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Optimization of Extraction Conditions and Development of a Sensitive HPTLC Method for Estimation of Wedelolactone in different extracts of *Eclipta alba*

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ABSTRACT
Eclipta alba has been used in Ayurvedic medicine for treatment of various ailments. An attempt has been made to optimize different extraction conditions for *Eclipta alba*. Further an accurate, precise, reproducible and sensitive accurate, precise and reproducible (HPTLC) method has been developed for the estimation of Wedelolactone in *Eclipta alba*. An attempt has been made to quantify wedelolactone in various extracts by HPTLC method. Precoated aluminum silica gel F plates were used as stationary phase and toluene: ethyl acetate (9:1) was used as mobile phase. This system was found to give compact spots for wedelolactone (RF value of 0.30 ± 0. 3) with a linearity range of 1 to 80 µg per spot. The proposed method was applied to evaluate efficiency of different methods of extraction i.e. percolation, maceration, hot solvent extraction (Soxhlet apparatus), supercritical fluid extraction, microwave, orbital shaker bath and sonication method for extraction of wedelolactone from *Eclipta alba*.

Keywords: Column chromatography, *Eclipta alba,* HPTLC, Phytochemical, Wedelolactone.

INTRODUCTION

The whole plant of Bhiringraj (*Eclipta alba* Hassk) belongs to the *Asteraceae* family and is widely cultivated throughout tropical and subtropical regions of the world, mainly in India and China. This is highly valued in traditional Indian medicine and is one of the most extensively used plants in Ayurvedic Encyclopedia. [1-2] Whole plant contains wedelolactone, demethylwedelolactone which are coumanstat derivatives, alkaloids and thiophene derivatives. Wedelolactone is the active principle of *Eclipta alba* and exhibits hepatoprotective, antiplasmodial activity, sedative, muscle-relaxant, anxiolytic, nootropic and anti-stress activities. [3-5]

Literature survey reveals that various chromatographic methods are available for quantitative estimation of wedelolactone. Moreover further literature search revealed that there are very few HPTLC [6-7] and HPLC [8-10] methods available for analysis of wedelolactone. Taking this objective in consideration an attempt has been made to develop a simple and precise HPTLC method for estimation of wedelolactone.

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In the present paper, an accurate HPTLC method for quantitative determination of wedelolactone in herbal extract was developed and validated. Also various extraction techniques have been tried and optimized to check the efficacy of extraction by various methods.

EXPERIMENTAL

Chemicals and Reagents

HPLC grades Methanol, Toluene and Ethyl Acetate were purchased from Merck (S. D. fine chemicals, Mumbai). The TLC aluminum sheets precoated with silica gel 60 F_{254} (20 × 20 cm; layer thickness, 0.2 mm) were purchased from Merck (Darmstadt, Germany) and used as stationary phase. CO_2 (99.99%) and N_2 (99.99%) were purchased from Alcheme gases Mumbai for SFE.

Plant material

The whole plant of *Eclipta alba* (Bhringaraaja, Bhringa, Bhangraa) was procured from the local market, Mumbai, India and authenticated according to Ayurvedic Pharmacopoeia. [1]

Equipment

The Liquid chromatographic system consisted of following components: Camag Linomat V automatic spotting device, Camag glass twin- trough chamber (10 × 10 cm) and Camag TLC Scanner 3. Chromatographic analysis was performed using Camag Wincats 1.2.2 software (Camag Sonnenmattstr., Muttenz, Switzerland). A 100µl HPTLC Syringe (Hamilton

Company, Reno, NV, USA), was used for chromatographic studies. The supercritical fluid extraction was performed on the instrument SFT-XW-100, supercritical fluid technology.

Sample Extraction

The plant of *Eclipta alba* was subjected to various methods of extraction. Extraction and comparison of extractive methods was performed with respect to wedelolactone content. [11]

Maceration followed by Percolation

The whole plant of *Eclipta alba* was air dried and ground to obtain coarse powder. About 100 g of whole plant was extracted using 500 ml methanol, by maceration for 24 h. The powder was then kept for percolation until the percolate was almost colorless. The methanolic extracts were combined and evaporated to dryness using rotary vacuum evaporator at 40°C under inert atmosphere to obtain dark green colored sticky mass. The extract obtained by this method was labeled as E1.

