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DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF POLYHERBAL FORMULATION (SJT-ONC-1).

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Abstract:

The polyherbal formulations were found to be rich in alkaloids, flavanoids, phenolics, steroids and terpenoids but saponins were present in SJT-ONC-1. The formulations was quantitatively estimated for all the phytoconstituents. The total phenolic content of SJT-ONC-1 was found to be 3.50 % w/w; total flavanoid content, 2.59 % w/w; total alkaloidal content,0.66 %w/w; total tannin content 5.9 %w/w. To achieve the best resolution of marker compounds, Lapachol (R_f : 0.74), Bacalein (R_f : 0.40) and Lupeol (R_f : 0.34) in SJT-ONC-1, specific solvent system i.e. Hexane: ethyl acetate: methanol: glacial acetic acid (7:2:1:0.4) was developed through TLC study.HPTLC methods were developed for estimation of Lapachol, Bacalein and Lupeol in SJT-ONC-1, Lapachol, Bacalein and Lupeol were estimated as 0.707, 1.992 and 1.886 % w/w respectively in the SJT-ONC-1

Key words: SJT-ONC-1, Lapachol, Bacalein, Lupeol, TLC, HPTLC

1.Introduction:

Preparation of highly standardized herbal products with respect to chemical composition and biological activity is considered to be a valuable approach in this field. Standardization is an essential factor for polyherbal formulation in order to assess the quality of the drugs based on the concentration of their active principle.

High performance thin layer chromatography (HPTLC) is an invaluable quality assessment tool for the standardization of herbal products. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. Additionally, numerous samples can be run in a single analysis thereby dramatically reducing analytical time. Thus, the aim of the present project was marker based standardization of the polyherbal formulations, SJT-ONC-1 developed at S. J. Thakkar Pharmacy college for the treatment of cancer and using HPTLC.

HPTLC is the most simple separation technique available today which gives better precision and accuracy with extreme flexibility for various steps (stationary phase, mobile phase, development technique and detection). The HPTLC was carried out using a Hemilton 100 µl HPTLC syringe, Camag Linomat V automatic spotting device, Camag twin trough chamber, Camag TLC Scanner-3, WINCAT integration software, aluminium sheet precoated with Silica Gel 60F254(Merck), 0.2 mm thickness. HPTLC finger printing technique is useful to identify and to check the purity of raw herbal extracts as well as finished product. Hence forth it is very useful tool in standardizing process of raw herbal extracts and finished products.

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SJT ONC (S.J. Thakkar Oncology) is a proprietary herbal blend. The name derives from SJT, for institute and ONC, word for Oncology. The preparation includes four different herbs, stem bark of Tecomella undulata Seem [9-13], Bauhinia variegata Linn[22-28], Oroxylum indicum Vent[37-44] and leaves of Indigofera tinctoria Linn[53-60]. So produced formulation can be able to act on multiple targets, produce synergistic effects and give safe and effective therapy for cure of cancer. Natural compounds are themselves having such properties and also having anti-cancer activity at lower concentration when they are used in combination due to synergism. These all selected plant drugs individually reported for their anticancer activity.

The present study deals with standardization of patented and proprietary polyherbal formulations SJT-ONC-1 using HPTLC method.

2.MATERIAL AND METHODS

2.1 Collection, Identification and Authentification of plant material

SJT-ONC-1:

Dried Stem barks of *Tecomella undulate*, *Oroxylum indicum* and dried leaves of *Indigofera tinctoria* were procured from the Prashant pharmaceuticals, Rajpipla, Gujarat, India. *Bauhinia variegata* was collected from the Junagadh, Gujarat, India and the plant material were identified and authenticated by Prof. Vishal Muliya, Botany Department, Christ Science College, Rajkot, and Gujarat, India.

2.2 Extraction

All crude drug powders of formulations, SJT-ONC-1(Table 1) was extracted with different solvents. The extracts were concentrated and dried.

Table 1 : Extracts of the selected plant parts of SJT-ONC-1

Sr. No.	Plant name	Extract	% Yield
1	*Tecomella undualta* stem bark	Chloroform extract	4.5
2	*Bauhinia variegata* stem bark	Alcoholic extract	2.2
3	*Indigofera tinctoria* Leaves	Alcoholic extract	18.62
4	*Oroxylum indicum* stemm Bark	Alcoholic extract	19.2

2.3 Preparation of polyherbal formulations

SJT-ONC-1:

Above mentioned extracts were mixed in different proportion and prepare different formulations.

Tecomella undulata stem bark 50 %

Bauhinia variegate Stem bark 17 %

Indigofera tinctoria leaves 17 %

Oroxylum indicum stem bark 16 %

2.4 Preliminary Phytochemical Screening

Preliminary phytochemical screening performed for both the formulations

(i) Test for Alkaloids

5 ml methanlic extract of the formulation were evaporated to dryness. The residue were dissolved in 15 ml of H_2SO_4 (2 N) and filtered. After making alkaline, the filtrate were extracted with chloroform. The residue left after evaporation, were tested for the presence of alkaloids with Dragendorff reagent[70].

(ii) Test for Flavanoids

5 ml methanlic extract of the formulation were evaporated to dryness. To the residue 0.3 ml boric acid solution (3 %w/v) and 1 ml oxalic acid solution (10 %w/v) were added. The mixture was evaporated and the residue were dissolved in 10 ml ether. The ethereal layer shows greenish fluorescence under UV indicating presence of flavonoids[70].

(iii) Tests for Saponins

0.1 g formulation was separately shaken with 5 ml of distilled water in a test tube for 30 Sec and was left undisturbed for 20 min Persistent foam indicate presence of saponins[70].

(iv) Test for Phenolic compounds

(a) Ferric chloride test

The formulation (1 g) taken separately in warm water and observed the formation of green or blue color on addition of 2 ml $FeCl_3$ solution[70].

(b) Lead acetate test

Lead acetate solution was added to 1 gm of formulation and observed the formation of precipitates[70].

(v) Test for tannins

0.5 g of the SJT-ONC-1 boiled in 20 ml of water in a test tube and then filtered separately. Few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration[71].

(vi)Test for steriods

2 ml of acetic anhydride was added to 0.5 g of formulation with 2 ml H_2S0_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids[71].

(vii) Test for terpenoids (Salkowski test)

5 ml of methanolic solution of the formulation was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was added carefully to form a layer. A reddish brown colouration of the inter face shows positive results for the presence of terpenoids[71].

2.5 Estimation of Phytoconstituents

Different phytoconstituents usually, alkaloids, flavonoids, phenolics, terpenoids and tannins were quantitatively estimated by different procedures

(i) Estimation of Phenolics

10 ml of distilled water and 1.5 ml of diluted (1:2) folin ciocalteu reagent (1 part of folin ciocalteu reagent in 2 part of distilled water) were added to 1 ml of the methanolic solution of polyherbal formulations and kept the mixture aside for 5 min. After adding 4 ml of 20 % Na_2CO_3 solution adjust the final volume to 25 ml using distilled water. Measured the absorbance at 765 nm at an interval of 30 min up to 2 h, against distilled water as a blank. Then standard curve was prepared using standard galic acid[70].

(ii) Estimation of Flavanoids

The flavanoids estimation was done by the aluminium chloride method (Bahorun et al., 1996) 180. 0.1 ml of methanolic solution of polyherbal formulation diluted upto 1 ml with methanol and then 1 ml of 2 % aluminum chloride in methanol was added. After 10 min the absorbance measured at 430 nm. Rutin served as standard[70].

(iii) Estimation of Alkaloids

Test Solution:

Dragendorff's reagent (2 ml) was added to 5 ml of methanolic solution. After centrifugation, the precipitate were washed with alcohol, and treated with 2 ml of 10 % w/v of sodium sulfide solution. Centrifuge brownish black precipitate formed and completion of precipitation was checked by sodium sulfide solution. Residue were dissolved in 2 ml concentrated nitric acid, with warming.

Standard Solution:

Solution of different concentration of atropine (1 mg/ 10 ml) were prepared in water.

1 ml of the test and standard solutions were treated with 5 ml of 10 % w/v solution of thiourea and the absorbance was measured at 435 nm against the blank containing nitric acid and thiourea. Alkaloid content was estimated using standard curve[72].

(V) Estimation of tannin

To 4.5 ml polyherbal formulations, 50 ml distilled water has been added and then filter it. To 1ml of that filter, 0.4 ml 1% ferric chloride and 0.4 ml 0.005 M pottasium ferrocynide were added and volume was adjusted to 10ml. Same way standard gallic acid panel was prepared and the absorbance measured at 720 nm within 10 minute[72].

2.6 Procurement of markers[73]

Marker compound means chemical constituents within a medicine that can be used to verify its potency or identity. Following chemical markers were procured from the different herbal drug suppliers (Table 2).

