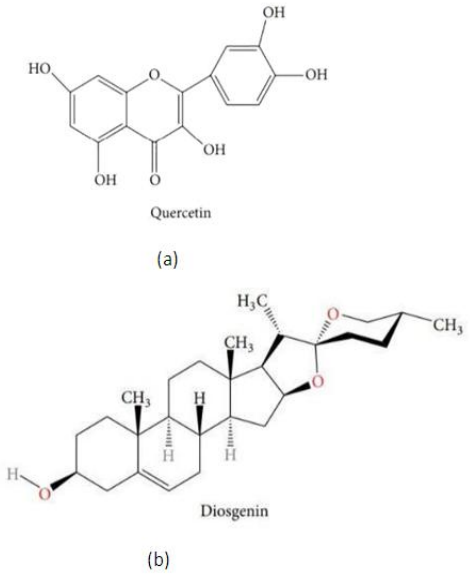

Fig.2. Structures of (a) Quercetin and (b) Diosgenin

Due to the innumerable health benefits of phytochemicals, recently, great attention is being given to determine the quality, efficacy, and standards of the herbal drugs. By considering the widespread use of fenugreek seeds by the general population and the expected increase in its therapeutic use for several diseases the review has been presented for the study of HPTLC, a validated sensitive and fast method using sophisticated instrumentation for determination and quantification of quercetin and diosgenin in fenugreek.

EXPERIMENTAL METHOD

Preparation of Extract of Plant Material

The seeds of fenugreek were collected and the powdered seeds were passed through Sieve No. 85, weighed, and then used for extraction. Powdered fenugreek seeds were extracted with water: Ethanol mixture (1:1) for 72 h at 70°C using soxhlet apparatus. The extract was then concentrated to dryness by removing the solvent in the rotary evaporator under reduced pressure.

Chemicals

1) Standard diosgenin (98%) and quercetin (98%) were procured for the development of the method.
2) Anisaldehyde-sulfuric acid spray reagent-To 0.5 mL of anisaldehyde reagent, 10 mL of glacial acetic acid, and 85 mL methanol were added along with an addition of 5 mL concentrated sulphuric acid.
3) All the other Analytical grade solvents and chemicals are ethyl acetate, acetone, methanol,
4) ethanol, chloroform, n-heptane, cyclohexane, phosphomolybdic acid, acetic acid, isopropanol, and sulphuric acid.

Preparation of Standard Solution

Standard solution of diosgenin (98%) and quercetin (98%) were prepared either separately or in a mixture at a concentration of 1 mg mL−1 each in absolute methanol. For the complete dissolution of the mixture, Ultrasonication was ensured.

Sample Preparation

Extracted samples are treated three times each with different solvents including dichloromethane (DCM), ethyl acetate, acetone, and hexane which gives a maximum yield of diosgenin and quercetin in the selected solvent [Figure 3]. The pooled extracts were washed in 2 mL of 0.1 M sodium hydroxide to remove the free fatty acids, and then by 1 mL of distilled water to remove the remaining hydrophilic contaminants. The washed extracts were quantitatively transferred to a 50 mL flask and then evaporated to dryness with a rotary at 50∘ C. The dry residue obtained was dissolved in 5 mL of methanol and a reconstituted solution was filtered and used as a test solution.

