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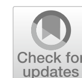

A developed high-performance thin-layer chromatography method for the determination of baicalin in *Oroxylum indicum* L. and its antioxidant activity

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Abstract
A simple, accurate, precise, and specific high-performance thin-layer chromatography (HPTLC) method for the quantitative determination and validation of baicalin in different extracts of *Oroxylum indicum* has been developed for the first time. The mobile phase of acetone—ethyl acetate—water—formic acid (2:10:0.5:0.5, *V/V*) was used for achieving good separation. Densitometric determination was carried out at 318 nm. The calibration curves were found to be linear in the range between 200 and 1000 ng per spot. During the analysis, the ethanolic extract of *O. indicum* showed higher content of baicalin than acetone, DMSO, and DMF extracts. Further, the antioxidant potential of different extracts of *O. indicum* were assessed with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The developed method of HPTLC was validated for specificity, accuracy, precision and linearity. The ethanolic extract has unveiled significant antioxidant activity with a percentage inhibition of 67.34%.

Keywords Baicalin · *Oroxylum indicum* · High-performance thin-layer chromatography (HPTLC) · Antioxidant activity

1 Introduction

Oroxylum indicum (L.) Kurz, tree of Damocles, is a widely distributed deciduous tree throughout the Asian continent [1]. It has been used as a prime ingredient in various ayurvedic medicinal preparations such as *Dasamula*, *Brahma Rasayana*, *Dantyadyarista, Dhanawantara Ghrita, Amartarista, Chyawanprash Awaleha,* and *Narayana Taila* [2, 3]. In folk medicinal practices, the plant has gained significant importance as a blood purifier, astringent, tonic, diuretic, carminative, laxative, and also used for other common problems like diarrohea, allergic dermatitis, and dysentery [4–9].

As per Ayurveda, diversified medicinal properties are ascribed to various parts of the Indian trumpet tree [10]. The plant exhibits antioxidant [11], anti-inflammatory [11], analgesic [11], antimicrobial [14–16], anticancer [10], photocytotoxic [10], antimutagenic [10], antiarthritic [10], immunostimulant [10], and antiproliferative activities [10]. Various other effects like antiallergic [13], antiasthmatic [12], antihelmintic [17], antidiabetic [25], cardioprotective [24], gastroprotective [23], hepatoprotective [18–21], antiobesity [22], and wound-healing [10] have also been explored. The potential against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also been reported [26].

O. indicum contains a broad variety of phytochemicals such as tannins, alkaloids, saponins, sterols, flavonoids, lignins, glycosides, phenols, fats and oils. The active constituent of the *O. indicum* is baicalin [10].

Baicalin, a bioactive natural glycosyloxyflavone having the formula

Syeda Nashvia Adin and Isha Gupta have equal contribution in this article and should be considered joint first author.

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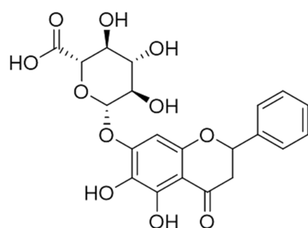

Fig. 1 The chemical structure of baicalin

5,6-dihydroxy-4-oxygen-2-phenyl-4H-1-benzopyran-7-β-D-glucopyranose acid (shown in Fig. 1), has been copiously explored both in vivo and in vitro [27]. It possesses a multifaceted biological profile, including cardioprotective, nephroprotective, hepatoprotective, and neuroprotective activity. It exhibits anti-viral, anti-tumor, anti-inflammatory, antibacterial, antioxidant, antiarthritic, and antipsoriatic effects [28]. Baicalin is also the main ingredient of flavocoxid, which is an approved medical food and classified under Generally Recognised as Safe (GRAS) category by the United States Food and Drug Administration (USFDA) [29]. Since it possesses several health-endorsing properties, it can be considered as a suitable chemical marker for the quality control of *O. indicum*.