Table 2: List of Markers and their suppliers

Name of the Formulation	Name of Plant	Marker	Marker Suppliers
SJT-ONC-1	*Tecomella undualta* stem bark	Lapachol	Sigma Aldrich
	Bauhinia variegata stem bark	Lupeol	Sigma Aldrich
	Oroxylum indicum stem Bark	Baicalein	Sigma Aldrich

2.7 Devlopment of solvent system by TLC Study

The TLC technique is used for qualitative determination of possible number of phytoconstituents from the formulation. Solvent systems were optimized in order to get maximum separation of various phytochemicals. Solvent system optimized in TLC study has been chosen for HPTLC study.

Mobile phase : SJT-ONC-1
For Lapachol, Baicalein and Lupeol:
Hexane: Ethyl acetate: Methanol: Glacial acetic acid (7:2:1:0.4)

Stationary phase : Pre-coated silica gel 60 F_{254} aluminum plate,0.2 mm thickness

Chamber saturation time : 30 min.

Derivatization : Vanillin-sulphuric acid (for 6-Gingerole)/
5 % aqueous sulphuric acid (for lupeol)

Visualization : 254 nm or 366 nm or visible light

Temperature : 25±1 ^{0}c

2.8 Development of HPTLC methods for each chemical markers[74]

HPTLC method was developed for polyherbal formulations SJT-ONC-1 taking procured marker compounds as working standards. All the markers were quantified in polyherbal formulation by co-chromatography using standard markers. Procedure for HPTLC method development is outlined as follow (Fig.1).

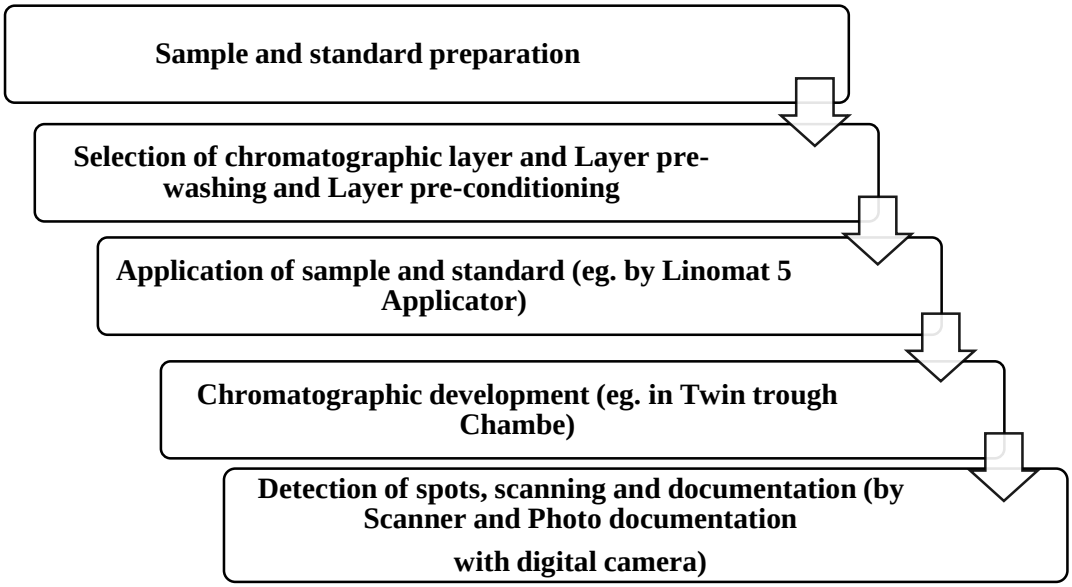

Fig. 1: Schematic procedure for HPTLC Method Development

2.8.1 Instrument

A Hamilton 100 µl HPTLC syringe

A Camag Linomat 5 (semiautomatic spotting device)

A Camag twin-trough chamber

A Camag TLC Scanner 3

A Camag data evaluation 32 bit software (latest version)

2.8.2 Spotting Parameters

Start position : 15 mm from bottom edge

Band width : 4 mm

Space between two bands : 5 mm

2.8.3 Chromatographic Conditions (Table 3)

Table 3: SJT-ONC-1

	Spotting vol. (µl)	Amt/band (µg)
A) For calibration curve		
Lapachol	1-5	0.5-2.5
Bacalein	1-5	0.5-2.5
Lupeol	3-7	1.5-3.5
B) For test samples		
Methanolic polyherbal Formulation	10 / 15 µl	50/ 75

2.8.4 Experimental Conditions for HPTLC

Stationary phase : pre-coated TLC plates of silica gel 60 F_{254} (E. Merck)

Separation technique : Ascending

Development chamber : Twin-trough chamber

Mobile phase : For Lapachol, Baicalein and Lupeol:
Hexane: Ethyl acetate: Methanol: Glacial acetic acid (7:2:1:0.4)

Derivatization : vanillin-sulphuric acid (for 6-Gingerole) /5 % aqueous sulphuric acid (for lupeol)

Chamber saturation time : 30 min.

Temperature : 25±2 °C

Migration distance : 60 mm

2.8.5 Densitometric Scanning (CAMAG scanner 3)

Mode : Absorbance / Reflectance

Wavelength : Lapachol 284 nm
Bacalein 279 nm
Lupeol 559 nm

Lamp used : Mercury

Slit dimension : 3 X 0.45mm

2.8.6 Preparation of Solutions

(i) Preparation of Standard Solution of Chemical Marker Compounds

Accurately weighed 2.5 mg of lapachol and Bacalein were dissolved in 5 ml of methanol(0.5mg/ml) in a volumetric flask separately and 2.5 mg of Lupeol was dissolved in 5 ml of n-hexane (0.5mg/ml) in a volumetric flask.

(ii) Preparation of Sample Solutions

Sample solutions were generated by dissolving 50 mg of above prepared polyherbal formulations i.e. SJT-ONC-1 in 10 ml of methanol (2.0 mg/ml) in a volumetric flask separately.

2.8.7 Calibration Curves of Chemical Markers

SJT-ONC-1

(i) Calibration Curve of Lapachol

Graded concentrations of standard Lapachol solution (0.5 mg/ml), 1, 2, 3, 4 and 5 µl volume were spotted. The plate was developed in mobile phase, Hexane: Ethyl acetate: Methanol: Glacial acetic acid (7:2:1:0.4). After development the plate was dried and scanned at 284 nm. Data of peak height and peak area of each Lapachol spot was rec-orded. Standard curve of peak area Vs concentration of Lapachol was plotted.

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(ii) Calibration Curve of Bacalein

Graded concentrations of standard Bacalein solution (0.5 mg/ml), 1, 2, 3, 4 and 5 µl volume were spotted. The plate was developed in mobile phase, Hexane: Ethyl acetate: Methanol: Glacial acetic acid (7:2:1:0.4). After development the plate was dried and scanned at 279 nm. Data of peak height and peak area of each Bacalein spot was recorded. Standard curve of peak area Vs concentration of Bacalein was plotted.

(iii) Calibration Curve of Lupeol

Graded concentration of standard Lupeol solution (0.5 mg/ml), 3, 4, 5, 6 and 7 µl volume were spotted. The plate was developed in mobile phase, Hexane: Ethyl acetate: Methanol: Glacial acetic acid (7:2:1:0.4). After development the plate was dried, derivatized by 5 % aqueous H_2SO_4 and scanned at 559 nm. Data of peak height and peak area of each Lupeol spot was recorded. Standard curve of peak area Vs concentration of Lupeol was plotted.

2.8.8 Estimation of Chemical Markers in polyherbal formulations

SJT-ONC-1

(i) Estimation of Lapachol

15 µl of test sample solution (4.0 mg/ml) was used for spotting.

The plate was developed in the mobile phase. After development the plate was dried and immediately scanned at 284 nm using the Camag scanner 3. Peak areas were noted and concentration of lapachol in the formulation was calculated from the calibration curve.

(ii) Estimation of Bacalein

10 µl of test sample solution (4.0 mg/ml) was used for spotting.

The plate was developed in the mobile phase. After development the plate was dried and immediately scanned at 279 nm using the Camag scanner 3. Peak areas were noted and concentration of Bacalein in the formulation was calculated from the calibration curve.

(iii) Estimation of Lupeol

10 µl of test sample solution (4.0 mg/ml) was used for spotting.

The plate was developed in the mobile phase, derivatized by vanillin-sulphuric acid and scanned at 559 nm. Peak areas were noted and concentration of Lupeol in the formulation was calculated.

