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HPTLC Chromatographic Studies and Evaluation of Ayurvedic Formulation

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ABSTRACT

Polycystic ovary syndrome is a disease that affects the female reproductive health and its prevalence amongst the adolescent population has shown alarming rise in recent years. Many Ayurvedic formulations have been prescribed and have been reported to be effective in clinical practice of managing female reproductive disorders. Pushyanuga Churna is one such preparation widely used in clinical practice by Vaidyas for treating female reproductive dysfunctions. Since the ingredients of Pushyanuga Churna are reported to be effective in various female reproductive disorders that are also associated symptoms of PCOS, it is hypothesized that this formulation will be an effective agent to manage PCOS and its associated symptoms. The different volumes from standard stock solution (10 µg/ml); 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 µl were spotted on HPTLC plate (20 x 10 cm) in order to deliver concentration of 120, 160, 200, 240, 280, 320, 360 ng/spot of berberine respectively using vial I followed by spotting of 2 µl of 3 sample stock solutions. Samples were applied as bands 4 mm wide, 12 mm apart, by CAMAG Linomat V applicator using 100 µl test syringe with a steady application rate of 150 nLs-1. After sample application plates were produced in a developing chamber that was pre-saturated with the mobile phase (20 ml) ethanol : glacial acetic acid : water (16:2:2, v/v) for 20min. The plate was developed in CAMAG horizontal twin tough glass developing chamber (20 x 10 cm) at the room temperature up to 8 cm (80% of the total plate size). Ascending mode was used for the development of Thin Layer chromatography. After development, plates were dried on CAMAG TLC plate heater at 120^0C temperature for 5 minutes. The plate was then observed under UV and fluorescence reflectance mode in CAMAG TLC visualiser photo documentation system at wavelength of 254 and 366 nm respectively. The developed plate was then scanned at 366 nm using CAMAG TLC densitometric scanner 3 incorporated with WINCATS 1.4.8 programming. The calibration curve was readied using standard concentration range of 120- 360 ng/spot. Each concentration peak area was plotted against the concentration of berberine spotted in densitometric analysis within the integrated software. Quantification of berberine was done as the peak area of sample spots was recorded and the amount of berberine was figured using standard curve. Method validation: ICH Q2B guidelines were used for developed HPTLC protocol (ICH, 2005) and the results were recorded. The present work, hence, aims to validate the use of Pushyanuga Churna in the treatment of PCOS. The research is an attempt to put forth some scientific evidences on the pharmacological and pharmacodynamic aspects of the therapeutic usage of Pushyanuga Churna, which in turn will rationalize its use in management of PCOS and assist its integration into the main stream health-care system. The aim of this study was to determine the concentration of Berberine in Pushyanuga churna brands available in India to ensure whether the berberine concentration in the follow churna as per FDA recommendation or not. There are few reputed brands like Patanjali, Dhoopapeshwar, Baidyanath were studied, by using HPTLC method. The minimum berberine level was observed in the dhoopapeshwar Pushyanugachurna, while Patanjali Pushyanuga churna sample showed the highest berberine content.

INTRODUCTION

Ayurveda, the name for the traditional Indian medical system, is based on prehistoric literature that emphasizes a "natural" and all-encompassing approach to physical and mental health. One of the oldest medical systems in the world, ayurvedic medicine is still used in India as a form of traditional medicine. Ayurvedic medicine includes many items (mainly derived from plants, but may also include animal, metal, and mineral), diet, exercise, and lifestyle.[1] Herbal and plant-based medicines have had a long history of the way these medicine schools have evolved and have been practiced worldwide. It has been proposed that in its early stages, these schools were widely practiced over many different countries including Greek and other European nations, which led to the rise of traditional systems of medicine such as the Ayurvedic School of medicine from India or the school of traditional Chinese medicine.[2] Traditional Medical knowledge containing a rich heritage of indigenous herbal practices have helped to sustain the health of most people of rural India.[3] Globally, 60-80% of people rely on traditional herbal medicines as a resource for primary health care system. Hence, traditional herbal medicines have gained more recognition in developing countries. This is also partly because of its affordability and accessibility.[4,5] Ayurveda is expected to have had an evidence-based origin. But, with reference to the current perspectives and practices, it is looked upon to be largely an experience-based system intermingled with myths and fallacies. The inappropriate commercial practices have also added a negative impact on its integration in to main stream medicine practices and philosophies.[6] Evidence based standardization of Indian traditional medicine in clinical practice has been sought after in order to help provide quality health care to all.[7]