Thin-layer chromatography (TLC) is an extensively utilized technique for assessing the identity, content, and purity of herbal drugs. Chromatographic fingerprinting analysis along with analytical method development for phytoconstituents are requisite approaches for the quality control of herbal drugs [30, 31]. Several studies have reported the developed high-performance thin-layer chromatography (HPTLC) method for baicalin from *Scutellaria radix* [32] and *Scutellaria lateriflora* [33]. However, no researcher has developed the HPTLC method for the identification and quantification of baicalin from *O. indicum*. Therefore, our present work deals with the development and validation of a simple, rapid, precise, sensitive, economical and specific HPTLC method for the identification and quantification of baicalin in *O. indicum* as there is a paucity of phytochemical research on this plant. In vitro antioxidant activities of baicalin using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method in different extracts of *O. indicum* were also been assessed.

2 Experimental

2.1 Collection, authentication, and preparation of plant specimen

The stem barks of *O. indicum* L. were procured from the medicinal plant market, Delhi, India. Identification and authentication were done by CSIR-National Institute of Science Communication and Policy Research (NIScPR), New Delhi, India, with authentication number NIScPR/RHMD/Consult/2021/3880-81.

2.2 Chemicals

Analytical grade methanol, ethanol, acetone, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF) were purchased from SD Fine Chem Ltd. (Mumbai, India). Precoated HPTLC plates 60 F254 were acquired from Merck (Mumbai, India). Standard baicalin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Preparation of the extract

The stem barks of *O. indicum* were shade-dried, powdered, and passed through the sieve having 40 mesh size sieve. An aliquot of 10 g of dried plant material was taken and imperiled to a reflux extraction using four different solvents: ethanol, acetone, DMSO, and DMF at a temperature of 50 °C for 60 min, with a drug-to-solvent ratio of 1:10 g/mL. After extraction, solid residue was separated and removed by filtration and the filtrate was being concentrated in a rotary evaporator; each extract was weighed, and the percentage yield was determined.

2.4 Prefatory phytochemical evaluations

The presence of various phytochemicals (alkaloids, carbohydrate, glycoside, phenolic compounds, flavonoids, phytosterols, triterpenoids, steroid, resins and tannin) in ethanolic, acetonic, DMSO and DMF extracts of *O. indicum* were assessed by prefatory phytochemical investigation.

2.5 HPTLC fingerprint analysis

2.5.1 Preparation of standard and sample solutions

The sample solutions were prepared by dissolving 1 g of the dried extracts of *O. indicum* in methanol. A standard baicalin solution (1 mg/mL) was prepared by dissolving 2 mg of baicalin in 2 mL of methanol. The standard solutions and sample solutions (extracts) were filtered by dint of a 0.22 µm Millipore (Burlington, MA, USA) syringe filter for analysis.

2.5.2 Development of a solvent system

For the elution, diverse solvent systems were utilized: chloroform−methanol−water−formic acid (10:2:0.5:0.5, *V/V*), chloroform−acetone−water−formic acid

(10:2:0.5:0.5, *V/V*), chloroform−methanol−water−formic acid (10:2:1.0:0.5, *V/V*), chloroform−acetone−water−formic acid (10:2:1.0:0.5, *V/V*), acetone−ethyl acetate−water−formic acid (2:10:0.5:0.5, *V/V*), and acetone−ethyl acetate−water−formic acid (2:10:1.0:0.5, *V/V*). The solvent system consisting of acetone−ethyl acetate−water−formic acid (2:10:0.5:0.5, *V/V*) was selected based on improved resolution.

2.5.3 Sample application and development of chromatogram

The standard solution and sample solutions were loaded on the 10 × 10 cm plates as 6 mm bands by using a 100 µL syringe with the assistance of a CAMAG (Muttenz, Switzer land) Linomat 5 automatic applicator with an application rate of 150 nL/s in the presence of nitrogen gas. The narrow interspaces between the bands were 10 mm. The plate was developed in a twin-trough critical glass chamber (CAMAG) pre-saturated with the mobile phase consisting of acetone−ethyl acetate−water−formic acid (2:10:0.5:0.5, *V/V*) and removed when the solvent reached up to 85 mm. To recognize the bands, the plate was further dried and heated at 60 °C in the oven for 5 min.

The densitometric study was carried out using CAMAG TLC Scanner 3 with winCATS software (Version 122.0) at a wavelength of 318 nm in reflectance mode by a tungsten (W) lamp. The slit dimension of 5.00 × 0.45 mm was used for scanning with a scanning rate of 20 ms^{-1}.

2.6 HPTLC method validation

The International Council for Harmonisation (ICH) guidelines were adopted for the validation of the parameters presented below.