Supercritical fluid extraction

The coarse powder of whole plant of *Eclipta alba* was extracted by supercritical fluid extraction method using different pressure and same temperature conditions. The extract obtained by this method was labeled as E2. The details of temperature and pressure are given in table T1. Each time 100 gm of sample was taken for extraction and the CO_2 flow rate was 23.98 ml/min. The optimized conditions for Supercritical Fluid Extraction are given in Table 1.

Soxhlet extraction

The coarse powder of whole plant of *Eclipta alba* was extracted with methanol in herb: menstrum ratio of 1:6 by hot solvent extraction by heating with it for 12 hr. The methanolic extract was concentrated under reduced pressure to obtain a dark green coloured sticky mass. The extract obtained by this method was labeled as E3.

Microwave assisted extraction

The coarse powder of whole plant of *Eclipta alba* was extracted with methanol in herb: menstrum ratio of 1:5 in microwave for 15 min at voltage of 100 W. The methanolic extract was concentrated under reduced pressure to obtain dark green colored sticky mass. The extract obtained by this method was labeled as E4.

Ultra sonication method

The coarse powder of whole plant of *Eclipta alba* was extracted with methanol in herb: menstrum ratio of 1:5 by ultra-sonicator method for 45 min. The methanolic extract was concentrated under reduced pressure to obtain a dark green coloured sticky mass. The extract obtained by this method was labeled as E5.

Orbital shaker bath

The coarse powder of whole plant of *Eclipta alba* was extracted with methanol in herb: menstrum ratio of 1:5 by keeping this flask in shaker bath for 3 h. The methanolic extract was concentrated under reduced pressure to obtain a dark green coloured sticky mass. The extract obtained by this method was labeled as E6.

Isolation of wedelolactone standard

The methanolic extract obtained by maceration followed by percolation method was subjected to column chromatography for isolation of wedelolactone. The concentrated dry material (3 g) was impregnated on 30 g of silica gel, loaded onto a column of silica gel [60–120 mesh] (100 g), and eluted with toluene. Isolated compound was further purified with the help of preparative HPTLC. The extract thus obtained was further characterized with the help of spectroscopic techniques to confirm the presence of wedelolactone.

Characterization of isolated wedelolactone

The UV spectrum of isolated spectra shows absorption λ_{max} at 351 nm. The peak at 351 nm confirms the presence of wedelolactone. The IR spectrum of isolated compound showed peak at 3458, 1639, 1055, 1010 cm^{-1}. The peaks at 1639 and 3458 confirm the presence of lactone ring and O-H stretching. The wedelolactone thus obtained and characterize was considered as standard S1 for further research work.

Preparation of standard and sample solution

Stock solution of wedelolactone (1 mg/ml) was prepared in methanol with the standard S1. The stock solution was applied in different volumes (5-80µl) to obtained final concentration of 5-80µl per spot, which was analyzed by developed and validated HPTLC method. The sample solutions of E1 to E6 (1 mg/ml) were prepared in methanol for further analysis.

Instrumentation and Optimized Chromatographic Conditions

Chromatography was performed on a pre-activated silica gel HPTLC plate (60 F 254, 20 × 10 cm). Samples and standard were applied on the plate as 6mm wide bands with an Automated Camag TLC applicator, Linomat 5 (Camag, Multenz, Switzerland) with N_2 flow at 150 nl/sec, positioned 15 mm from the bottom of the plate and 20 mm from side of the plate. The mobile phase of the system was toluene: ethyl acetate (9:1 v/v). The volume of mobile phase was 10 ml with a chamber saturation of 25 min. The temperature and relative humidity were 25 ± 1^oC and 30-45% respectively. After development, the plate was removed and dried and spots were visualized in Camag UV chamber (366 nm) and wedelolactone were quantified with a Camag TLC scanner model 3 equipped with WINCATS software 1:2:2 under the following conditions [Slit width – 5 × 0.45 mm, Wavelength – 366 nm, absorption/reflection detection mode. The HPTLC analysis of wedelolactone showed single peak at RF 0.30 ± 0.3 respectively Fig. 1. The application parameters were identical for all the analysis performed.