THE NEED FOR STANDARDIZATION

Standardization and quality assessment of natural products is a complex task due to their heterogeneous composition and inherent variability of the constituents of plant-based drugs. It is a process of evaluation carried out to ascertain the quality and purity of crude drugs using various evaluative parameters.[8] In recent years, the increase in awareness regarding the subsistence of Ayurvedic and traditional formulation-based medicines has created an upheaval in the demand. This demand has increased by the disclosure regarding the dangers and shortcomings of modern medicine. Leading to sudden increase in the load on herbal industry. In such a scenario, it is now the prime responsibility of the regulatory authorities to ensure the purity, safety, claimed potency and efficacy of traditional medicines.[9,10] Low

extraction yields and poor-quality output might also result from inefficient processing. One of the most critical conditions for the development of a quality drug is a well-defined and consistent drug composition.[11]

As a result, standardization of herbal medicine in terms of its safety and efficacy is important. In terms of organoleptic evaluation, pharmacognostic evaluation, volatile matter, quantitative evaluation (ash values, extractive values), and phytochemical evaluation, standardization contributes to the quality of herbal remedies by establishing their identity and purity. Phytochemical fingerprints aid in the identification of chemical ingredients present in a sample, which can be used to assess the drug's quality. Bioactive indicators add to the formulation's overall therapeutic efficacy. As a result, the measurement of plant-specific marker chemicals can be utilized as a reference standard for standardizing herbal formulations employing analytical processes. Adulteration or substitution in commercial markets is possible. Hence, reproducible standards for each plant are required for effective quality control.[12,13]

AYURVEDA IN WOMEN REPRODUCTIVE DISORDERS

According to Ayurveda, a woman's health begins during the fetal period, which specifies the procedures to produce a healthy female child. The ancient Ayurvedic sages carefully researched the anatomical and physiological characteristics of women of various ages. Just as the river is cleansed by its flow the women are purified by the menstrual flow. This explains why women are immune to a wide range of diseases. Ayurveda strongly recommends following certain regimens throughout menstrual and postmenstrual cycles. Many gynaecological and systemic disorders in women are caused by disobedience to these regimens. In addition to the systemic diseases, 20 gynecological diseases are described, which are explained in the classics under the entity of Yonivyapad and all the Yoni Roga are the disease of anatomical components of ArtavavahaSrotas (Reproductive system).[14] Woman’s life is associated with drastic physiological, anatomical and psychological changes during menarche, reproductive period, pregnancy, menopause and post menopause.[15] The oldest medical system, Ayurveda, calls diseases of the complete female reproductive system "Yonivyapada." It has demonstrated promising outcomes in the treatment of reproductive health issues. Simple herbal remedies combined with a healthy food and lifestyle. Modifications can aid in keeping a healthy reproductive health status for women.[16] Though no one can deny the revolutionary changes and differences that modern medicine,

sophisticated treatment regimens, and techniques have made in improving the understanding and management of female reproductive disorders, there are still disorders like polycystic ovarian syndrome (PCOS) that modern medicine has not been able to completely manage the treatment of. In such circumstances, Alternative and complementary medications have been favoured.[17]

PCOS CURRENT STATUS IN INDIA

In women who are fertile, polycystic ovarian syndrome (PCOS) is a hormonal condition. Infertility in women is a common cause for this diagnosis. A syndrome, not a disease, is PCOS. It is a condition that lasts for the rest of one's life, long beyond childbirth. One in five (20%) Indian women are thought to have PCOS. The disorder can have adverse effects on one's health if it is not treated promptly. Dr. Duru Shah, a gynaecologist and the founder of the PCOS Society of India, stated that PCOS is a condition that can manifest itself in several ways rather than a disease. "While younger women may experience irregular periods, hirsutism (unwanted male-pattern hair growth), and obesity, it may cause infertility, increase the risk of miscarriage, and more in the somewhat older age group.PCOS can make it challenging to get pregnant, according to Dr. Shah, who also noted that there is a 40% chance that a mother with PCOS will have a female kid who also has the condition. Diabetes, high blood pressure, and other health issues are very likely to occur in women with PCOS.

Dr. Anita Soni, a gynaecologist at Powai's Hiranandani Hospital, stated that the hormonal imbalance is more pronounced the more peripheral fat a person has. She claimed that while a person's optimal Body Mass Index (BMI) is 25, when they are fat, their BMI rises to over 27 or 28, which is alarming, Dr. Soni emphasized the need of a diet with low carbohydrates, high protein, and healthy fats, stating that although PCOS is a lifelong health problem, it can be managed with good diet and an ideal body weight and daily exercise.

According to experts, most women overlook the typical signs of PCOS and only seek medical attention when they are having problems getting pregnant. Even though the condition's prevalence has been rising year, according to Dr. Anjali Talwalkar, who runs a clinic in Kurla, many women don't suspect PCOS when they experience symptoms. "They only blame it on lifestyle. Although PCOS does have negative impacts and should be treated, it does result from bad lifestyle choices. People are not concerned enough about it because it is not life-threatening, the speaker claimed.

