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HPTLC Analysis of Neem Oil Extract in Herbal Dosage Form

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The aim of present study is to develop a simple, specific, accurate and reproducible TLC method for the quantitative estimation of neem oil extract in capsule/tablet dosage form. Chromatographic analysis was performed using Camag HPTLC system, in marketed herbal formulation of neem oil extract on precoated silica gel aluminium TLC plate $60F_{254}$ with a solvent system of chloroform:ethyl acetate containing 1 % acetic acid 8:2 (v/v) and detection was done by densitometric analysis at a wavelength of 265 nm without any derivatization. Samples of tablet and capsule formulation revealed peaks at the R_f 0.33 ± 0.02, 0.55 ± 0.02 (substance 1 and substance 2). The amount of substance in the sample was determined by using standard calibration curve which was found good linear in the range 4 µg to 100 µg with r^2 0.9936 and 0.9931 (with respect to peak area) and r^2 0.9931 and 0.9916 (with respect to peak height) of substance 1 and substance 2, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were 1 µg and 4 µg, respectively for both substances.

Key Words: Neem oil extract, HPTLC, Quantification, Herbal analysis.

INTRODUCTION

Traditional systems of medicine like Ayurveda, Unani and Siddha mainly use herbal formulations for the treatment of various ailments. These formulations require quantification of its constituents to ensure the quality of herbal drugs which should be maintained in each preparation batch to batch. The finger printing technique may be useful to the formulations for routine quality control test. In this study, highly sophisticated HPTLC[1-4] method is used to quantify the amount of neem oil extract. Neem oil extract is a bitter solid alcoholic extract obtained from the oil of seeds of *Azadirachta indica* (Neem) (Meliaceae), possesses anti gastric ulcer[5-8], anti-arthritic[9] and antiinflammatory[10] activities. The major problem associated with the herbal formulations and dietary supplements is its quality control. Hence, in the present communication, we proposed an approach for standardization and quality control of those drug formulations in which the marker compounds yet have not been identified. The present investigation deals with the development of TLC finger print profile of neem oil extract which indicates the presence of different constituents as a separate spot in chromatogram and as a separate peak in densitometric analysis. Each peak can be considered as a separate entity (compound) and can be used for standardization purpose. In the present analysis, two major peaks of the neem oil extract has been considered as standards out of ten peaks and used for quantification/assay of neem oil extract in formulations. The method was found to be accurate, simple, specific and reproducible[11,12]. The method proposed may be useful for routine quality control of formulations, *in vitro* drug release, content uniformity and stability testing.

EXPERIMENTAL

Neem oil extract obtained from The Central Council for Research in Ayurveda & Siddha (New Delhi, India). All other chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals (Mumbai, India). Aluminium TLC plates precoated with 0.2 mm layers of silica gel 60 F_{254} (20 cm × 10 cm) were purchased from E. Merck (Darmstadt, Germany).

Sample preparations: Dried powdered alcoholic extract of neem oil was dissolved in methanol to get a concentration of 2 mg/mL and used as standard neem oil extract solution for standardization and quality control of neem oil extract in formulations.

HPTLC instrumentation and general condition: The samples were spotted in the form of band width 6 mm with a Camag microliter syringe on precoated silica gel aluminium plate $60F_{254}$ (20 cm × 10 cm with 0.2 mm thickness, E. Merk, Germany) using Camag Linomat V (Switzerland) sample

applicator. A constant application rate of 120 nL/s was employed and space between two bands was 6.6 mm. Linear ascending development was carried out in twin through glass chamber, saturated with mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature. The length of chromatogram run was 80 cm subsequent to the development TLC plates were dried in a current of air with the help of an air drier. Scanning was performed using Camag TLC scanner III in the absorbance mode. The source of radiation utilized was deuterium and tungsten lamp. The slit dimension was kept 6X0.45 mm Micro and 20 mm/s scanning speed was employed.

Finger printing of neem oil extract: 2 µL of neem oil extract (20 mg/mL) was applied in duplicate on TLC plate (20 cm × 10 cm) for finger printing. It was developed in the solvent system chloroform: ethyl acetate containing 1 % acetic acid 8.0:2.0 (v/v) after a proper selection and optimization. The developed chromatogram was then scanned in multi wavelength scanner in the range of 250-365 nm at an interval of 5 nm, to find the best suitable wavelength (the wavelength of scanning showing maximum number of spots with maximum area) for finger printing of neem oil extract. Further, it was scanned on the best suitable wavelength (265 nm) in single wavelength mode and the data obtained was analyzed by using Wincats software. The two major peaks in densitometric analysis showing better linearity over the concentration range in the chromatogram were selected as standard 1 and standard 2. The peaks of standards representing the respective concentrations of neem oil extract.