2.6.1 Precision

5 simulates of pre-determined concentrations of baicalin (1 µg) were spotted on the plate for determining the instrumental precision. Reproducibility (inter-day), repeatability (intra-day) and precision were analyzed at five varying concentrations (0.2−1.0 µg) levels by assessing three individual spots of baicalin spotted on the plate consequently for three days. Apropos of relative standard deviation (RSD), estimated values were expressed.

2.6.2 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for baicalin marker were determined using the following expression:

$$\text{LOQ} = 10 \times \sigma/\text{S}$$
$$\text{LOD} = 3 \times \sigma/\text{S}$$

where S represents the slope of the calibration curve, and σ represents the standard deviation.

2.6.3 Range and linearity

For estimating the linearity, the concentration range of baicalin was taken between 0.2 and 1.0 µg/band. Peak area against concentration was focused on for the analysis of intercept and the least square linear regression. From the calibration plot, values of regression and slope were deduced, and a linear correlation between test concentration and peak area was deduced. Densitometric scanning was carried out to estimate the concentration of baicalin in different extracts of *O. indicum* using a calibration plot.

2.6.4 Specificity

The process specificity was assessed by investigating the test sample, standard, diluent, and solvent system. By matching the R_F values of the developed bands, a spot of baicalin in the extract was established. The spectra at three sundry levels of apex peak, end peak, and start peak of the spot were tallied and the purity of the standard peak was assessed.

2.6.5 Accuracy

By assessing the recovery of baicalin in the sample solution, the accuracy for the process was assessed. Fixed amounts of standard solution were impaled to 50%, 75%, and 100% with a pre-measured amount of the sample solution, and peak area was reckoned. Percentage recovery was determined using the following formula:

$$\text{Recovery} = 100 \times \frac{(\text{Amount found} - \text{Original Amount})}{\text{Amount impaled}}$$

2.6.6 Robustness

The robustness was assessed by using the five kindred concentration levels (0.2, 0.4, 0.6, 0.8 and 1.0 µg/mL). By changing the ratio of the solvent system, amount of solvent system, time duration of saturation, a run-up of mobile phase on the plate, spotting time of standards and development time, the variations were observed in the baicalin chromatogram run.

Table 1 Baicalin content determined by the developed HPTLC−densitometric method in the samples

Sample	HPTLC Baicalin (mg/g)
E1	26.498 mg
E2	8.631 mg
E3	13.883 mg
E4	20.529 mg

E1 Ethanolic extract of *Oroxylum indicum*, *E2* Acetonic extract of *Oroxylum indicum*, *E3* DMSO extract of *Oroxylum indicum*, *E4* DMF extract of *Oroxylum indicum*

2.7 Quantification of baicalin

The amount of baicalin in different extracts was quantified by employing the developed HPTLC method. Standard solutions of baicalin and sample solutions (different extracts of *O. indicum*) were spotted on the TLC plate, further, the plate was placed in the twin-trough chamber for the development. The amount of baicalin was quantified using the winCATS software (Table 1).

2.8 Antioxidant activity using DPPH

The antioxidant potentials of different extracts of *O. indicum* were determined by employing the DPPH method [34] in assistance with ultraviolet (UV) spectrophotometer at 517 nm. The aliquots of 20, 40, 60, 80, and 100 µg/mL of different extracts of *O. indicum* were taken in different tubes, to which methanol (5 mL) and 1 mM DPPH (0.5 mL) were added. Ascorbic acid (vitamin C) was availed as standard in a concentration of 20, 40, 60, 80, and 100 µg/mL. A blank solution containing methanol (5 mL) and 1 mM DPPH (0.5 mL) was made, and all the solutions were incubated for 0.5 h at ambient temperature. The antioxidant potential was assessed using the following equation:

$$\%\ \text{Scavenging} = \frac{\text{Absorbance of blank solution} - \text{Absorbance of test sample}}{\text{Absorbance of blank solution}} \times 100$$

Data are represented as mean of 4, IC_{50} values and sample size were assessed using linear regression analysis.