According to doctors, PCOS is typical in obese women. Obesity affects about 80% of PCOS patients, according to Dr. Shah, who also suggested that urban Indian women may be more vulnerable. A recent pan India study of 27,411 samples carried out by Metropolis Healthcare in 2015 has reported an alarming 25.88 % positive cases in east India and 18.62 % in north India which has been largely attributed to lack of awareness and ignorance.[19]

A recent cross-sectional survey analysis undertaken by Joshi et al, on 778 adolescent and young girls in Mumbai reported 22.5 % positive cases by Rotterdam method and 10.7 % by Androgen Excess Society Criteria.[20] A very recent study of prevalence of PCOS in adolescent girls in Kashmir valley reports 46.4 % cases with probable PCOS conditions, showing the prevalence of PCOS in Kashmiri women highest in the world.[21]

Recent studies have suggested that complementary and alternative medicines (CAM) have the potential to alleviate symptoms and improve quality of life of women with PCOS. Consequently, the use of complementary and alternate medicine has increased in the past years ranging from about 26 % to 91 %.[22]

AYURVEDA IN PCOS

Many Ayurvedic formulations have been prescribed and are being reported to be effective in clinical practice of managing female reproductive disorders.[23] The management strategies of PCOS primarily aims at reducing testosterone, improving estrogen levels controlling tendencies of hyperlipidemia and type II diabetes. Several medicinal plants have been reported to have estrogenic, antiandrogenic, hypoglycemic and hypolipidemic properties.[24] Earlier studies have proved that plants like *Cyperus rotundus, Saraca indica, Symplocos racemosa* and *Terminalia arjuna*,[25] are effective estrogenic agents while *Aegle marmelos*,[26] *Berberis aristata* have been found to be efficient hypoglycemic agents.[27]

Despite the fact that several medicinal plants are prescribed for the treatment of PCOS, either as traditional formulations or proprietary herbal formulations, these have not been integrated into healthcare systems due to a lack of scientific studies on their rationalized use. There is also a scarcity of data on the effects of medicinal plants and other herbal formulations prescribed for PCOS on lipid regulation and sugar control in PCOS patients.

The majority of the plants mentioned above are included in many reported Ayurvedic formulations that are specifically designed and prescribed for the treatment of female reproductive disorders. Traditional formulations' components, which individually

demonstrate a consistent impact with minimum adverse effects, may show promise in treating PCOS by enhancing immunity and regulating menstrual cycle without fluctuating hormone levels. Ayurvedic formulas such as Pushpadhanwa Rasa, PathadiKwatha, Ashokarishta, and other herbs have been used to heal various ailments. Several gynaecological diseases have been documented to be caused by such compositions. Patients suffering from PCOS are prescribed by local Vaidyas. One promising example is Pushyanuga Churna is an Ayurvedic formulation.

PUSHYANUGA CHURNA'S ROLE IN PCOS MANAGEMENT

Ayurvedic texts and references prescribe Pushyanuga Churna for a wide range of female reproductive disorders such as menorrhagia and metrorrhagia, leucorrheoa, disorders of female genital tract and menstrual disorders which are also associated symptoms of PCOS. Clinical evaluation of Pushyanuga Churna on 46 patients with dysfunctional uterine bleeding showed significant positive effect after treatment for fifteen days. In India, due to the popular therapeutic use of Pushyanuga Churna in the female reproductive disorders, the formulation is being prepared and marketed by various manufacturers like Dabur, Patanjali , Dhoopapeshwar, Baidyanath, Arkashala and by local Vaidyas.[28] Moreover, many of the medicinal plants mentioned earlier in this chapter such as *Hemidesmus indicus, Salmalia malabarica, Glycyrrhiza glabra, Cyperus rotundus, Saraca indica, Symplocos racemosa, Terminalia arjuna, Aegle marmelos, Berberis aristata, Myrica esculenta, Mimosa pudica* and *Woodfordia fruticosa* are all ingredients of Pushyanuga Churna.[29] The medicinal plants thus collected get special properties imbibed in them during this nakshatra. These properties may lead to healthy conception and treatment, and would lead to the production of a formulation with maximum potency. Thus the current research work proposes the use of Pushyanuga Churna as a single treatment in the management of PCOS.[30]

EXPERIMENTAL

Materials and methods

Chemicals and reagents

Berberine standard were obtained from Yucca Enterprises, Mumbai, India. HPLC grade solvents were used in chromatographic separation of Beberine. Market formulations of Pushyanuga Churna was obtained by Patanjali, Baidyanath and Dhootpapeshwar respectively.