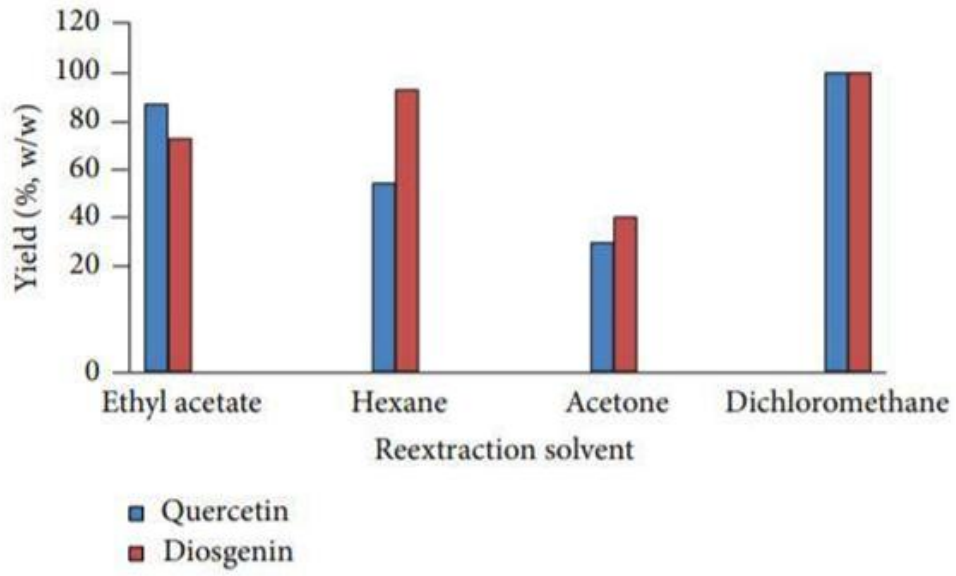

Fig. 3. Re-extraction of Solvents for Maximum yield of Quercetin and Diosgenin.

HPTLC Instrumentation and Experimental Conditions:

1) Sample Application:-The samples were spotted in the form of a band with automated instrumentation CAMAG 100-µL syringe on pre-coated silica gel or aluminum plate which was prewashed before use by dipping in methanol and plates were activated at 60∘ C for 5 min prior to chromatography.
2) Development of Chromatogram:-The plates were developed twin trough glass containing 25 mL of mobile phase as a mixture of toluene: ethyl acetate: formic acid (5: 4: 1, v/v/v). The optimized chamber saturation of the mobile phase was done. The length of the chromatogram run was up to 80 mm from the point of application (10 mm). After the development of the chromatographic plates, they were dried for 5 minutes in a current of air with the help of a hairdryer in normal mode.

Quantitative Evaluation

For quantification of quercetin, the plates were directly scanned within 10 minutes using densitometric scanner III with WinCATS software (Camag) in the UV mode with the deuterium source set at 275 nm. The spots corresponding to quercetin were observed at = 0.57 ± 0.02. For diosgenin, the dried plates were dipped into the anisaldehyde-sulphuric acid detection reagent and dried for 10 minutes under hot air followed by placing in an oven at 105∘ C for 10 minutes. The plates were scanned within 10 minutes using densitometric scanner III with WinCATS software (Camag) in the visible mode with the tungsten-halogen source set at 450 nm. The spots corresponding to diosgenin were observed at = 0.69 ± 0.02.

Validation of Method

The established HPTLC method was primarily validated in terms of specificity, linearity, precision, limits of detection (LOD) and quantification (LOQ), and robustness. Validation parameters for two wavelengths, 426 and 590 nm, were compared. Validation was performed with the aid of the obtained hydrolyzate of the methanol extract from exhaustive extraction in a Soxhlet apparatus as well as using diosgenin standard solutions. Specificity was checked by analyzing standards and extract samples of equivalent concentrations. The bands for diosgenin and quercetin in the sample

were confirmed by comparing the value and spectra of the band from the sample and standard solution. Accuracy of the method was assessed by spiking pre-analyzed samples with known amounts of standard quercetin and diosgenin solution and then reanalyzed by the HPTLC method. After developing and visualizing the chromatogram, densitometric analysis was performed. Instrumental precision, expressed as RSD (%), was determined by linear scanning of bands of both the standard solution (n = 9) and sample (n = 9) after derivatization using a modified anisaldehyde reagent at 426 and 590 nm. Intermediate precision of the method was tested by performing slight changes in the procedure; analyses were made by two different analysts using chemicals from different containers, whereas robustness testing was based on small deliberate changes in mobile phase composition and development distance. The compounds were detected based on value as well as UV/Visible spectral overlaying of respective standard compounds. Standards were diluted and applied on HPTLC plates to plot the calibration curves. The LOD was determined based on the lowest concentration detected by the instrument from each of two standards, while the LOQ was determined based on the lowest concentration quantified in the samples. The robustness of the method was studied by introducing small deliberate changes in experimental conditions and results examined. The robustness of the method was performed in triplicate and the results were expressed as RSD (%) of the peak.