2.9 Validation of developed HPTLC Mehods[75]

The developed HPTLC methods was validated using standard validation parameters as per ICH guidelines. Validation parameters typically monitored are:

- Linearity of the calibration graph
- Precision (indication of random errors)
- Accuracy (indication of systematic errors)
- Limit of quantification (within which the analyte can be quantified)
- Limits of detection
- Specificity

SJT-ONC-1

(i) Validation of HPTLC Method for Lapachol

Linearity

Linearity of the method was evaluated by constructing calibration curves at different concentration levels. Calibration curve was plotted over a concentration range of 0.5-2.5 µg/spot of pure Lapachol solution (0.5 mg/ml). The calibration curve was developed by plotting peak area Vs. concentrations. The results were expressed in terms of correlation co-efficient of the linear regression analysis.

Precision

Precision was evaluated in terms of intraday and interday precisions. Intraday precision was determined by analyzing sample solutions of analyte from formulations at three levels covering low, medium, and higher concentrations of calibration curve (1,1.5,2 µg/spot) for 3 times on the same day. Interday precision was determined by analyzing sample solutions of analyte at three levels covering low, medium, and higher concentrations (1,1.5,2 µg/spot) over a period of 3 days. The peak areas obtained were used to calculate mean and % CV (Coefficient varience) values.

Repeatability

Repeatability of measurement of peak area (RSD<1 % based on seven times measurement of same spot) was determined by analyzing one concentration of the calibration curve (1.5 µg/spot of 0.5 mg/ml standard solution) seven times without changing the position of plate. Repeatability of sample application (RSD<3 % based on application of equal volume of seven spots) was assessed by spotting sample covering similar concentration of calibration curve (1.5 µg/spot of 0.5 mg/ml standard solution) seven times and analyzing them once.

Accuracy

Accuracy of the method was evaluated by carrying the recovery study at three levels. Recovery experiments are performed by adding three different amounts of standard drug, i.e., 50, 100, and 150% of the drug, to the preanalyzed formulation (SJT-ONC-1), and the resultant is reanalyzed 3 times and %Recovery is calculated.15µl spots of 5 mg/ml formulation were spiked with 0.5,1,1.5 µl of pure lapachol solution (0.5mg/ml).

Limit of Detection

The limit of detection is the lowest amount of the analyte, which can be detected but not necessarily quantitated as an exact value. LOD was determined at signal-to-noise ratio of 3:1. Determination of the signal to noise ratio was performed by comparing measured signals from samples with known low concentration of analyte can be reliably detected.

The LOD can be calculated as

$$LOD = 3.3 \times (SD/Slope)$$

Where,

SD = Standard deviation of the Y- intercepts of the 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

Limit of Quantification (LOQ)

Quantification Limit is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. LOQ was determined at signal-to-noise ratio of 10:1.

The LOQ can be calculated as

$$LOQ = 10 \times (SD/Slope)$$

Where,

SD = Standard deviation of the Y- intercepts of the 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

Specificity

It is an ability of the developed analytical method to detect the analyte quantitatively in presence of other compounds, which are expected to be present in the same matrix. The specificity of the method was ascertained by analyzing standard drug and sample. The R_f value and absorption spectra for standard and sample were compared. The peak purity of the analyte was assessed by comparing the spectra at three different regions of the spot.

(ii) Validation of HPTLC Method for Bacalein

HPTLC method for estimation of the Bacalein was validated as mentioned earlier. Linearity (concentration range of 0.5-2.5 µg/spot of 0.5 mg/ml pure Bacalein solution), interday and intraday precision (at three concentration levels, 1, 1.5, 2 µg/spot), repeatability of measurement of peak area and sample application (by analyzing 1.5 µg/spot of 0.5 mg/ml standard solution), accuracy (by spiking 0.5, 1, 1.5 µl of pure Bacalein solution to the 5µl spots of 5 mg/ml formulation), specificity, limit of detection and limit of quantification were determined.

(iii) Validation of HPTLC Method for Lupeol

HPTLC method for estimation of the Lupeol was validated as mentioned earlier. Linearity (concentration range, 1.5-3.5 µg/spot of 0.5 mg/ml pure Lupeol solution), interday and intraday precision (at three concentration levels, 2, 2.5, 3 µg/spot), repeatability of measurement of peak area and sample application (by analyzing 2.5 µg/spot of 0.5 mg/ml standard solution), accuracy (by spiking 0.9, 1.8, 2.7 µl of pure Lupeol solution to the 10µl spots of 5 mg/ml formulation), specificity, limit of detection and limit of quantification were determined.

6. RESULTS AND DISCUSSION

6.1 Preliminary Phyto-chemical Screening

The polyherbal formulations, SJT-ONC-1 and SJT-OB-1 were screened for the presence of phytoconstituents. Both the formulations were found to be rich in alkaloids, flavanoids, phenolic compounds, steroids and terpenoids. Further, saponins were present only in SJT-ONC-1(Table 4).

Table 4: Phyto-chemical screening of the roots

Sr. no.	Phytochemical Test	SJT-ONC-1
1.	Test for Alkaloids	+ve
2.	Test for Flavanoids	+ ve
3.	Test for Saponins	+ ve
4.	Test for Phenolic compounds	
	(a) Ferric chloride test	+ ve
	(b) Lead acetate test	+ ve
5.	Test for Steroids	+ ve
6.	Test for Terpenoids	+ ve

Estimation of Phytoconstituents

Total phenolics, flavanoids, alkaloids and tannin content were estimated in both the polyherbal formulation, SJT-ONC-1 and expressed as % w/w value (Table 5).

Table 5: Estimation of phytoconstituents

Sr. no.	Phytoconstituents	% w/w value (Mean ± S.E.M., n=3)
		SJT-ONC-1
1.	Phenolics	3.50±0.037
2.	Flavanoids	2.59±0.083
3.	Alkaloids	0.66±0.067
4.	Tannins	5.9±0.168

6.3 Preliminary TLC study for Solvent System Development

Solvent systems were optimized to achieve best resolution of the marker compounds from other components of the formulation through TLC study. The specific solvent system, Hexane: Ethyl acetate: Methanol: Glacial acetic acid (7:2:1:0.4) gave best resolutions of Lapachol with R_f value 0.74 (Figure 2a,b,c), Bacalein with R_f value 0.40 (Figure 3a,b,c) and Lupeol with R_f value 0.34 (Figure 4a,b,c) in the presence of other compounds in the polyherbal formulation SJT-ONC-1.

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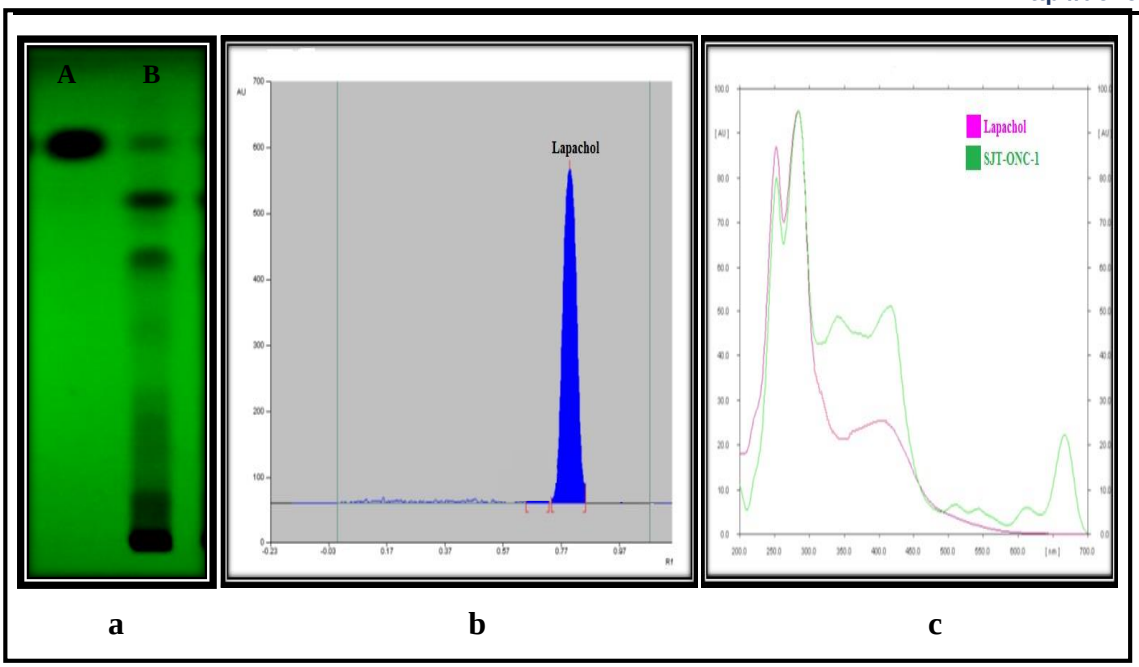