Instrument

Table no. 1 Instruments and equipments used for the analytical method development and validation

Name of Instrument	Use	Make/ Model
UV VISIBLE SPECTROPHOTOMETER	For selection of analytical wavelength	Shimadzu UV- 1800
		Software: UV probe
Electronic Weighing Balance	For weighing the components	Aczet (Sensitivity 0.0001 gm)
		Shimadzu (Sensitivity 0.001 gm)
Ultra Sonicator	For solubility purpose	Life Care Equipment 2.25L70H
Centrifuge	To centrifuge sample	BioEra LRE099A

Table no. 2 Instruments and equipments used for the analytical method development and validation by HPTLC method

SR.NO.	HPTLC SYSTEM	MODEL NUMBER	APPLICATION
1	CAMMAG Automatic TLC sampler 4	250243	Application of samples
2	CAMMAG TLC Visualizer	150503	Scanning at R white, 255, 366 nm
3	TLC Scanner 4	170422	Scanning at 366 nm
4	CAMMAG Twin-Trough Chamber	NA	Development of plate

Identification of drugs

Organoleptic properties Visual identification was carried out to inspect colour, nature of drugs obtained.

Solubility studies

Various solvents like Ethanol, methanol and water were tried to check the solubility of Berberine.

Selection of Analytical Wavelength by UV Analysis

Blank/Diluent:Methanol

Preparation of standard solution for UV analysis

10 mg of Berberine was weighed and transferred into two different 10 ml volumetric flask. Added 6 ml of diluent and sonicated well to dissolve. Volume was made up to the 10 ml using diluent and mixed well (1000 µg/ml). Further 1 ml was diluted from above stock to 100 ml volumetric flask using diluent (10µg/ml).

Selection of working wavelength

Baseline was taken by scanning at 800 to 200 nm range.

Standard solution of Berberine was scanned at 800 to 200 nm wavelength for determination of working wavelength by recording the spectra. After scanning, working wavelength was selected by overlaying both the UV Spectra.

HPTLC Method

Selection of diluent

The diluent was selected on the basis of its solubility studies performed on different solvents. So, depending on Solubility study, Mobile Phase composition Retention factor and reproducible peaks, diluent was selected.

Preparation of Standard solution

An accurately weighed quantity (10 mg) of berberine was dissolved in methanol and volume was made up to 10 ml with methanol in a volumetric flask. Stock solution of berberine was prepared by diluting 1 ml of this solution with methanol up to 100ml in volumetric flask to give 10 µg/ml concentration of berberine.

Preparation of sample solution

Weighed accurately 10 g of each marketed formulation churna. It was then transferred to 100 ml Diluent into round bottom flask. As it was separated and extracted through Soxhlet extraction method. The semi solid extract was concentrated through water bath. Allowed to cool at room temperature. Centrifuged it at 1000 rpm for 15 mins and collected the supernatant. The residue was dissolved in 10 ml of methanol and 1 ml of this stock solution was diluted upto 10 ml in a volumetric flask with methanol. The same procedure was performed for other market formulations of churna Mixed well to achieve final concentration of solutions to be 100 µg/ml. The Chromatograms were recorded and % Assay was calculated.

The amount of analytes present in the formulation were calculated by using the formula as given below,

Formula: % Assay = $ATAS \times WSDS \times DTWT \times P$ 100 × $AWLC \times 100$

Where, AT: Peak area response of test sample

AS: Peak area response of standard sample

WS: Weight of standard sample

DS: Dilution factor of standard sample

DT: Dilution factor of test sample

WT: Weight of test sample

AW: Average Weight LC: Label Claim P: Potency

Selection of Mobile Phase

Various trials were taken for optimization of mobile phase composition which will result in sharp, defined peaks.

Following Mobile phase compositions were tried:

Table No. 3 Mobile phase composition TLC trails for HPTLC method development

Sr no.	Mobile phase composition
1	n-Hexane: Ethyl acetate: Methanol: Formic Acid (8:2:1: 2-3 drops %v/v/v)
2	Chloroform: Methanol (8:2 %v/v)
3	n- Hexane: Ethyl Acetate: ethanol (7:2:1 %v/v/v)
4	n-Propanol :Methanol: Acetic acid (7:2:1 %v/v/v
5	Ethanol: Acetic Acid: Water (16:2:2%v/v/v)

The solvent system for separation was selected on the basis of good separation, Rf value etc. So, number of trails was performed to select a mobile phase.

Table No. 4 Optimized chromatographic condition for HPTLC Method

1	Mobile Composition	Ethanol: Acetic Acid: Water (16:2:2%v/v/v)
2	Saturation Time	20 min
3	Wavelength	366 nm (BER)
4	Diluent	Methanol
5	Stationary Phase	TLC Al Plates Silica Gel 60 F 254

Validation of HPTLC method

The developed analytical method was validated to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2 (R1).