RESULTS

A new quantitative HPTLC-densitometric method for diosgenin and quercetin estimation was performed together with the procedures of efficient extraction and acid hydrolysis of steroidal saponins and flavonoids in fenugreek seeds. The quantitative HPTLC method provides a dense and compact spot with significant values for the determination of diosgenin and quercetin in fenugreek seeds. For successful development, the important step is to optimize the solvent system for good extraction efficiency. To optimize the efficient re-extraction of diosgenin and quercetin from acidified ethanol extracts, different solvent including n-hexane, acetone, dichloromethane, and ethyl acetate were evaluated. The identity of bands of quercetin and diosgenin in sample extracts was confirmed by overlaying their UV-Vis absorption spectrum. A chromatogram was developed and bands were separated (Figure 4). The purity (98%) of the bands of diosgenin and quercetin in sample extracts was confirmed by comparing the spectra at various points including peak start, peak apex, and peak end positions of the spot. A good correlation was obtained between the overlaid spectra of diosgenin and quercetin in standard and sample extracts (Figure 5). The method reported here is simple, rapid, and suitable for the quantitation of Diosgenin from the extract of seeds of fenugreek.

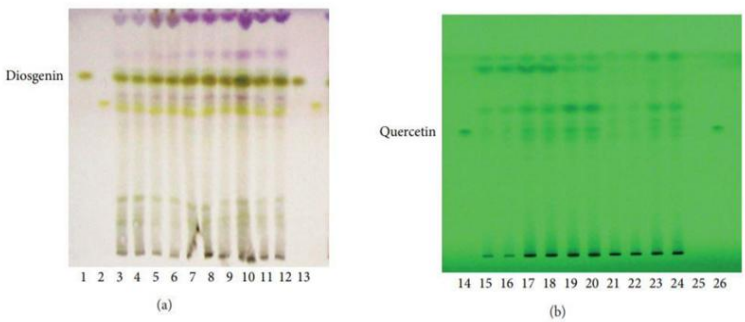

Fig.4. Images of HPTLC Separation of Extracts of Samples of Fenugreek Seeds: (a) Diosgenin and (b) Quercetin

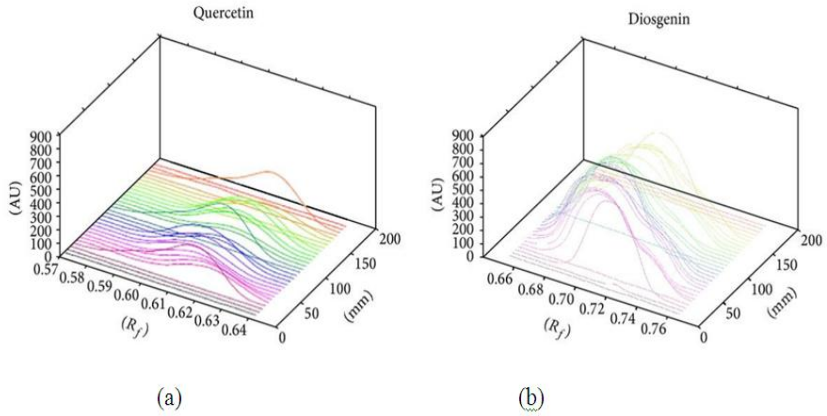

Fig.5. Overlaid in situ spectra of diosgenin and quercetin acquired by the proposed method from standards and samples (peak purity of the sample).

Fingerprint Profile for Some Other Herbal Preparations by HPTLC is given in the Following Table below:

Table 1: Fingerprint profile of other Herbal Preparations.

S.No.	Ayurvedic Preparation	Botanical Name	Family	Constituent	Solvent System	Uses
1	Chyawanprash	*Agele marmelos*	Orchid	Sugar, honey, ghee, Indian gooseberry, jam, sesame oil, berries, and various herbs and spices.	HPTLC:- Toluene: ethyl acetate: formic acid: ethanol (6:4:0.3:0.4) at 254 and 366 nm	Booster the immune system, Promote digestion, Slows the aging process, enhances fertility.
2	Ashwagandha capsule	*Withania somnifera*	Solanaceae	*Withania Somnifera (ashwagandha)*	HPTLC: Chloroform: Methanol (9:1) at 254 and366nm	Reduce anxiety and stress, fight depression, booster fertility.
3	Sanjivani Vati	*Selaginella Bryopte.*	Selaginellacea	*Embelia Ribes, Zingiber Officinalis, Terminalia chebula, Terminalia belerica, Tinospora cordifolia, Aconitum heterophyllum, Emblica Officinalis*	HPLC:- Acetonitrile: water (20:80), acetonitrile: water: acetic acid (48:52:1) at 254 nm at a Flow rate 1ml/min	Fever management, indigestion, Typhoid fever, snake bite.
4	Triphala ashi	*Terminalia chebula, Terminalia belerica*	Combretaceae,	*Terminalia chebula, Terminalia belerica, Phyllanthus Emblica.*	*Terminalia chebula, Terminalia belerica, Phyllantus Emblica*	Antimicrobial agents