Fig. 2a: TLC plate of Lapachol (A) and formulation SJT-ONC-1 (B)
Fig. 2b: HPTLC chromatogram of Lapachol scanned at 284 nm
Fig. 2c: Overlain UV spectra of spot of Lapachol

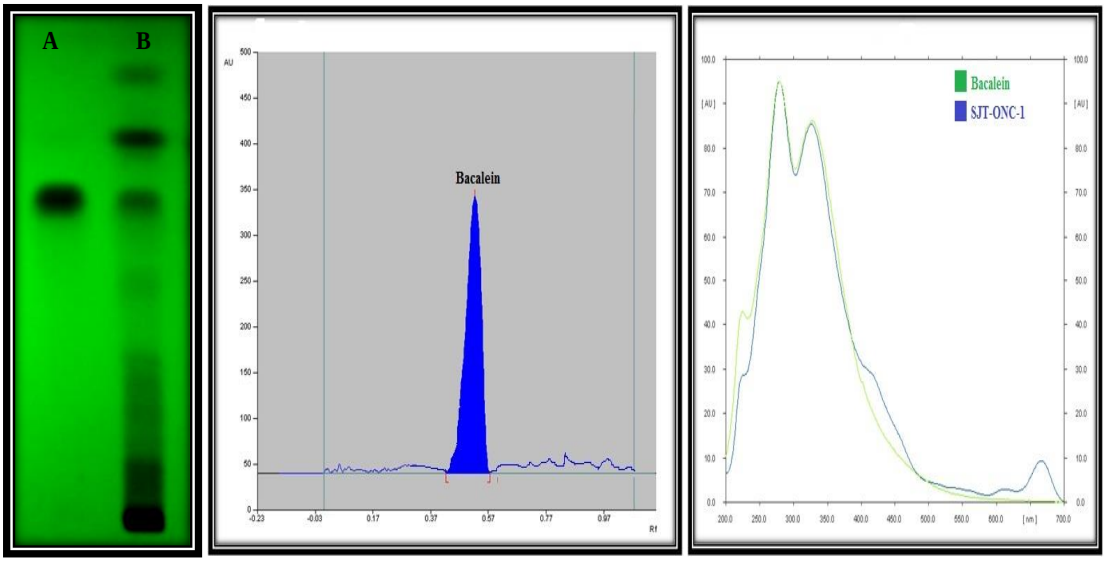

Fig. 3a: TLC plate of Bacalein (A) and formulation SJT-ONC-1 (B)
Fig. 3b: HPTLC chromatogram of Bacalein scanned at 279 nm
Fig. 3c: Overlain UV spectra of spot of Bacalein

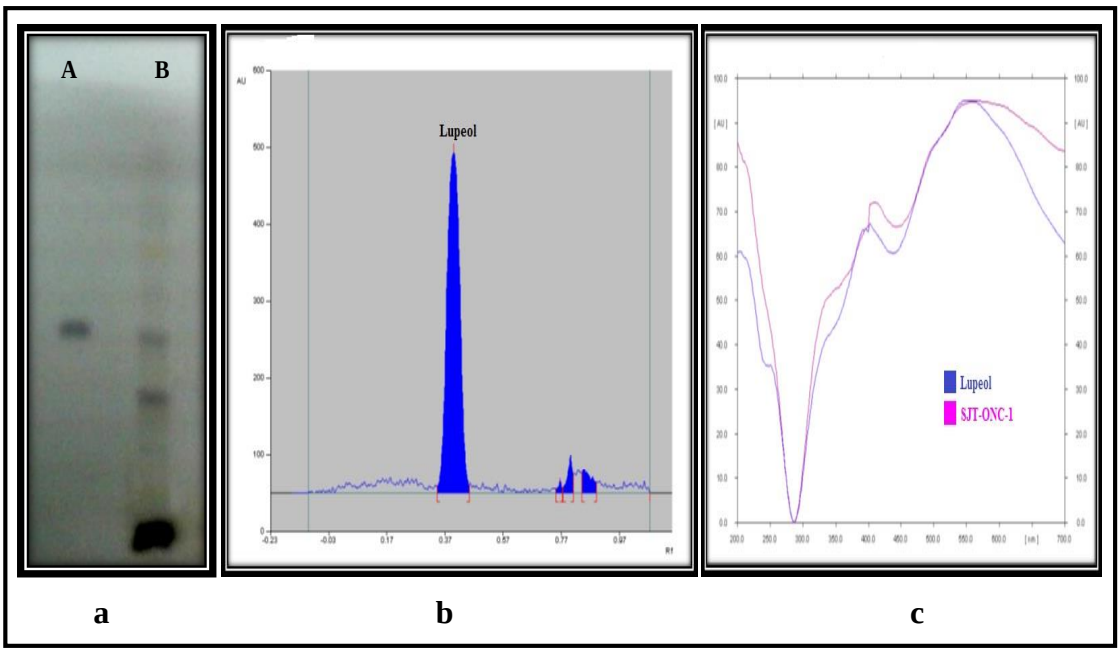

Fig. 4a: TLC plate of Lupeol (A) and formulation SJT-ONC-1 (B)
Fig. 4b: HPTLC chromatogram of Lupeol scanned at 559 nm
Fig. 4c: Overlain UV spectra of spot of Lupeol

3.4 Method Development and Validation of HPTLC method for each marker (SJT-ONC-1)

(i) Lapachol

Calibration Curve of Lapachol

Standard Lapachol showed single peak in HPTLC chromatogram (Fig. 2b). The calibration curve of Lapachol as obtained by spotting standard solutions of Lapachol on TLC plate. After development, the plate was scanned at 284 nm. Calibration curve was obtained by plotting concentration Vs average peak area of Lapachol. Linearity was obtained for Lapachol in the range of 0.5-2.5 µg/spot with correlation coefficient of 0.9965 (Fig. 5a,b,c Table 6).

Table 6: Calibration data of standard Lapachol

Sr. No.	Concentration of Lapachol (µg/spot)	Mean peak area ± S.D. (n=3)	% C.V.
1	0.5	15138.65 ± 72.33	0.47
2	1	18710.4 ± 78.48	0.42
3	1.5	23523.5 ± 55.15	0.23
4	2	27500.15 ± 91.57	0.33
5	2.5	30811.3 ± 64.20	0.20

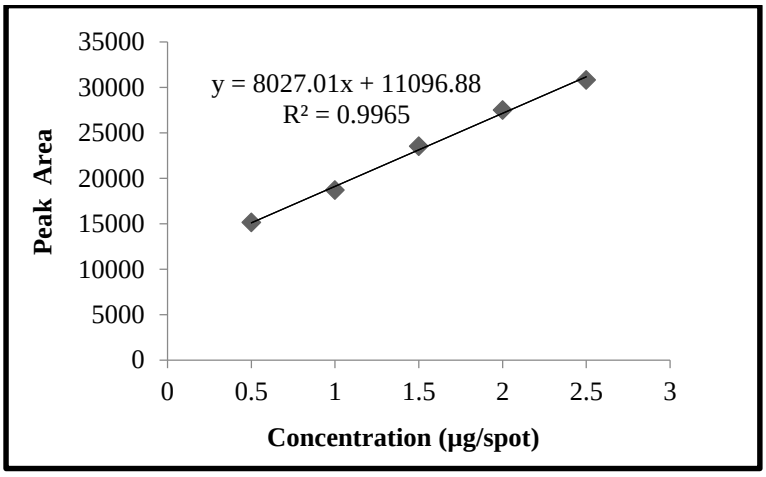

Figure 5a: Calibration curve of Lapachol

Estimation of Lapachol in SJT-ONC-1 by HPTLC method

The amount of Lapachol was computed from calibration curve (Fig. 5a,d Table 7). The amount of Lapachol found in SJT-ONC-1 was 0.707 % w/w respectively (Fig 5b,d).

Table 7: Estimation of Lapachol in SJT-ONC-1

Sample	Mean Peak Area (n = 3)	Average amount of Lapachol (µg/spot)	Average % of Lapachol ± S.D.	% C.V.
SJT-ONC-1	15352.87	0.530	0.707 ± 25.38	0.16

Validation of HPTLC Method:

Linearity

Linearity was obtained for Lapachol in the range of 0.5-2.5 µg/spot with correlation coefficient of 0.9965 (Fig.5a,b Table 6).

Precision

The intra-day and inter-day coefficient of variation (% CV) for Lapachol varied from 0.307-0.416 and 0.410-0.519 % respectively (Table 8). The low value of % CV indicated the precision of the developed method.