System Suitability: System suitability test was carried out to verify that the analytical system is working properly to give accurate and precise results. Six replicates of standard solution were applied and calculate the % RSD, Rf values.

Specificity: Checked any interference from Blank and placebo at the main drug peaks. Specificity was ensured by the use of a reference standard and is demonstrated by the lack of Interference from other components present in the matrix. The specificity of the method was ascertained by analyzing standard drugs, sample, diluent and solvents used. The spot for

Berberine in the sample was confirmed by comparing the Rf and spectra of the spot with that of standards. The possibilities of interference in the analysis were studied.

Linearity: Linearity is performed by preparing a standard solution at six different concentration levels of the drugs. The average peak area is recorded and a calibration curve was constructed between concentrations versus peak area. Linearity was established by applying 1.0 -3.5 µl/band (1.0, 1.5, 2.0, 2.5, 3.0, 3.5) of standard solution of Berberine on the TLC plate from 100 µg/ml stock solution with the help of microliter syringe using an automatic sample applicator. The plates were developed, dried and scanned densitometrically at 366 nm. The drug peak-area was calculated for each concentration level and a graph was plotted of drug concentration against the peak area.

Accuracy: Accuracy of the developed method was confirmed by doing a recovery study as per ICH guidelines at three different concentration levels (80 %, 100 % and 120 %) i.e., by multiple level recovery studies by replicate analysis (n=3). The percent recovery was calculated at different levels.

Precision: System reproducibility was assessed by spotting 1.0 µl/band of Berberine solution six times on a TLC plate, followed by development of plate and recording the peak area for 6 spots. The method reproducibility was determined by analyzing standard solution in the concentration of 100 ng/band of Berberine for 3 times on the same day and interday precision was determined by analyzing corresponding standards for next day.

Robustness: Robustness was determined by introducing small changes in the different parameters such as mobile phase composition, mobile phase saturation time and their effects on the results were examined. The 100 ng/band for Berberine and the effects of changes were examined on the results of peak areas and Rf value. The duration of saturation (±10 mins) was investigated.

Limit of Detection and Limit of Quantitation: Limit of Detection is the lowest amount of analyte in a sample which can be detected but not quantitated, and Limit of Quantitation is the lowest amount of analyte in the sample that can be quantitatively determined and were calculated by using the formula;

LOD=3.3×σ/S, LOQ = 10 × σ/

Where,

σ = Standard deviation of the response S = Slope of the calibration curve.

RESULTS AND DISCUSSION

Identification of Drugs

Phytochemical analysis was performed through standard official procedure for the identification of different class of secondary metabolites present in different methanolic extract of marketed Pushyanuga churna samples i.e., Patanjali, Baidyanath and Dhootpapeshwar respectively.

Table No. 5 Phytochemical tests

Phytochemical constituents	Test	Methanolic Extract
Flavonoids	$(CH_3COO)_2Pb$	+
	Increasing amount of NaOH	
Alkaloids	Mayer's reagent	+
	Wagner's reagent	+
	Dragendroff's reagent	+
Tannins	5% $FeCl_3$	-
	$KMnO_4$	-
	$K_2Cr_2O_7$	-
Terpenoids	Chloroform + concentrated H_2SO_4	+
Saponins	Water with vigorous shaking	+
Resins	concentrated H_2SO_4	-
Glycosides	Water + NaOH	-

Solubility studies

Table No. 6 List of the solvent used solubility test for Berberine

Sr no.	Solvent	Solubility
1	Distilled water	Sparingly soluble
2	Propanol	insoluble
3	Butanol	Sparingly soluble
4	Ethanol	soluble
5	Methanol	soluble

Selection working of wavelength

Berberine showed absorbance at 366 nm. Hence working wavelength for further study was selected as 366 nm.

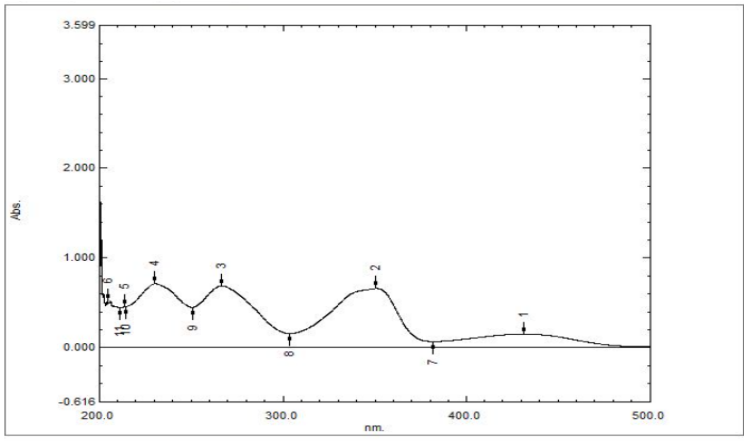

Fig no. 1 Beberine UV spectra

HPTLC METHOD

Selection of Suitable Solvent

The mobile phase in chromatography has to be polar and it also has to provide a reasonable competition for the adsorption sites for the analyte molecules. The Solvent was selected on the basis of its solubility studies performed on different solvents. So, depending on solubility study, various trials were taken with different proportion of solvents.