5	Vatari Guggulu	*Guggulu-suddha (Commiphora mukul Eng.*	Burseraceae	*Commiphora wightii, Ricinus communis, Terminalia chebula, Terminalia belerica, Emblica Officinalis, sulfur.*	HPTLC:- Toluene: acetone (9:1) at 250 nm.,	Body toxifier, Recover appetite, Treatment for arthritis
6	Kalmegh Ikshvadi	*Andrographis paniculata*	Acanthaceae	*Andrographis paniculate*	HPTLC:- Benzene: ethyl acetate (5:5) at 222nm.	digestive stimulant, hepatoprotective , Improve liver function, blood purifier.
7	Ashwagandh arishta	*Piper longu*	Solanaceae	*Withania somnifera.*	HPTLC:- Toluene:ethyl a cetate: formic acid at 540nm	Used in anemia, Increase height, Improve fertility.
8	Turmeric	*Curcuma longa*	Zingiberaceae	*Amomoum curcuma, Anlatone (constituent), artumerone, Curcuma, Curcuma aromatica, Curcuma aromatica Salisbury, Curcuma domestica, Curcuma domestica Valet, Curcuma longa, Curcuma longa Linn., Curcuma longa rhizoma, curcuma oil, curcumin, diferuloylmethan*	HPTLC:- acetonitrial and water(50:50) Curcumin 425nm.	Cosmetic, reduce pain and inflammation, anti-bacterial and antifungal.
9	Neem oil	*Azadirachta indica.*	Meliaceae	Meliantriol, Nimbin, nimbidin, nimbinin, nimbolides, fatty acids (oleic, stearic, and palmitic), and salannin	HPTLC:- chloroform : ethyl acetate containing 1% acetic acid 8:2(v/v).	Stimulate collagen Production, reduce scars, heal Wounds, treat acne, minimize warts and moles, Treat dry skin.
10	Shatavari	*Asparagus racemosus*	Liliaceae	Steroidal saponins 0.2%. Shatavarin I sarapogenin+3glu+1 Rha Shatavarin Iv Sarapogenin+2glu+2 Rha	Water, ethanol, methanol	Galactogogu to promote the flow of milk. Used as tonic and diuretic. Management of nervine and rheumatism. Treat the threatened abortion.

Chromatograms for some of the herbal preparations listed above

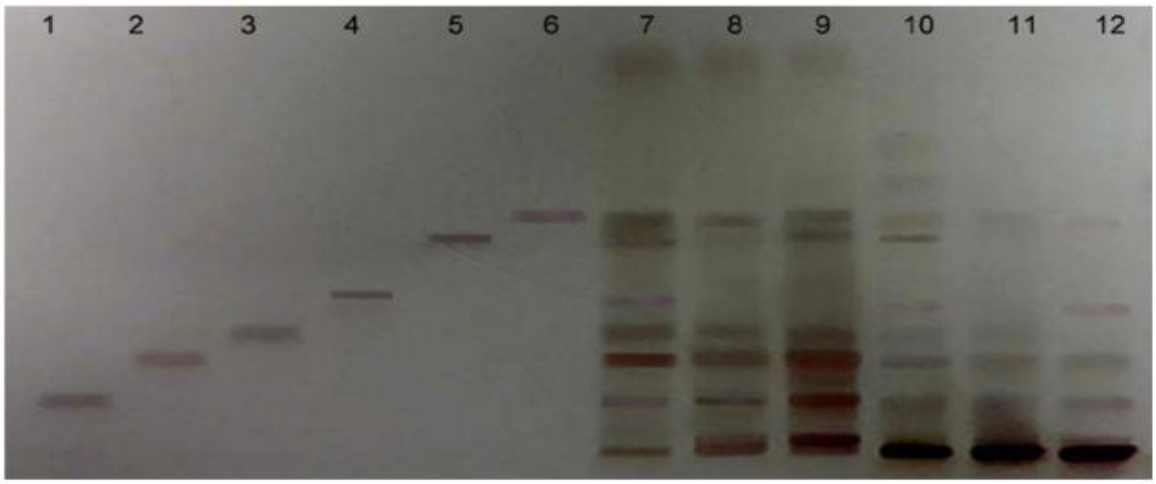

Fig. 6. HPTLC chromatogram for Withania somnifera (Ashwagandha).Visualization was made under UV light of wavelength 231 nm.