Validation of the method

The analytical method was validated for linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), specificity, robustness, and ruggedness, in accordance with ICH guidelines. Further calculations were performed. [12]

Limits of Detection (LOD) and Quantification (LOQ)

The limits of detection and quantitation were calculated by the method based on the standard deviation (σ) of responses for triplicate blank injections and the slope (S) of the calibration plot, using the formulae

$$LOD = 3.3\sigma/S \text{ and } LOQ = 10\sigma/S.$$

Linearity

Linearity was studied by preparing standard solutions at different concentrations from 1 to 80 µg/ml, plotting a graph of concentration against peak area, and determining the linearity by least-squares regression.

Accuracy as Recovery

Accuracy was evaluated in triplicate, at three different concentrations equivalent to 50, 100, and 150 % of the wedelolactone, by adding a known amount of wedelolactone standard to a sample of known concentration and calculating the recovery of wedelolactone, RSD (%), and standard error (SE) for each concentration.

Precision

Precision was studied by measuring intra-day (repeatability) and inter-day (by injection of samples over three consecutive days).

Robustness and Ruggedness

The robustness of the method was investigated by making small deliberate changes in the chromatographic conditions at three different levels. The ruggedness of the method was assessed by comparison of intra-day and inter-day results for assay of wedelolactone performed by two analysts in the same laboratory.

Repeatability of measurements of peak

15µl of standard wedelolactone (1 mg/ml) was spotted on a TLC plate developed, dried and each spot was scanned seven times without changing the plate position and %CV for measurement of peak area was estimated.

Repeatability of sample application

15µl of standard wedelolactone was applied six times on a TLC plate by automatic spotting device. The plate was developed and analyzed as described above and % CV for peak area for different peaks was estimated.

Peak purity

The purity of isolated wedelolactone was confirmed by HPTLC analysis and melting point determination. The isolated wedelolactone showed single spots on HPTLC plate and gave a single peak on scanning at λ_{max} 366 nm.

Table 1: Optimized Condition for Supercritical Fluid Extraction

S. No	Pressure (psi)	Temperature (°C)	Crude yield (mg)
1	4000	40	89
2	5000	40	109
3	6000	40	148
4	4000	50	98
5	5000	50	127
6	6000	50	178

Table 2: Precision and Accuracy

	Interday			Intraday		
Concentration Applied ng/ml	Mean ± SD	%CV	Accuracy	Mean ± SD	%CV	Accuracy
4000 ng	8499.27 ± 238.22	2.80	100.24	8400.85 ± 135.91	1.61	100.43
6000 ng	11133.7± 84.83	0.76	100.99	11066.7 ± 112.30	1.01	101.59
8000 ng	12675.8 ± 73.52	0.58	101.01	12686.8 ± 54.54	0.42	100.31

Table 3: Percentage of Wedelolactone in *Eclipta alba* by Different Method of Extraction

S. No	Method of extraction		% of Wedelolactone
1	Maceration fallowed by percolation		0.38 %
2	E1		
	Temperature (°C)	Pressure (psi)	
2 a	40	4000	0.002%
2b	40	5000	0.008%
2c	40	6000	0.007%
2d	50	4000	0.004 %
2e	50	5000	0.010%
2f	50	6000	0.013%
3	E2		0.48%
4	E3		0.27%
5	E4		0.36%
6	E5		0.33%

RESULTS AND DISCUSSION

HPTLC method development

Comparison of TLC methods for the separation of wedelolactone

Different compositions of mobile phase for HPTLC analysis of wedelolactone were tested in order to obtain high resolution, symmetrical and reproducible peaks. The desired resolution of compounds was achieved using toluene and ethyl acetate (9:1) as the mobile phase. On this system separation between wedelolactone and other compounds is larger and the spots are well defined. Spot of this compound showed florescence when observed under UV light. The scanning wavelength of 366 nm was found to be optimal for highest sensitivity of wedelolactone spot. Peaks corresponding to wedelolactone, checked via addition of standard, were well resolved for identification. It was found that pre-washing of TLC plates with methanol (followed by drying and activation) and pre-saturation of TLC chamber with mobile phase for 25 min ensure good reproducibility and peak shape of wedelolactone.