Selection of mobile phase

Different solvent systems were tried for the separation of Berberine in marketed formulation and Std Berberine on the TLC plates. The optimized mobile phase system was found to be Ethanol: Acetic Acid: Water (16:2:2%v/v/v). The Rf value found with this mobile phase composition was 0.47. for Berberine and respectively for all the samples. The resolution between spots of standard and depredates appeared better. So, the attempt has been taken to develop and validate a cost-effective simple and robust HPTLC technique to quantify thymoquinone and curcumin. The developed method was found to be quite selective.

Table No. 7 Mobile Phase composition trails for HPTLC method development

Sr no.	Mobile phase composition	Observation
1	n-Hexane: Ethyl acetate: Methanol: Formic Acid (8:2:1: 2-3 drops %v/v/v)	Tailing was observed
2	Chloroform: Methanol (8:2 %v/v)	Sample did not run far on the plate
3	n- Hexane: Ethyl Acetate: ethanol (7:2:1 %v/v/v)	Large broad bands were observed
4	n-Propanol :Methanol: Acetic acid (7:2:1 %v/v/v)	Minimal broad bands
5	Ethanol: Acetic Acid: Water (16:2:2%v/v/v)	Optimized bands including sample were also observed

Assay Method

Table No. 8 Data of Assay method

Parameters	SAMPLE 1	SAMPLE 2	SAMPLE 3
Standard area	10360	10360	10360
Sample peak area	9300	9530	7550
Label claim(mg)	3840	358	358
Standard weight(mg)	10	10	10
Average weight of sample(mg)	10000	10000	10000
Mean % Assay	102.7	98.3	88.9

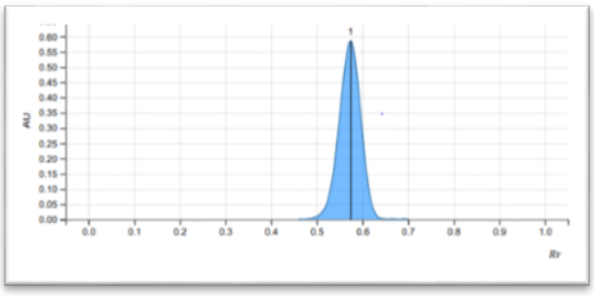

Fig no. 2 Chromatogram of Standard for Assay

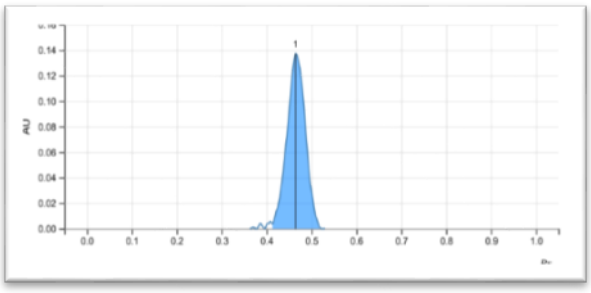

Fig no.3 Chromatogram of Sample1 for Assay

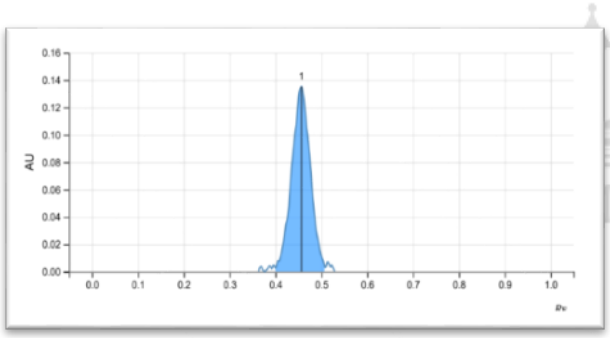

Fig no.4 Chromatogram of Sample1 for Assay

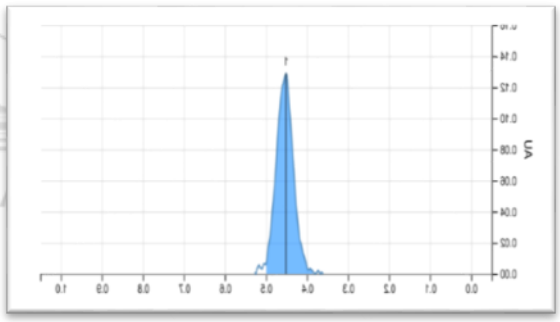

Fig no.5 Chromatogram of Sample1 for Assay

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Observation: The amount of Berberine present in analyzed dosage form was found to 102.7%, 98.3% and 88.9 % respectively.