Table 8: Data for intra-day and inter-day precision for Lapachol

Concentration (µg/ spot)	Intra-day Precision (n = 3)		Inter-day Precision (n = 3)	
	Peak area (Mean ± S.D)	% C.V.	Peak area (Mean ± S.D)	% C.V.
1	18849.4 ± 78.43	0.42	18882.41 ± 98.13	0.52
1.5	23439.7 ± 72.54	0.31	23494.39 ± 99.77	0.42
2	27706.87 ± 85.06	0.32	27729.19 ± 113.94	0.41

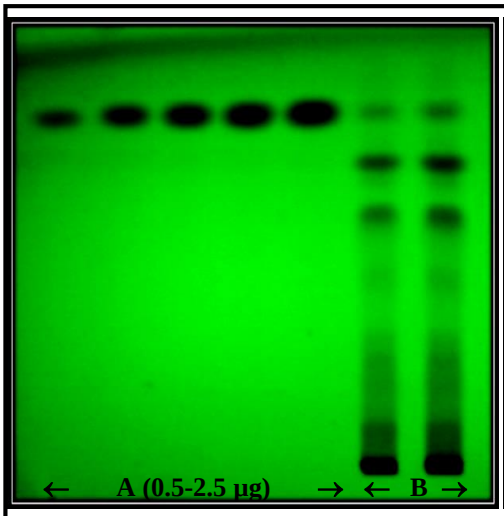

Fig. 5 b: Co-Chromatography of Lapachol (A) and Formulation SJT-ONC-1 (B)

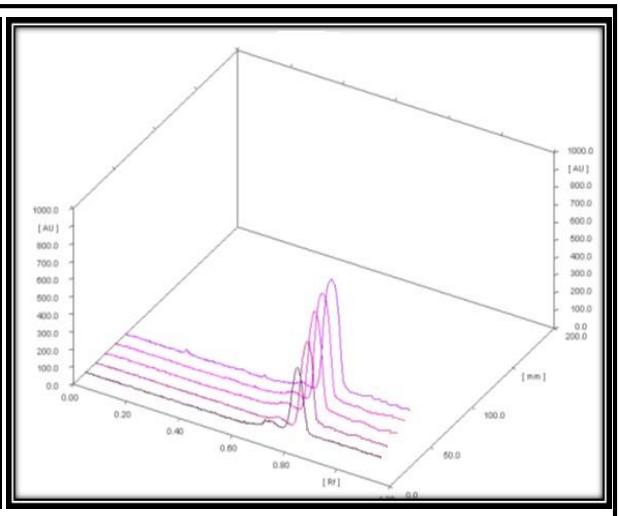

Fig. 5c: 3D-Chromatogram for linearity of Lapachol scanned at 284 nm

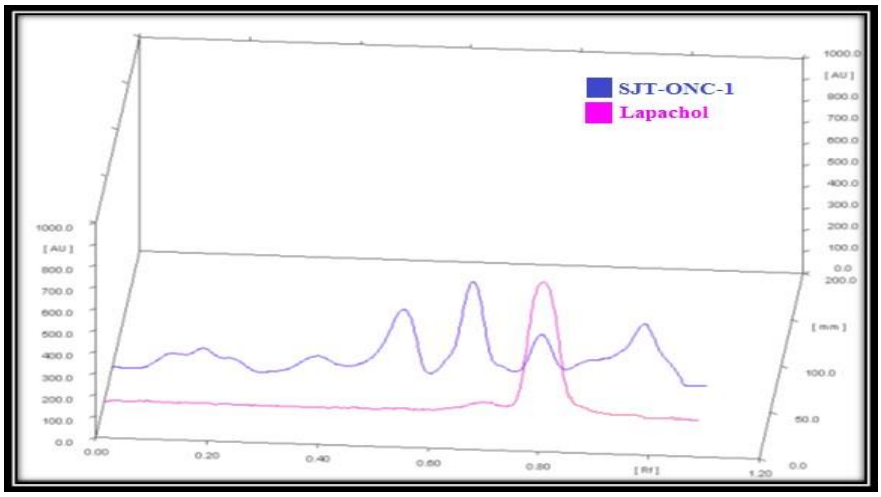

Fig. 5d: Densitometric chromatogram of Lapachol with formulation SJT-ONC-1 scanned at 284 nm

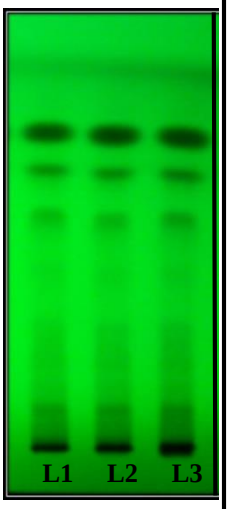

Fig. 5e:Accuracy of Lapachol (L1: 50%, L2: 100%, L3: 150%)

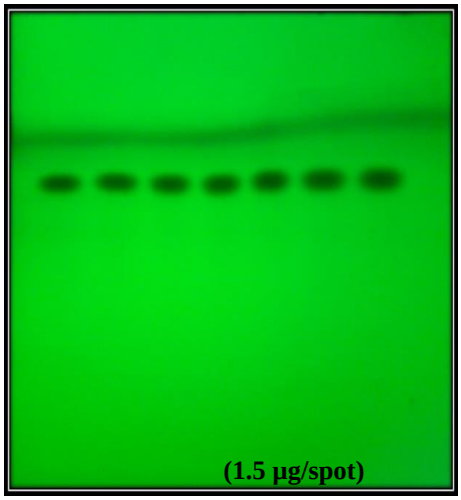

Fig. 5f: Repeatability of sample application of Lapachol

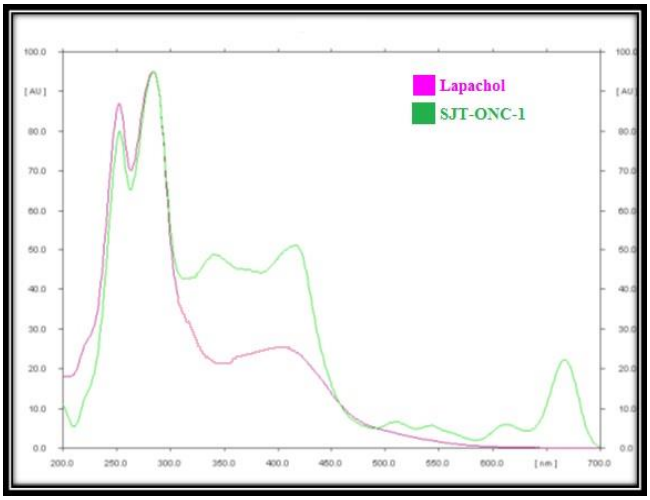

Fig. 5g: Overlain UV spectra of Lapachol with the spot resolving at the same R_f in the formulation SJT-ONC-1

Repeatability

Coefficient of variance for repeatability of measurement of peak area of 7 times measurement of the same spot was found to be 0.204 (Table 9).Similarly the coefficient of variance for repeatability of sample application for seven times was to be 0.301 (Fig. 5f,Table 10).

Table 9: Data of repeatability of measurement of peak area of Lapachol

No. of measurement	Area	Mean peak area ± S.D.	% C.V.
1.	23469.5	23423.94 ± 47.93	0.20
2.	23378.4		
3.	23466.8		
4.	23384.4		
5.	23426.2		
6.	23365.4		
7.	23476.9		

Table 10: Data of repeatability of sample application

No. of measurement	Area	Mean peak area ± S.D.	% C.V.
1.	23419.3	23407.53 ± 70.59	0.30
2.	23496.7		
3.	23366.8		
4.	23454.1		
5.	23336.4		
6.	23468.1		
7.	23311.3		

Accuracy (% recovery)

The % recovery of Lapachol was found to be 99.14%, which is satisfactory as shown in Table 11 (Fig. 5e).

Table 11: Data of accuracy for Lapachol

Concentration of Lapachol (µg/spot)		Amount of Lapachol found Mean ± S.D. (n=3)	% Recovery (n=3)	Average % Recovery
Taken	Added			
0.5	0.25	0.7399 ± 0.023	98.65	99.14
0.5	0.5	0.9923 ± 0.0255	99.23	
0.5	0.75	1.244 ± 0.032	98.52	

Specificity

It is observed that the other constituents of formulation did not interfere with the peak of Lapachol. The spectra of standard Lapachol spot and Lapachol in formulation were found to be similar (Fig. 5d,g). Therefore the method was specific.

Limit of Detection

The minimum detectable limit was found to be 0.044 µg/spot.

Limit of Quantification

The minimum quantification limit was found to be 0.132 µg/spot.