Preparation of calibration curves: Different volumes of neem oil extract solution (2 mg/mL), 1.0, 2.0, 4.0, 5.0, 10.0, 20.0, 40.0 and 50.0 µL were spotted in triplicate on TLC plate to obtain concentration of 2.0, 4.0, 8.0, 10.0, 20.0, 40.0, 80.0, 100.0 µg per spot. The data of peak area of substance 1 and substance 2 *vs.* drug concentration were treated by linear least-square regression by Wincats software and equation obtained was used for quantification of neem oil extract in formulations.

RESULTS AND DISCUSSION

Optimization of mobile phase: Chloroform: ethyl acetate mixture in different proportion was investigated for their suitability as mobile phase. A ratio of 8:2 (v/v) resulted in good resolution of neem oil extract constituents but with broadening. Eventually, chloroform:ethyl acetate containing 1 % acetic acid (8:2 v/v) was found to give a sharp well resolved and well defined peaks in neem oil extract.

Finger printing of neem oil extract: The chromatograms scanned at different wavelength showed that the peaks were sharp and well defined with maximum area at wavelength 265 nm; hence it was selected for fingerprinting analysis. The fingerprinting analysis showed 10 distinct peaks at different R_f values *i.e.* at 0.13, 0.19, 0.26, 0.33, 0.38, 0.48, 0.55, 0.63, 0.69 and at 0.78, which were tested for linearity (Table-1).

Calibration: Ten distinct peaks at different R_f values were obtained but two peaks (Fig. 1) at R_f 0.33 and 0.55 (substance 1 and substance 2) showed good linearity by increasing concentration 4 µg to100 µg. The correlation coefficient r^2 with respect to peak area and peak height were found 0.9936, 0.9931 for substance 1 and 0.9931, 0.9916 for substance 2, respectively (Table-3). The two peaks thus obtained with different concentration of neem oil extract were calibrated for its linearity which was used for quantification of neem oil extract in different formulations. The results of concentration and respective area and height as obtained in densitometric analysis are shown in Table-2.

TABLE-1
LINEARITY OF ALL SUBSTANCE OBTAINED FROM THE CHROMATOGRAM

R_f of peaks	Linearity
0.13	Non-linear
0.19	Non-linear
0.26	Non-linear
0.33*	Linear
0.38	Non-linear
0.48	Non-linear
0.55**	Linear
0.63	Non-linear
0.69	Non-linear
0.78	Non-linear

*Substance 1, **Substance 2.

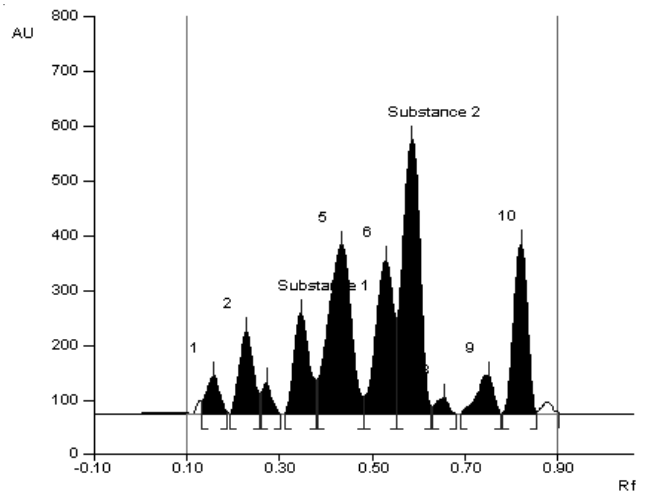

Fig. 1. HPTLC finger printing of neem oil extract (4 µg) at 265 nm showing peaks of all 10 spots

Accuracy as recovery: The recovery of the method was, determined by spiking a previously analyzed test solution of formulation with additional neem oil extract, which was found to be 97.4-98.66 % with respect to substance 1 and 98.6-99.7 % with respect to substance 2. The value of recovery and % RSD as depicted in Table-4 shows that the method is accurate.

Precision: Inter-day, intra-day, inter-analyst and inter-system precisions were carried out by applying six sample and assay for each analysis is calculated and for this % RSD was calculated and found to be within 3 % (Table-5). The low value of RSD (%) indicates the precision of the method.

Limit of detection and limit of quantification: The LOD and LOQ of the method, determined by the signal to noise ratio and found 1 µg and 4 µg, respectively with respect to both the substances, which indicates the method can be used for detection and quantification of neem oil extract over a very wide range of concentrations.