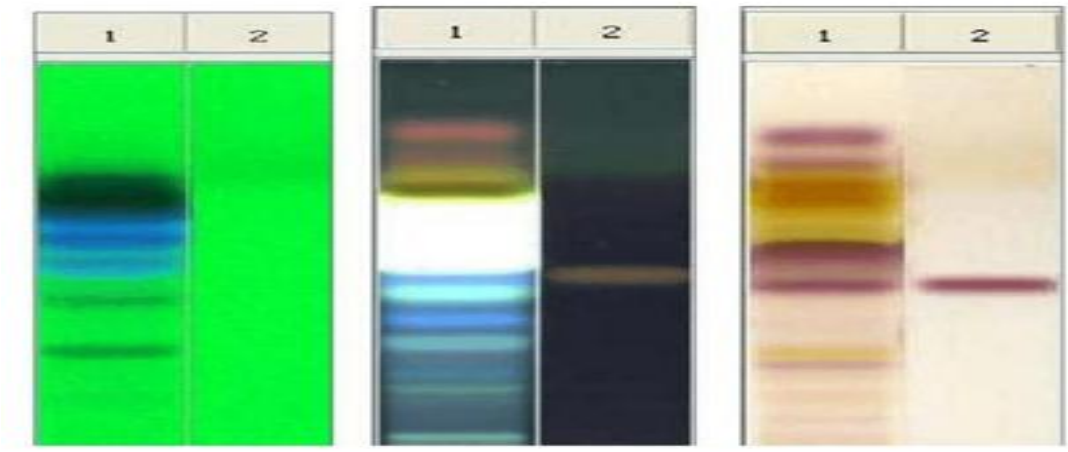

Fig. 7. HPTLC analysis of neem oil EtOAc extract.
Visualization: plate a (on the left) UV lamp 256 nm; plate b (in the middle) UV lamp 366 nm; plate c (on the right) UV lamp at 366 nm

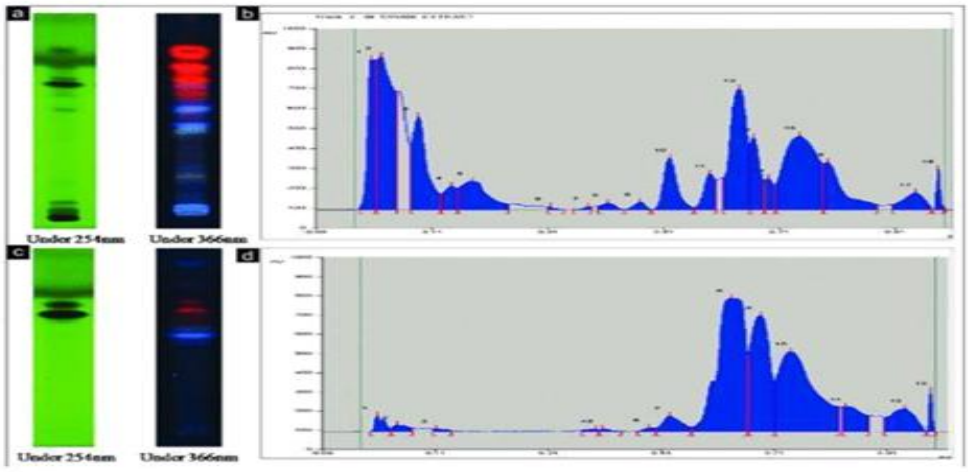

Fig. 8. High-performance-thin-layer chromatography Aegle marmelos (Chawanprash) under 254 nm and 366