There were no interfering peaks observed at the RF of the wedelolactone, thus ensuring the specificity of the developed method (Fig. 2).

Validation

Using the optimized extraction method and chromatographic conditions, the HPTLC method developed was validated in terms of linearity, limit of detection, limit of quantitation, precision, accuracy and specificity.

Linearity

The peak area of standard wedelolactone was found to be linear in the range of 5-80µl/ spot (i.e. 5-80µg/ml) with correlation coefficient of 0.999. The average linear regressed equation for the corresponding curve was Y= 2375.8 x-1257.1. The chromatograms for standard concentrations are shown in Fig. 3.

Limit of detection and limit of quantitation

The minimum detectable quantity was found to be 40 ng, while the limit of quantitation was 100 ng/spot for standard wedelolactone.

Precision

Repeatability of sample application three times and repeatability of measurement of the same spot showed very low RSD (% CV) which, in turn ensured reproducibility performance of the instruments, the intraday variation for determination of wedelolactone in extract was in the range of 0.42-1.61, while inter day variation was from 0.58- 2.80.

Accuracy

The percentage accuracy for analysis of wedelolactone in extract, determined using the standard addition method, was found to be between 100.24 - 101.59. The results for precision and accuracy are shown in Table 2.

Table 4: Results for Robustness

Condition	Modification	Mean	SD
Distance travels	70 mm	0.29	0.01
	80 mm	0.30	0.01
	90 mm	0.30	0.01
Mobile phase composition	8.9:1.1	5029.05	220.12
	9:1	5066.17	237
	9.1:0.9	5050.35	214.48

Peak purity

Peak purity test of wedelolactone was done by comparing peak of wedelolactone in standard and sample tracks. Peak purity results (obtained by scanning at 366 nm) were satisfactory. Correlations of the peak start spectrum with the peak centre spectrum were 0.998 for standard and sample