TABLE-2
STANDARD CALIBRATION CURVE AT 265 nm

Concentration (µg)	Area of peak (area ± SD)		Height of peak (height ± SD)	
	Substance 1	Substance 2	Substance 1	Substance 2
4	299.9 ± 19.0	712.7 ± 1.0	13.40 ± 0.46	25.30 ± 0.82
8	481.6 ± 8.3	1337.2 ± 155.4	23.07 ± 1.55	48.13 ± 5.02
10	586.3 ± 18.3	1492.2 ± 176.0	24.83 ± 5.59	52.57 ± 5.88
20	828.1 ± 98.5	1680.3 ± 195.0	30.20 ± 4.01	62.17 ± 6.67
40	1343.0 ± 33.1	2783.0 ± 151.5	46.77 ± 0.71	95.20 ± 4.11
80	2768.0 ± 19.3	5227.0 ± 123.7	90.23 ± 06.5	165.77 ± 2.85
100	3666.3 ± 321.0	6181.3 ± 855.1	115.27 ± 7.41	193.33 ± 19.93

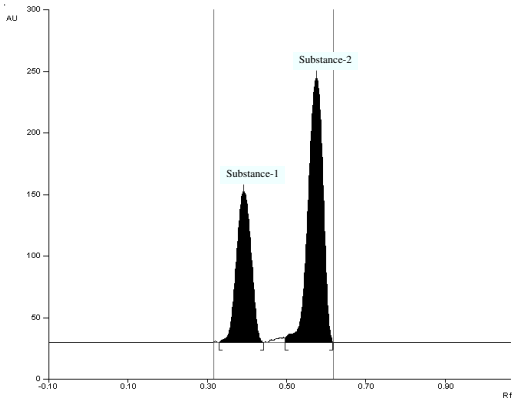

Fig 2. HPTLC chromatogram showing peak of substance 1 and substance 2 (at R_f 0.33and 0.55) in neem oil extract at 265nm (4 µg)

TABLE-3
LINEAR REGRESSION DATA FOR CALIBRATION PLOT (AREA)

Parameters	Substance 1	Substance 2
Linearity range (µg/spot)	4-100	4-100
Regression equation	Y = 33.735x + 162.07	Y = 55.058x + 712.66
Correlation coefficient ± SD	0.9936 ± 0.008	0.9931 ± 0.011
Slope ± SD	33.735 ± 0.22	55.058 ± 0.15
Intercept ± SD	162.07 ± 0.50	712.66 ± 0.44

TABLE-4
RECOVERY STUDIES

Parameters	Substance 1	Substance2
Percentage of standard spiked	80	80
	100	100
	120	120
Amount of standard spiked (µg)	48	136
	60	170
	72	204
Amount recovered (µg) mean ± SD	47.36 ± 0.11	135.6 ± 0.89
	62.18 ± 0.12	168.5 ± 0.24
	70.12 ± 0.29	201.2 ± 1.67
Percentage recovery	98.66	99.7
	103.66	99.1
	97.40	98.6

Assay of capsule/tablet: The capsule and tablet formulations were dissolved in methanol to get 2 mg/mL equivalent of neem oil extract and filtered through 0.45 µm membrane filter. 4 µL of same was applied in duplicate on TLC plate (20 cm × 10 cm) for quantification. The neem oil extract was quantified with respect to substance 1 and substance 2 by Wincats software using regression equation obtained from calibration curve. The mean of duplicate samples were calculated, which showed presence of 96.3-103.2 % of label claim of neem oil extract in capsule and tablet dosage form (Table-6). The present manuscript provides a way for standardization and quantity control of herbal formulation in which the chemical markers yet, have not been identified.

TABLE-5
PRECISIONS

Precisions	Percentage RSD of area		Percentage RSD of R_f		Percentage RSD of assay	
	S1	S2	S1	S2	S1	S2
Inter day precision	2.22	1.76	0.52	0.66	2.30	2.65
Intra day precision	2.65	2.11	0.67	0.75	2.55	2.48
Inter analyst precision	2.90	2.91	0.99	0.92	2.86	2.88
Inter system precision	2.44	2.75	1.00	1.52	2.47	2.79

S1: Substance1, S2: Substance 2.

TABLE-6
ASSAY OF NEEM OIL EXTRACT IN CAPSULE/TABLET

With respect to substances	R_f ± SD	Content of neem oil extract in capsule mean % ± SD (lable claim 200 mg/capsule)	Content of neem oil extract in tablet mean % ± SD (lable claim 400 mg/tablet)
1	0.33 ± 0.02	198.2 ± 2.01 mg	385.2 ± 0.45 mg
2	0.55 ± 0.02	206.4 ± 2.45 mg	401.1 ± 3.56 mg

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