Table 12: Summary of Validation Parameters

Sr. No.	Parameters	Results
1	Range	0.5-2.5 µg/spot
2	Correlation Coefficient (R^2)	0.9965
3	Precision (% C.V.) Repeatability of Measurement Repeatability of Application Intraday Interday	 0.204 0.301 0.307-0.416 0.410-0.519
4	Accuracy	99.14 %
5	Limit of Detection	0.044 µg/spot
6	Limit of Quantification	0.132 µg/spot
7	Specificity	Specific

(ii) Bacalein

Calibration Curve of Bacalein

Standard Bacalein showed single peak in HPTLC chromatogram (Fig.3b). The calibration curve of Bacalein as obtained by spotting standard solutions of Bacalein on TLC plate. After development, the plate was scanned at 279 nm. Calibration curve was obtained by plotting concentration Vs average peak area of Bacalein. Linearity was obtained for Bacalein in the range of 0.5-2.5 µg/spot with correlation coefficient of 0.9962 (Fig. 6a,b,c Table 13).

Table 13: Calibration data of standard Bacalein

Sr. No.	Concentration of Bacalein (µg/spot)	Mean peak area ± S.D. (n=3)	% C.V.
1	0.5	10045.95 ± 46.17	0.45
2	1	13076.25 ± 44.05	0.33
3	1.5	15809.3 ± 32.66	0.20
4	2	19639.75 ± 50.27	0.25
5	2.5	23134.55 ± 67.52	0.29

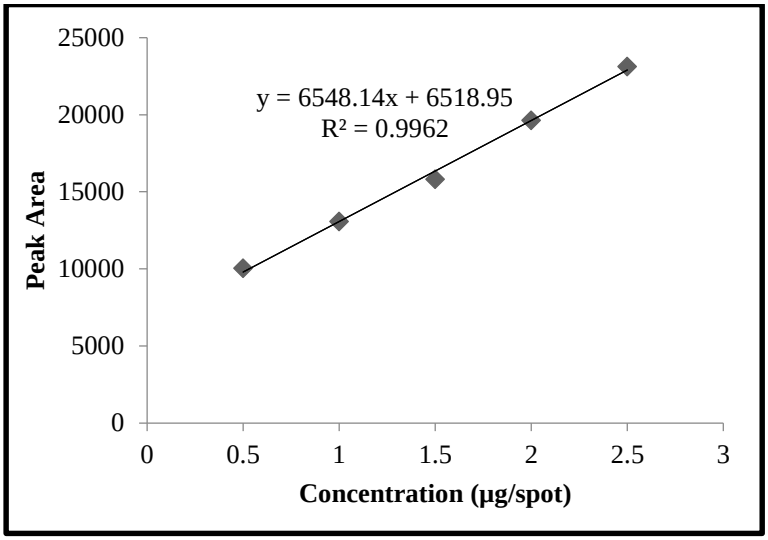

Figure 6a: Calibration curve of Bacalein

Estimation of Bacalein in SJT-ONC-1 by HPTLC method

The amount of Bacalein was computed from calibration curve (Fig. 6a,d Table 14). The amount of Bacalein found in SJT-ONC-1 was 1.992 % w/w respectively (Fig. 6b, d).

Table 14: Estimation of Bacalein in SJT-ONC-1

Sample	Mean Peak Area (n = 3)	Average amount of Bacalein (µg/spot)	Average % of Bacalein ± S.D.	% C.V.
SJT-ONC-1	13044.07	0.99	1.992 ± 52.04	0.39

Validation of HPTLC Method:

Linearity

Linearity was obtained for Bacalein in the range of 0.5-2.5 µg/spot with correlation coefficient of 0.9962 (Fig. 6a,b Table 13).

Precision

The intra-day and inter-day coefficient of variation (% CV) for Bacalein varied from 0.233-0.480 and 0.502-0.667 % respectively (Table 15). The low value of % CV indicated the precision of the developed method.

Table 15: Data for intra-day and inter-day precision for Bacalein

Concentration (µg/ spot)	Intra-day Precision (n = 3)		Inter-day Precision (n = 3)	
	Peak area (Mean ± S.D)	% C.V.	Peak area (Mean ± S.D)	% C.V.
1	13257.63 ± 50.55	0.38	13241.56 ± 88.43	0.66
1.5	15894.97 ± 76.31	0.48	15833.68 ± 90.32	0.57
2	19544.9 ± 45.62	0.23	19479.59 ± 97.91	0.50

http://doi.one/10.1729/Journal.25984

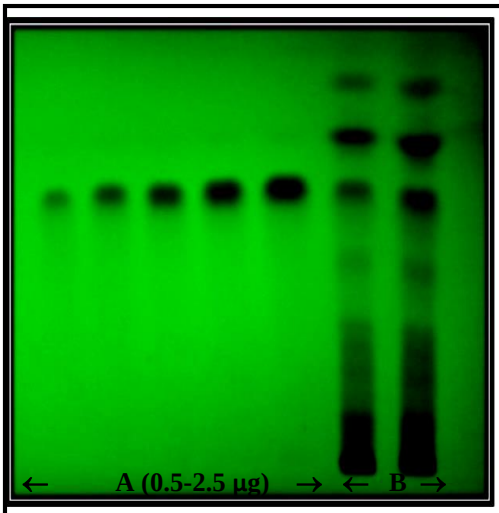

Fig. 6b: Co-chromatography of Bacalein (A) and formulation SJT-ONC-1 (B)

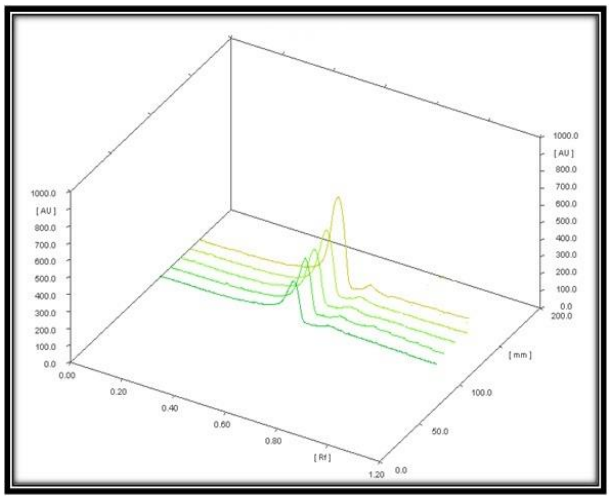

Fig. 6c: 3D-Chromatogram for linearity of Bacalein scanned at 279 nm

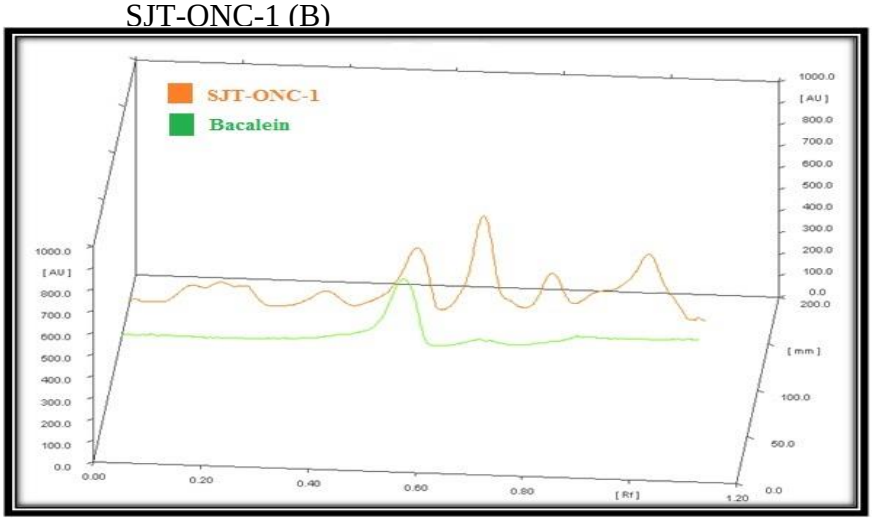

Fig. 6d: Densitometric chromatogram of Bacalein with formulation SJT-ONC-1 scanned at 279 nm

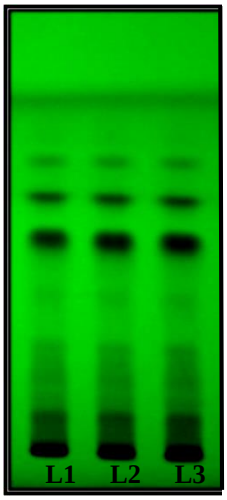

Fig. 6e: Accuracy of Bacalein (L1: 50%, L2: 100%, L3: 150%)