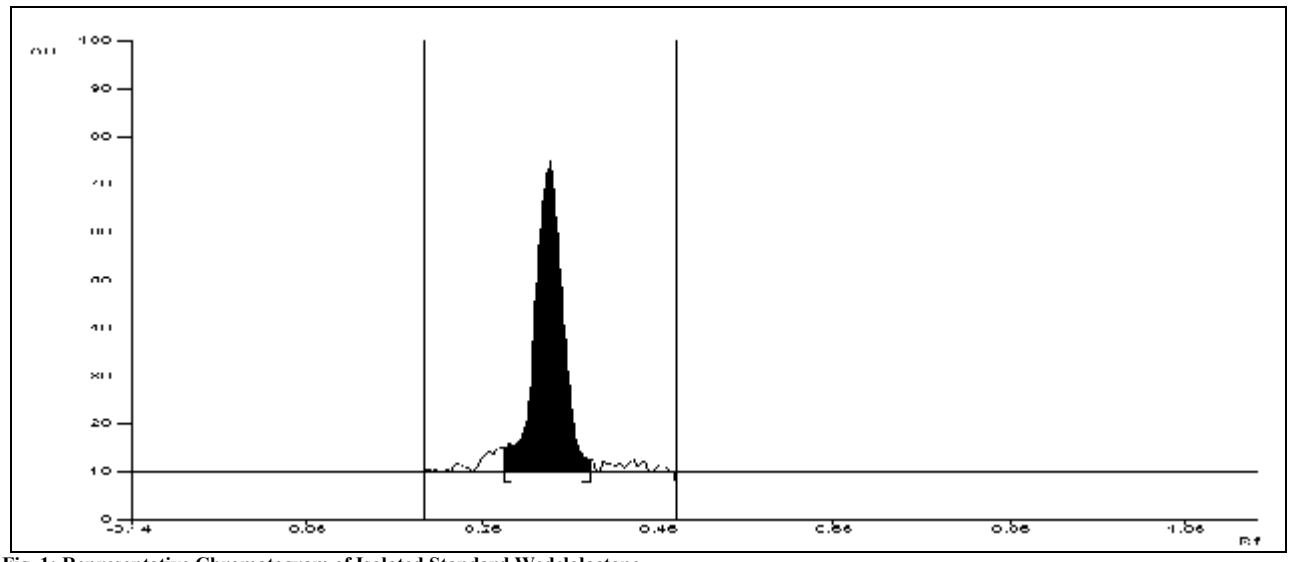


Fig. 1: Representative Chromatogram of Isolated Standard Wedelolactone

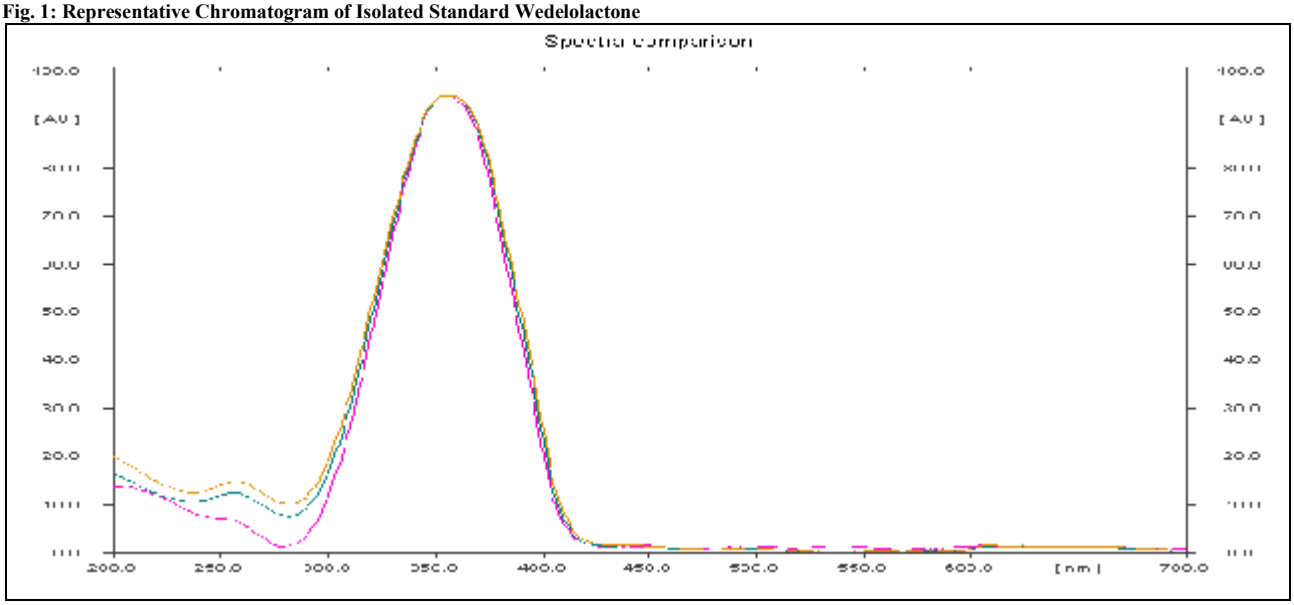