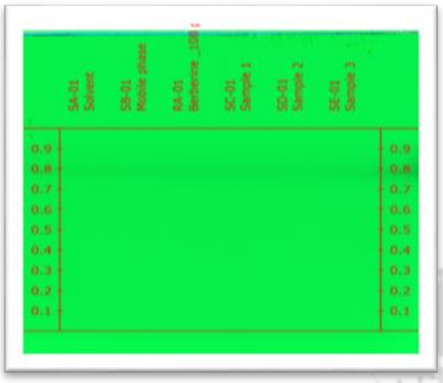
Validation Parameters

1. Specificity

No interference was observed from the blank, solvents with that of the standard and sample.

Table No. 9 Specificity study for HPTLC Method validation

Track no.	Band spotted
1	Solvents
2	Mobile Phase Composition
3	Berberine Standard
4	SAMPLE 1
5	SAMPLE 2
6	SAMPLE 3



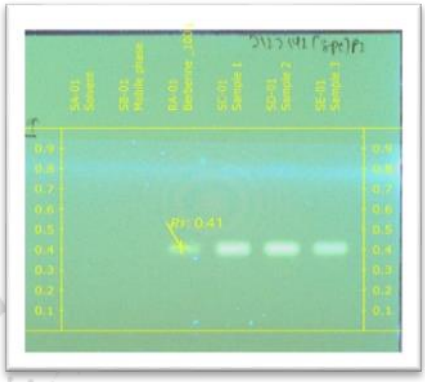


Fig no. 6 Specificity at 254 nm **Fig no. 7 Specificity at 366 nm**

Linearity

The correlation co-efficient for linear curve obtained between concentrations vs. area for standard preparations of Berberine was found to be 0.999. The concentration of the drug was linear in the range examined.

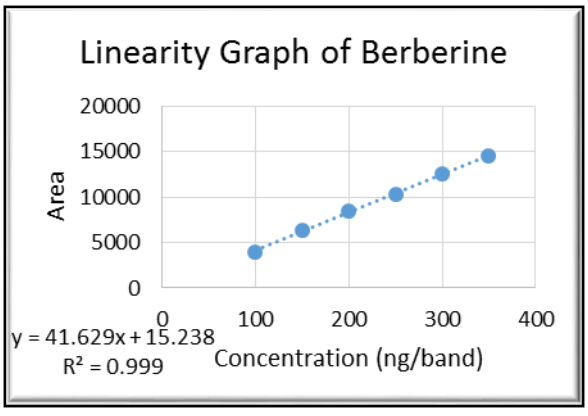


Fig no. 8 Linearity of Berberine

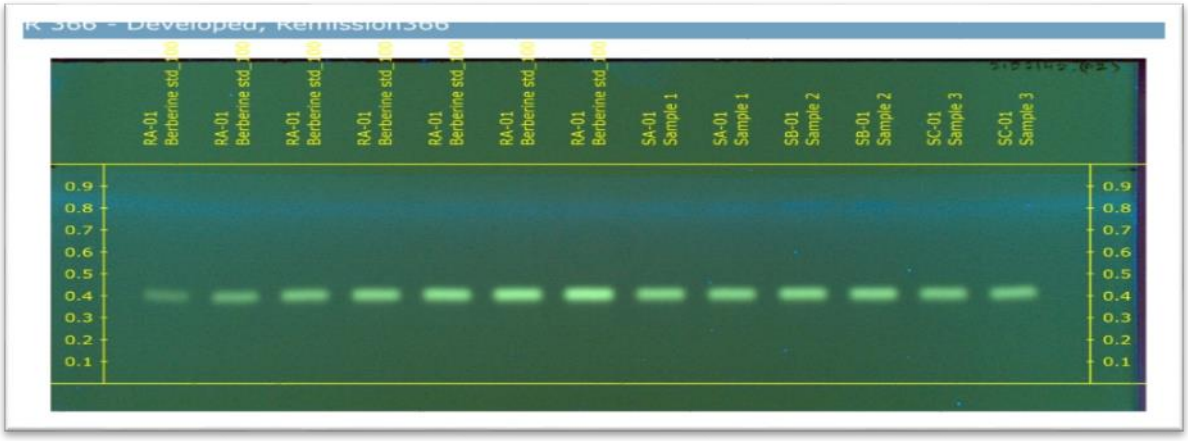


Fig no. 9 Developed TLC Plate images captured in TLC Visualizer at 366 nm for Linearity study

Table No. 10 Linearity curve for Berberine

Track no.	Concentration (ng/band)	Peak Area Response
1	100	4010
2	150	6400
3	200	8480
4	250	10360
5	300	12490
6	350	14550

Observation: The correlation co-efficient for linear curve obtained between concentrations vs. area for standard preparations of Berberine was found to be 0.999. The concentrations of drug was linear in the range examined since all the points lie in a straight line.