3 Results and discussion

3.1 Prefatory phytochemical screening

The chemical test and phytochemical screening of *O. indicum* extracts in ethanol, acetone, DMSO, and DMF signifies the presence of flavonoids and phenolic compounds (Table 2). The extractive value of baicalin in different solvents indicates the character and extent of phytobioactive constituents in each solvent (shown in Table 1). The ethanolic extract of *O. indicum* revealed the presence of triterpenoids, flavonoids, glycosides, phenolic compounds, alkaloids, reducing sugar and phytosterols as the major secondary metabolites which may be responsible for its therapeutic potential.

Table 2 Prefatory phytochemical screening of the extracts of *Oroxylum indicum* stem barks in different solvents

Plant extract + test reagent	Ethanol	Acetone	DMSO	DMF
Alkaloids	+	−	−	−
Carbohydrate	+	+	−	−
Glycoside	+	+	−	−
Phenolic compounds	++	+	+	+
Flavanoids	++	+	+	+
Phytosterols	+	+	−	−
Triterpenoids	+	−	−	−
Steroid	+	−	−	−
Resins	−	−	−	−
Tannin	+	+	−	−

+: Present, −: Absent

Table 3 Optimized HPTLC conditions for the analysis of baicalin

HPTLC instrument	CAMAG Linomat 5
Stationary phase	Silica gel 60 G F_{254}
Mobile phase	Acetone−ethyl acetate−water−formic acid (2:10:0.5:0.5, *V/V*)
Observed R_F values	Baicalin (0.49)
Band width	6 mm
Saturation time	30 min
Solvent front distance	85 mm
Plate activation time	15 min
Detection lamp	Deuterium
Detection wavelength	318 nm
Scanning rate	20 mm/s

3.2 HPTLC method development and validation

To quantify the flavonoid compounds in the different extracts of *O. indicum*, a validated HPTLC method was

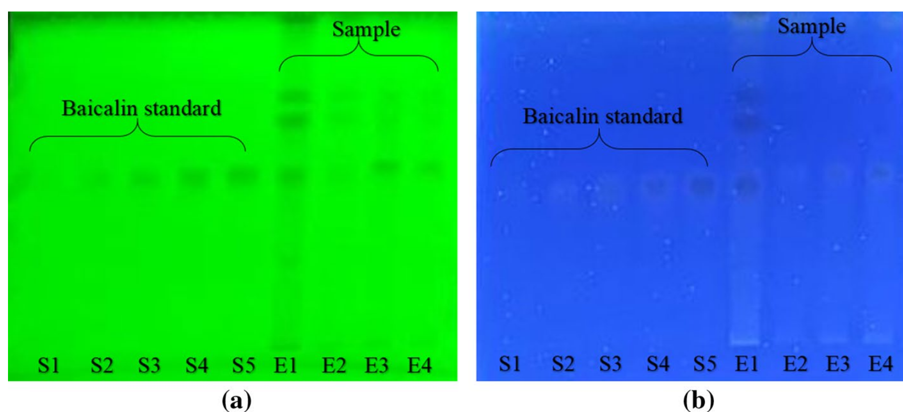

Fig. 2 Developed HPTLC plate **a** at λ_{max} 254, and **b** at λ_{max} 366 nm. Lanes S1−S5: baicalin standard; Lane E1: Ethanolic extract of *Oroxylum indicum*; Lane E2: Acetonic extract of *Oroxylum indicum*; Lane E3: DMSO extract of *Oroxylum indicum* and Lane E4: DMF extract of *Oroxylum indicum*

established for the concurrent quantification of baicalin. ICH guidelines were followed for process validation. The experimental circumstances like the movement of the solvent front, band size, chamber saturation time, and slit length were significantly varied. Further, the quintessential circumstances were selected (shown in Table 3).

The standard and sample solutions were spotted on the 10 × 10 cm HPTLC plate and further developed using the above-established method. The developed HPTLC plate is shown in Fig. 2 and the chromatograms of standard baicalin (Fig. 3) and extract of *O. indicum* in ethanol, acetone, DMSO, and DMF are shown in Fig. 4.