Fig. 2: Spectra for Specificity

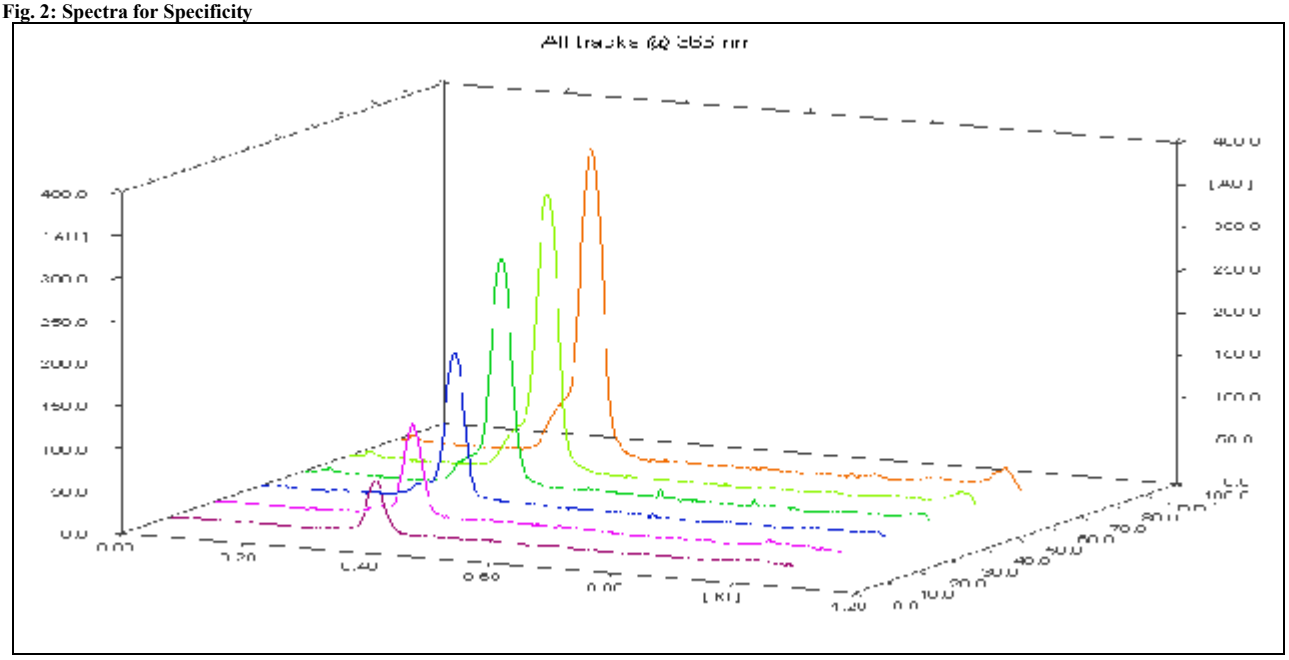


Fig. 3: Chromatogram of Standard at Different Concentration