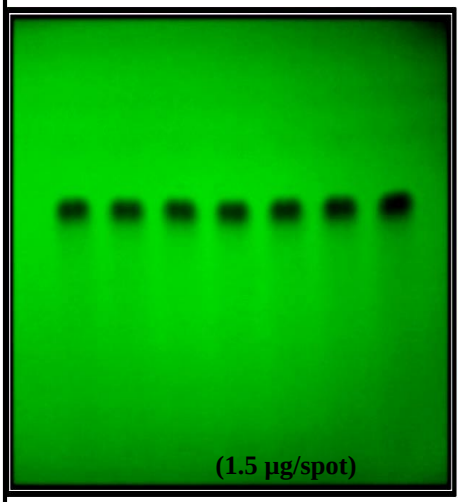

Fig. 6f: Repeatability of sample application of Bacalein

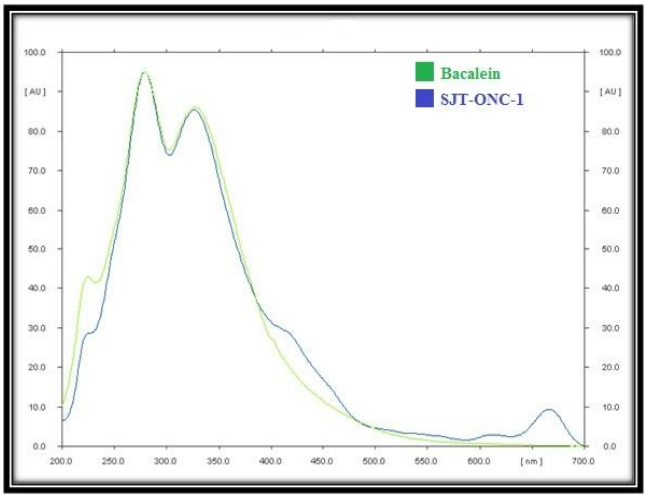

Fig. 6g: Overlain UV spectra of Bacalein with the spot resolving at the same R_f in the formulation SJT-ONC-1

Repeatability

Coefficient of variance for repeatability of measurement of peak area of 7 times measurement of the same spot was found to be 0.325 (Table 16). Similarly, the coefficient of variance for repeatability of sample application for seven times was to be 0.44 (Fig. 6f, Table 17).

Table 16: Data of repeatability of measurement of peak area of Bacalein

No. of measurement	Area	Mean peak area ± S.D.	% C.V.
1.	15956.4	15912.61 ± 51.86	0.32
2.	15825.1		
3.	15883.4		
4.	15976.4		
5.	15886.9		
6.	15943.7		
7.	15916.4		

Table 17: Data of repeatability of sample application

No. of measurement	Area	Mean peak area ± S.D.	% C.V.
1.	15956.4	15830.06 ± 69.65	0.44
2.	15843.3		
3.	15783.4		
4.	15756.4		
5.	15776.4		
6.	15878.1		
7.	15816.4		

Accuracy (% recovery)

The % recovery of Bacalein was found to be 99.14%, which is satisfactory as shown in Table 18 (Fig 6e).

Table 20: Data of accuracy for Bacalein

Concentration of Bacalein (µg/spot)		Amount of Bacalein found Mean ± S.D. (n=3)	% Recovery (n=3)	Average % Recovery
Taken	Added			
0.5	0.25	0.7422 ± 0.008	98.96	99.51
0.5	0.5	1.0064 ± 0.014	100.64	
0.5	0.75	1.2366 ± 0.027	98.93	

Specificity

Specificity of the proposed It was observed that the other constituents of formulation did not interfere with the peak of Bacalein. The spectra of standard Bacalein spot and Bacalein in formulation were found to be similar (Fig 6d,g). Therefore the method was specific.

Limit of Detection

The minimum detectable limit was found to be 0.053 µg/spot.

Limit of Quantification

The minimum quantification limit was found to be 0.177 µg/spot.

Table 19: Summary of Validation Parameters

Sr. No.	Parameters	Results
1	Range	0.5-2.5 µg/spot
2	Linearity	0.9962
3	Precision (% C.V.) Repeatability of Measurement Repeatability of Application Intraday Interday	 0.325 0.44 0.233-0.48 0.502-0.667
4	Accuracy	99.51 %
5	Limit of Detection	0.053 µg/spot
6	Limit of Quantification	0.177 µg/spot
7	Specificity	Specific

(iii) Lupeol

Calibration Curve of Lupeol

Standard Lupeol showed single peak in HPTLC chromatogram (Fig. 4b). The calibration curve of Lupeol as obtained by spotting standard solutions of Lupeol on TLC plate. After development, the plate was scanned at 559 nm. Calibration curve was obtained by plotting concentration Vs average peak area of Lupeol. Linearity

was obtained for Lupeol in the range of 1.5-3.5 µg/spot with correlation coefficient of 0.9957 (Fig. 7a,b,c, Table 20).

Table 22: Calibration data of standard Lupeol

Sr. No.	Concentration of Lupeol (µg/spot)	Mean peak area ± S.D. (n=3)	% C.V.
1	1.5	11962.75 ± 14.77	0.12
2	2	12829.7 ± 19.94	0.15
3	2.5	14050.25 ± 25.52	0.18
4	3	14856.4 ± 28.14	0.19
5	3.5	15761.05 ± 30.75	0.20

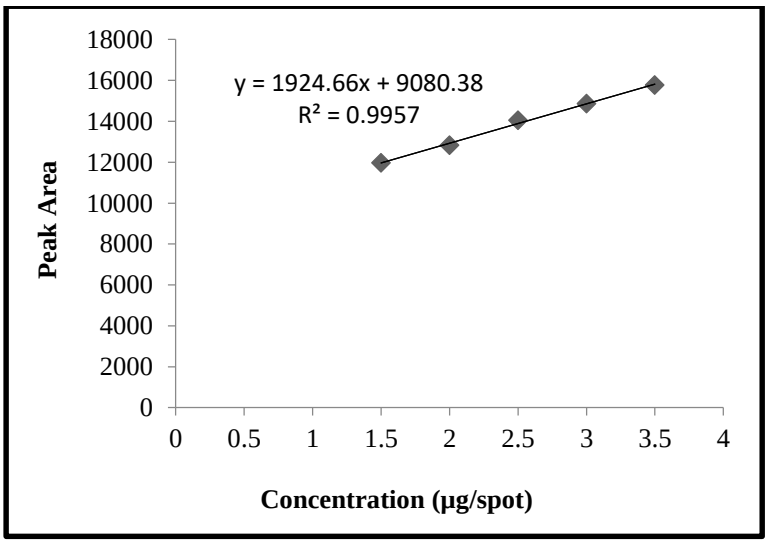

Figure 7a: Calibration curve of Lupeol

Estimation of Lupeol in SJT-ONC-1 by HPTLC method

The amount of Lupeol was computed from calibration curve (Fig. 7a,d Table 21). The amount of Lupeol found in SJT-ONC-1 was 1.886 % w/w respectively (Fig. 7 b,d).

Table 23: Estimation of Lupeol in SJT-ONC-1

Sample	Mean Peak Area (n = 3)	Average amount of Lupeol (µg/spot)	Average % of Lupeol ± S.D.	% C.V.
SJT-ONC-1	12750.93	0.94	1.886 ± 13.86	0.10

Validation of HPTLC Method:

Linearity

Linearity was obtained for Lupeol in the range of 1.5-3.5 µg/spot with correlation coefficient of 0.9957 (Fig. 7a,b Table 20).

Precision

The intra-day and inter-day coefficient of variation (% CV) for Lupeol varied from 0.238-0.490 and 0.416-0.626 % respectively (Table 22). The low value of % CV indicated the precision of the developed method.

Table 22: Data for intra-day and inter-day precision for Lupeol

Concentration (µg/ spot)	Intra-day Precision (n = 3)		Inter-day Precision (n = 3)	
	Peak area (Mean ± S.D)	% C.V.	Peak area (Mean ± S.D)	% C.V.
2	12788.97 ± 46.69	0.36	12801.77 ± 80.15	0.63
2.5	14096.7 ± 33.58	0.23	14103.97 ± 58.73	0.42
3	14799 ± 72.58	0.49	14757.59 ± 79.59	0.54

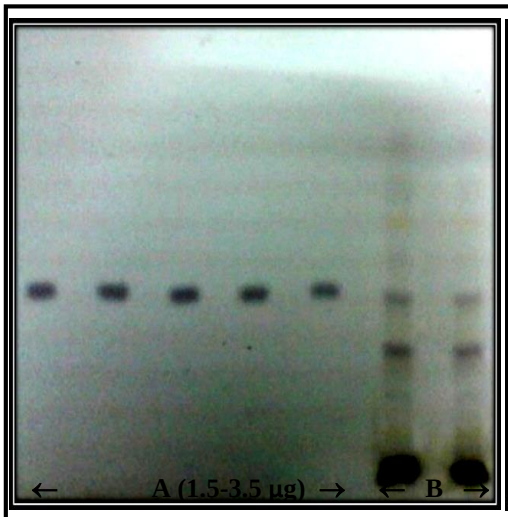

Fig. 7b: Co-chromatography of Lupeol (A) and formulation SJT-ONC-1 (B)