3.2.1 Precision

The method reproducibility was determined using the sample from the same homogenous batch by different analysis, and inter-day and intra-day precision was used to determine the repeatability. To ascertain the method's effectiveness, suitability tests were done on a freshly prepared mixture of pre-analyzed ethanolic extract of *O. indicum* spiked with standard baicalin solution. The measurement of peak area and repeatability of sample application were denoted by *%RSD*. The *%RSD* of inter-day and intra-day analysis are shown in Table 4. Inter-day precision based on baicalin content was found to be 0.418*%RSD*, whereas intra-day precision based on baicalin content was found to be 0.396*%RSD*, respectively. Finally, the TLC−densitometric method was

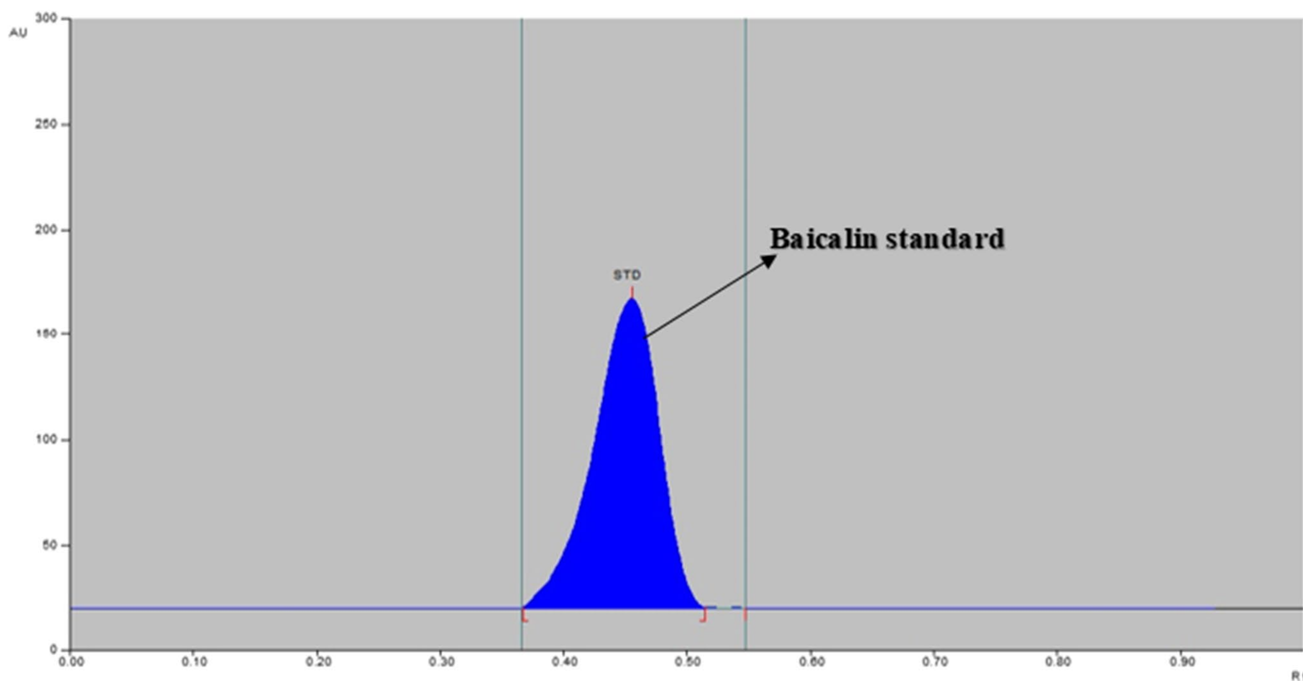

Fig. 3 HPTLC chromatogram of standard baicalin at λ_{max} 318 nm

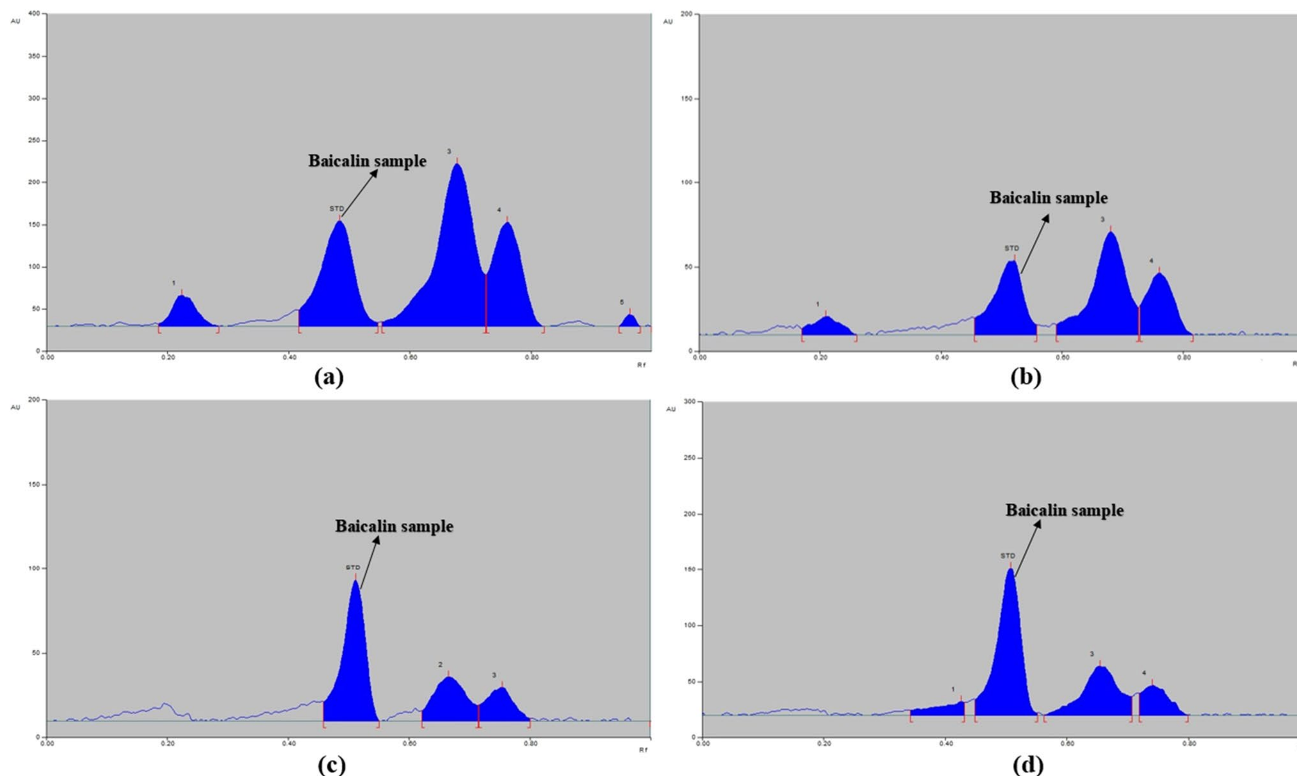

Fig. 4 HPTLC chromatogram at λ_{max} 318 nm of **a** Ethanolic extract of *Oroxylum indicum*; **b** Acetonic extract of *Oroxylum indicum*; **c** DMSO extract of *Oroxylum indicum* and **d** DMF extract of *Oroxylum indicum*

reckoned to be precise based on the results obtained from the inter-day and intra-day precision evaluation study.

3.2.2 LOD and LOQ

The LOD and LOQ were estimated using standard deviation of blank and slope of the calibration curve acquired from the standard solution of baicalin. The LOD and LOQ for baicalin were estimated to be 0.056 and 0.188 µg/band, respectively (shown in Table 4). The superimposed in situ UV spectra of baicalin and different extracts of *O. indicum* showed a good correlation (shown in Figs. 5 and 6). The peak clarity of baicalin was recognized by matching the in situ UV spectra of bands at different peaks.

3.2.3 Range and linearity

A good linear correlation was established between quantity achieved and peak area for baicalin at 318 nm having a concentration range between 0.2 and 1.0 µg/spot and R^2 correlation coefficient of 0.99499 (shown in Table 4). Linearity specifies a linear correlation between the analyte concentration and its signals in the test sample range (shown in Fig. 7).

3.2.4 Specificity

The peak purity of baicalin was determined by matching the spectra at three sundry levels of apex peak, end peak and start peak of the spots. A good correlation was acquired between the sample and standard. Therefore the method can be reckoned specific as the peak of baicalin in different extracts of *O. indicum* did not interfere with the peak of standard baicalin.

3.2.5 Accuracy (recovery studies)

For recovery studies, the method of standard addition was employed. At two different levels, the recoveries of added standards were determined. The results of recovery studies and content estimation of baicalin from ethanolic extract of *O. indicum* after impaling it with 200–400 ng/spot of the auxiliary standard are shown in Table 5. At two different levels, the average percent recoveries were found in the range of 98.96–99.52%, which indicates the reproducibility and reliability of the method, respectively.