Recovery (Accuracy)

The recovery studies were carried out three times, chromatogram was recorded and % Recovery and Mean % Recovery was calculated.

Table No. 11 Recovery Data

Level %	Amount of std added (µl/band)	Amount of sample added (µl/band)	Final concentration added (µl/band)
80	0.4	0.5	0.9
100	0.5	0.5	1
120	0.6	0.5	1.1

Table No. 12 Recovery Data for Sample 1

Level %	Expected Area Response	Recovered Area response	Avg % Recovery
80	9172	9034	98.49
100	8911	9763	109.55
120	9612	9612	99.99

Table No. 13 Recovery Data for Sample 2

Level %	Expected Area Response	Recovered Area response	Avg % Recovery
80	6411	6368	99.32
100	6712	6862	102.22
120	7189	6716	93.4

Table No. 14 Recovery Data for Sample 3

Level %	Expected Area Response	Recovered Area response	Avg % Recovery
80	9066	8204	90.48
100	10134	9067	89.46
120	11020	9587	86.99

Observation: %Recovery for Sample 1, Sample 2 and Sample 3 was found to be 98.49% (80), 109.55 (100), 99.99 (120), 99.32 (80), 102.22 (100), 93.4 (120) and 90.48 (80), 89.46 (100), 86.99 (120) respectively.

Reproducibility

Table No. 15 Precision Data for Berberine

Sr no.	Area	Area	Area
	1	2	3
1	6350	6320	6490
2	6450	6580	6290
3	6660	6610	6370
4	6590	6470	6470
5	6600	6520	6580
6	6630	6630	6600
Average	6546.667	6521.667	6466.667
Std Dev	120.4436	115.1376	119.7776
%RSD	1.83977	1.765463	1.85223

Observation:

Mean %RSD for Berberine was found to be 1.81 %.

Robustness

Robustness was determined by introducing small changes in the different parameters such as saturation time, mobile phase saturation way and their effects on the results were examined. % RSD for all the parameters examined was found to be less than 2%.

Table No. 16 Robustness Data

	1	2	3	Mean	SD	%RSD
AREA	5350	5400	5340	5363.333	32.1455	0.599357
AREA	5360	5320	5510	5396.667	100.1665	1.856081
AREA	5470	5450	5560	5493.333	58.59465	1.06665
AREA	5480	5430	5500	5470	36.05551	0.65915

Acceptance Criteria:

%RSD should not be more than 2%

LOD And LOD

Th e LOD and LOQ are observed as follows below:

LOD (ng	14.09
LOD (ng	42.7

CONCLUSION

This method development was aimed at developing a precise, specific and accurate HPTLC method for estimation of Berberine was developed to be used for routine quality monitoring and compared with marketed formulations. As this drug is very effective and is used in Ayurvedic formulation which is commonly prescribed for the clinical treatment of female reproductive disorders. The developed method was simple, linear, accurate, precise and robust for quantitative analysis. In order to achieve phenomenal retention factor, peak asymmetry; mobile phase selected was of composition Ethanol: Acetic Acid: Water (16:2:2%v/v/v). The retention factor for Berberine was found to be 0.47 at 366 nm wavelength. The correlation coefficient of regression was found to be 0.999 for berberine, equivalent to the range of 100- 350 ng/band which states that the method was good linear to the concentration versus peak area replications. The comparison of chromatograms of blank, standard and sample, there was no interference observed. It shows that the method is specific. The reproducibility studies were performed and the % RSD of the determinations was found to be 1.81% which is within the limit which indicate that the proposed method was found to be precise. %Recovery for Sample 1, Sample 2 and Sample 3 was found to be 98.49% (80), 109.55 (100), 99.99 (120), 99.32 (80), 102.22 (100), 93.4 (120) and 90.48 (80), 89.46 (100), 86.99 (120) respectively. For recovery at 80 %, 100 % and 120 % levels were all within the constraints which denote that the proposed method was found to be precise. %RSD was within 2% even after changes in saturation time (±10 mins) and saturation method (with and without filter paper). The method was found to be robust even though on remote deliberate variation in the method conditions did have a minute effect on the peak asymmetry, retention factor and all are within the constraints which indicate that the method is robust. HPTLC

were validated for system suitability, specificity, linearity, precision, accuracy (Recovery) and robustness was found to be within the specified limit. The proposed method can be successfully used for the routine analysis.

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