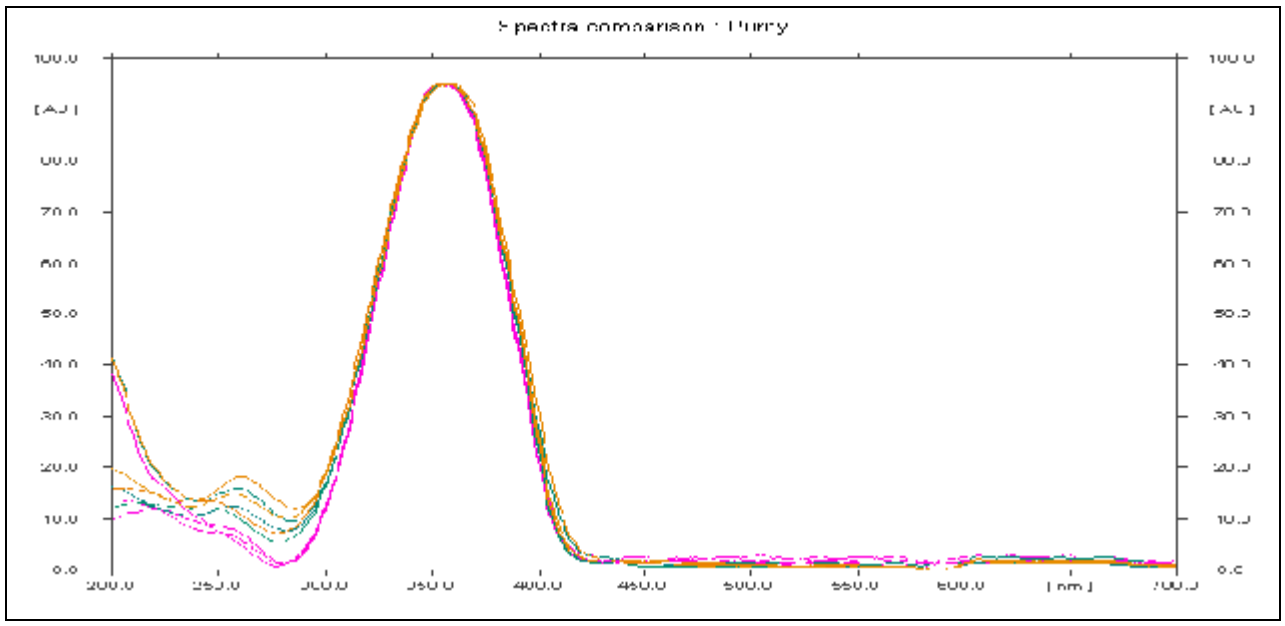


Fig. 4: Spectra for Peak Purity

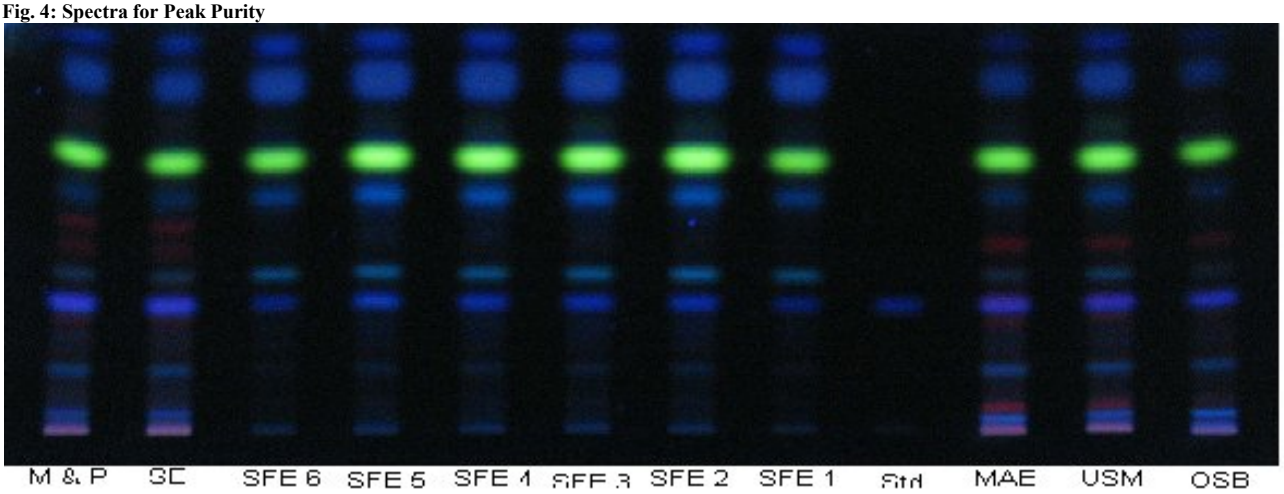
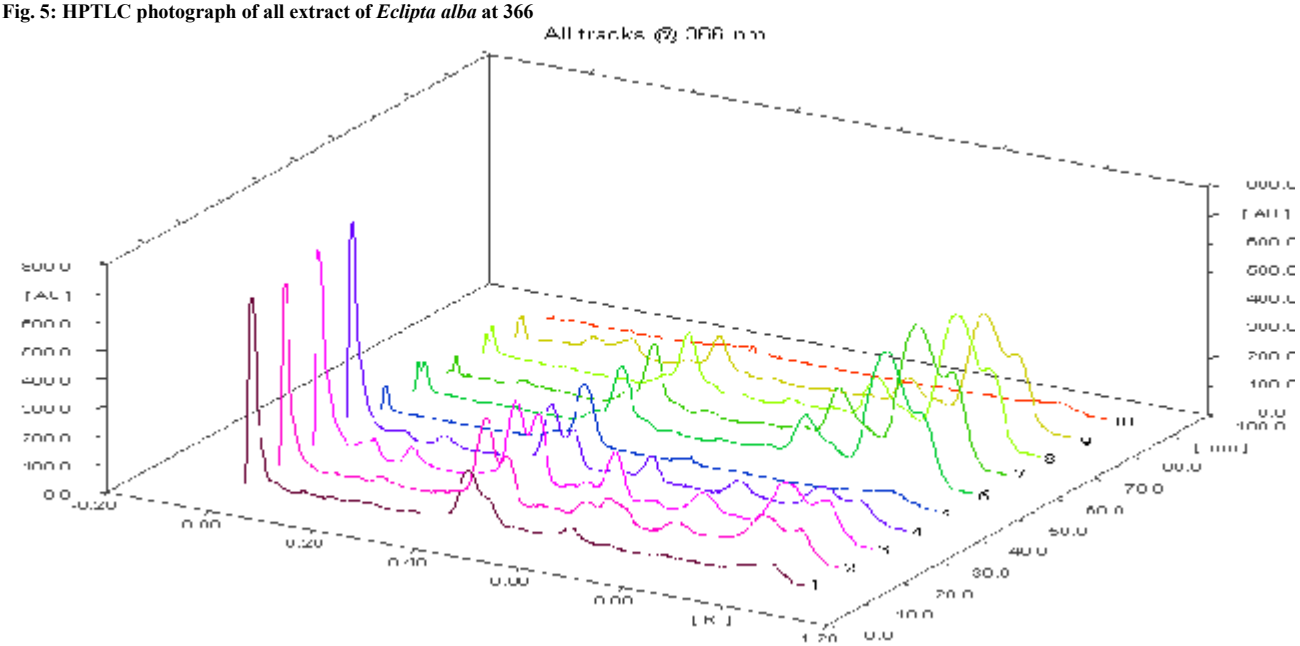