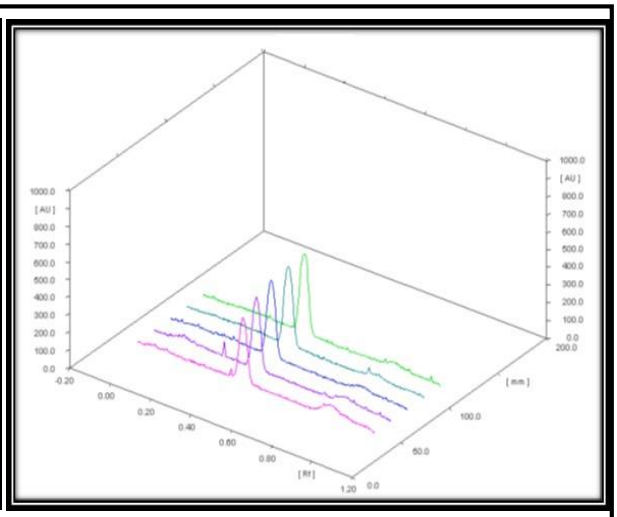

Fig. 7c: 3D-Chromatogram for linearity of Lupeol scanned at 559 nm

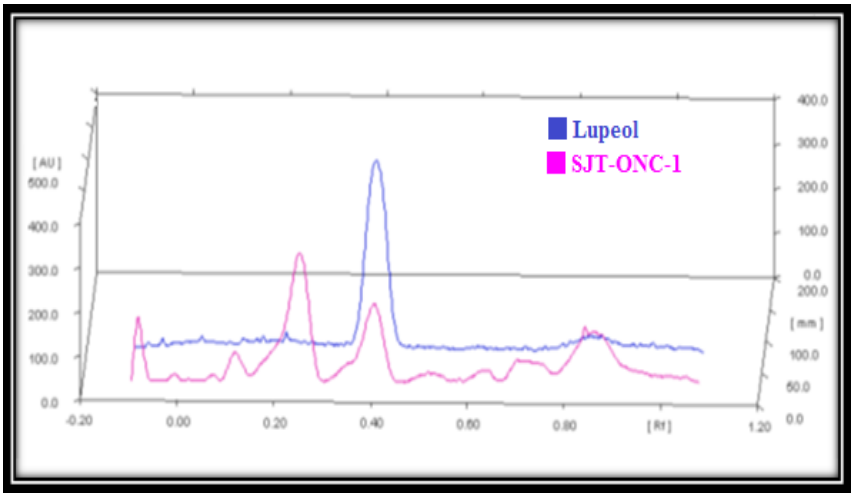

Fig. 7d: Densitometric chromatogram of Lupeol with formulation SJT-ONC-1 scanned at 559 nm

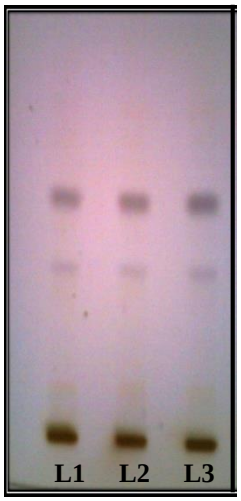

Fig. 7e: Accuracy of Lupeol (L1: 50%, L2: 100%, L3: 150%)

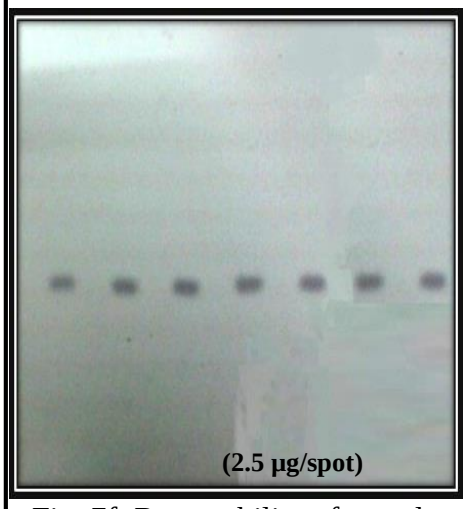

Fig. 7f: Repeatability of sample application of Lupeol

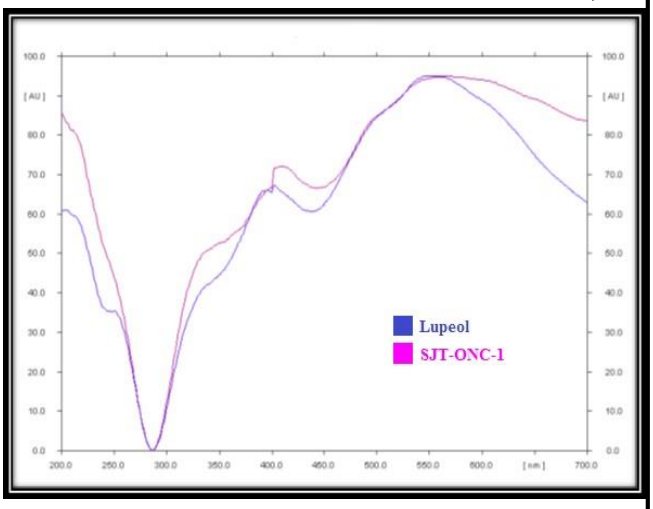

Fig. 7g: Overlain UV spectra of Lupeol with the spot resolving at the same R_f in the formulation SJT-ONC-1

Repeatability

Coefficient of variance for repeatability of measurement of peak area of 7 times measurement of the same spot was found to be 0.246 (Table 23). Similarly the coefficient of variance for repeatability of sample application for seven times was to be 0.302 (Fig 7f, Table 24).

Table 23: Data of repeatability of measurement of peak area of Lupeol

No. of measurement	Area	Mean peak area ± S.D.	% C.V.
1.	14039.9	14050.03 ± 34.59	0.246
2.	14082.2		
3.	14034.5		
4.	14015.3		
5.	14110.2		
6.	14049.3		
7.	14018.8		

Table 24: Data of repeatability of sample application

No. of measurement	Area	Mean peak area ± S.D.	% C.V.
1.	14039.4	14052.77 ± 42.46	0.302
2.	14112.2		
3.	14034.5		
4.	14005.3		
5.	14110.4		
6.	14049.3		
7.	14018.3		

Accuracy (% recovery)

The % recovery of Lupeol was found to be 98.47%, which is satisfactory as shown in Table 25 (Fig. 7e).

Table 25: Data of accuracy for Lupeol

Concentration of Lupeol (µg/spot)		Amount of Lupeol found Mean ± S.D. (n=3)	% Recovery (n=3)	Average % Recovery
Taken	Added			
0.9	0.45	1.3329 ± 0.028	98.73	98.47
0.9	0.9	1.7612 ± 0.018	97.84	
0.9	1.35	2.2242 ± 0.040	98.85	

Specificity

Specificity of the proposed It was observed that the other constituents of formulation did not interfere with the peak of Lupeol. The spectra of standard Lupeol spot and Lupeol in formulation were found to be similar (Fig. 7d,g). Therefore the method was specific.

Limit of Detection

The minimum detectable limit was found to be 0.135 µg/spot.

Limit of Quantification

The minimum quantification limit was found to be 0.407 µg/spot.

Table 26: Summary of Validation Parameters

Sr. No.	Parameters	Results
1	Range	1.5-3.5 µg/spot
2	Linearity	0.9957
3	Precision (% C.V.) Repeatability of Measurement Repeatability of Application Intraday Interday	 0.246 0.302 0.238-0.490 0.416-0.626
4	Accuracy	98.47 %
5	Limit of Detection	0.135 µg/spot
6	Limit of Quantification	0.407 µg/spot
7	Specificity	Specific

Discussion:

The developed HPTLC methods were validated in terms of linearity, precision, repeatability, accuracy, specificity, limit of detection and limit of quantification. Accuracy was validated by analysis of spiked blank and standard addition of samples and precision by performing replicate analyses on a single day and on different days. It is apparent from the results that validation data for the quantitative HPTLC methods for analysis of Lapachol, Bacalein, and Lupeol in SJT-ONC-

So, the simple, precise, accurate and reproducible HPTLC methods were successfully developed and validated for analysis of polyherbal formulations, SJT-ONC-1 and SJT-OB-1 by using multiple markers.

Conclusion

This is the first report for the standardization of polyherbal formulations, SJT-ONC-1 which is going to be available in the market for the treatment of cancer. Incorporation of this will authenticate quality thereby reducing further problems. Quality is inspected at right starting point then it will eliminate all bottlenecks in quality control of the polyherbal formulations to obtain better formulations.

These methods also can be applied by the herbal manufacturers to estimate all the markers like Lapachol, Bacalein, Lupeol in their products as routine quality control and to keep a check on to the batch to batch variation.

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