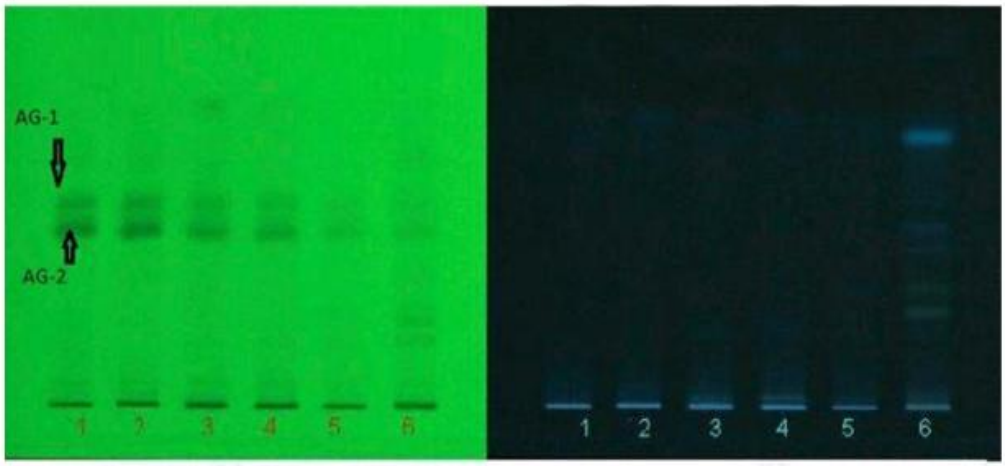

Fig. 9. HPTLC Chromatograms of Piper Longa (Ashwagandharista). (a) at 254nm and (b) at 366 nm.

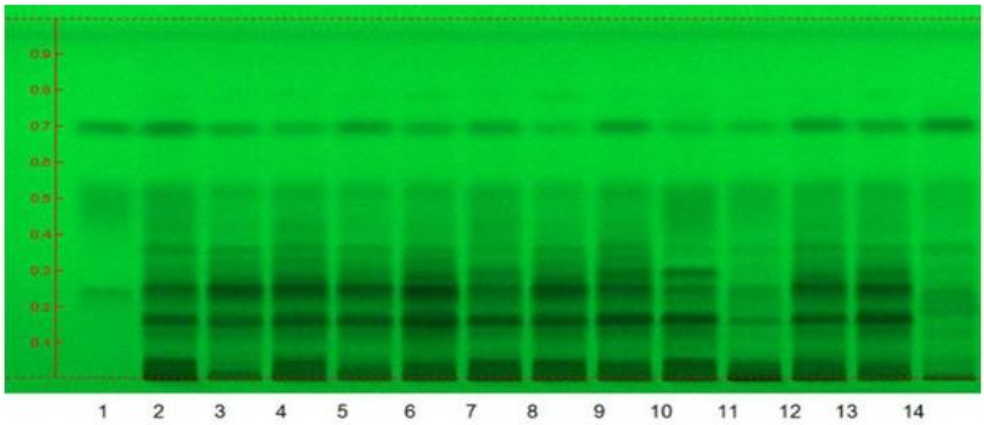

Fig. 10. Chromatogram of Terminalia chebula (Triphala Mashi).

HPTLC Superiority over Other Techniques

1) **Resolution-** In other chromatographic techniques, it becomes difficult to differentiate between overlapping bands and spots. HPTLC is a highly flexible analytical technique and allows to adapt the analytical method to the individual need in each process step.
2) **Reproducible analysis and reliable quantification-** Quantitative evaluation of analytes can be achieved based on hyperspectral data, densitograms. HPTLC is an analytical technique involving sophisticated instrumentation, standardized and documented procedures, as well as validated methods. This allows user to achieve reproducible results.
3) **Multiple detections of separated analytes-** The strength of HPTLC are the possibility to use multiple detection methods on the same sample and plate. In contrast to other chromatographic techniques, separate analyses of the sample remaining on the plate.
4) **Analysis of multiple samples in parallel without cross-contamination-** HPTLC allows for parallel instead of sequential analysis with little to no sample preparation. At least 15 samples can be developed under identical conditions and analyzed at the same time. HPTLC is highly efficient because of several reasons: short run time, low solvent consumption, and cross-contamination due to single-use of plates.
5) **Coupling possibility to mass spectrometry-** HPTLC-MS is a strong additional detection possibility and allows the structural confirmation

of targeted analytes and also elucidation by coupling HPTLV yo high-resolution MS.

CONCLUSION

Fingerprint profile is quite helpful in setting up standards and keeping a check on intentional/unintentional adulteration. It can be concluded that HPTLC is an ideal tool for the identification of herbal materials. The reported HPTLC method is an attractive simple, rapid, and selective method for the simultaneous quantitative determination of flavonoid quercetin and steroid diosgenin in fenugreek extract. This method could be widely applied for direct routine analysis and quality assurance of related extracts and drugs. HPTLC is playing an important role in today's analytical world, not in competition to any other chromatographic techniques but also as a complementary method used till date. The uses in the validation of new uprising herbal products and its introduction into the regulatory systems are of much importance towards the future of HPTLC. Also, it has widespread applications in numerous other fields.

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