Fig. 5: HPTLC photograph of all extract of *Eclipta alba* at 366



1: Maceration; 2: Soxhlet apparatus; 3: orbital shaker; 4: ultra sonication; 5: Standard wedelolactone; 6-10 (supercritical fluid extraction)
Fig. 6: HPTLC 3D picture of all extract of *Eclipta alba* at 366nm

tracks. Correlation of the peak centre spectrum with the peak end spectrum was 0.999 respectively. Fig. 4 represents the purity of wedelolactone by the validated method.

Percentage of wedelolactone in the extracts of *Eclipta alba* obtained by different method

Estimation of wedelolactone, extracted from *Eclipta alba* by six different methods of extraction using HPTLC was found to be 0.38% w/w for Maceration fallowed by Percolation, about 0.002%-0.013% w/w for Supercritical fluid extraction at different temperature (40-50°C) and pressure (4000-6000 psi), 0.27% w/w for Microwave assisted extraction, 0.36% w/w for Ultra sonication method, 0.33% w/w for Orbital shaker bath. The highest percentage of wedelolactone was found in extract prepared by Soxhlet extraction (0.48% w/w) and lowest in case of SFE. This can be attributed to polar nature of the wedelolactone. The comparative separation and resolution of wedelolactone from all extracts of *Eclipta alba* at 366 nm can be observed in HPTLC photograph shown in Fig. 5. Also an HPTLC 3D picture of all extracts of *Eclipta alba* at 366nm are shown in Fig. 6. The percentage amounts of wedelolactone obtained from *Eclipta alba* by six different method of extraction *Eclipta alba,* estimated by HPTLC are given in Table 3.

Ruggedness and Robustness

The ruggedness and robustness studies confirm the method to be suitable for industrial application to a less than 2% low RSD value. The robustness data is shown in Table 4.

The HPTLC method proposed for determinations of wedelolactone in the herbal extract is accurate, precise, rapid, and selective. It can, therefore, be easily and conveniently adopted for routine quality control (QC) analysis. The proposed method is also useful in determining bhirngraj in polyherbal formulations. The method has a great industrial applicability.

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