Table 4 Regression data of baicalin and intra-day and inter-day precision of the HPTLC method developed for *Oroxylum indicum*

Parameters	Value baicalin
Linearity range (µg/spot)	0.2−1.0 µg/spot
λ_{max}	318 nm
Regression coefficient	$Y=-354.303+6.934x$
Correlation coefficient (R^2)	0.99499
Slope (µg/spot)	354.303
Intercept	6.934
Limit of detection (µg/spot)	0.056
Limit of quantification (µg/spot)	0.188
R_F value	0.49
% Recovery of baicalin	99.17%

Intra-day precision ($n=3$)

Compounds	Conc. (µg/uL)	Area	Mean area	±SD	*%RSD*
Baicalin	0.2	1274.84	1286.27	10.41	0.81
	0.4	2142.92	2155.63	12.92	0.60
	0.6	3912.62	3925.53	11.63	0.30
	0.8	5052.78	5062.82	8.76	0.17
Mean *%RSD*	1.0	6758.76	6754.82	6.94	0.10 0.396

Inter-day precision ($n=3$)

Compounds	Conc. (µg/uL)	Area	Mean area	±SD	*%RSD*
Baicalin	0.2	1251.35	1260.68	8.82	0.7
	0.4	2126.03	2140.57	12.64	0.59
	0.6	3892.85	3909.51	14.47	0.37
	0.8	5025.26	5040.32	13.08	0.26
Mean *%RSD*	1.0	6736.10	6747.26	11.40	0.17 0.418

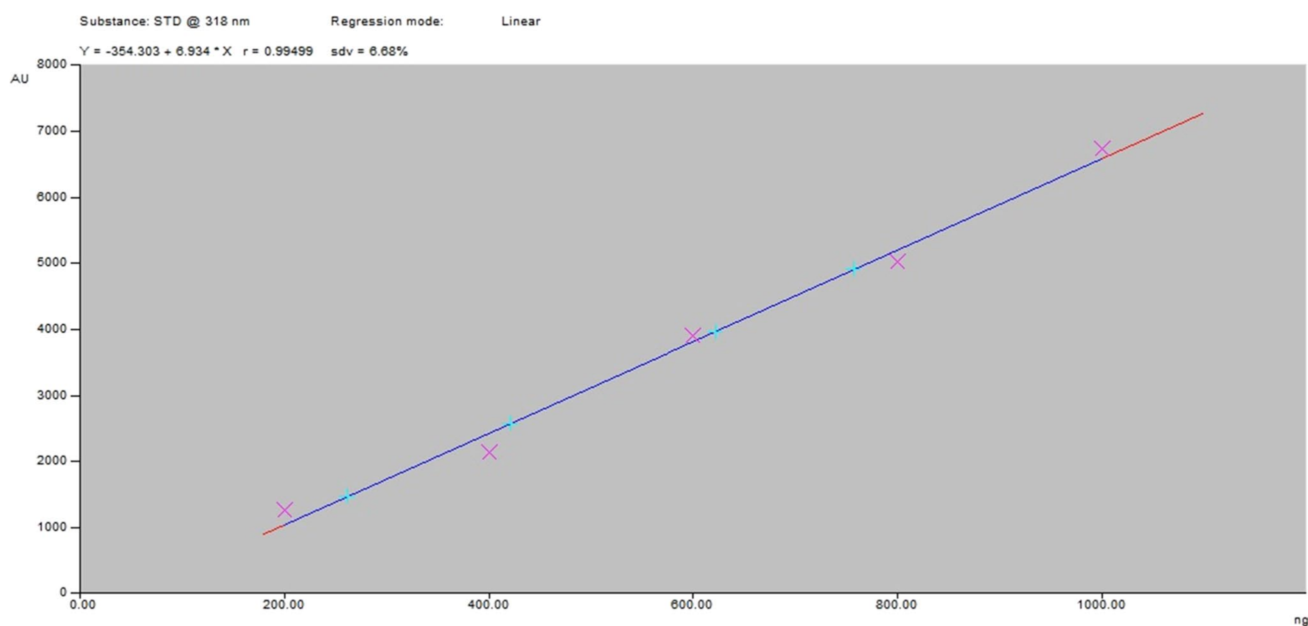

Fig. 5 3D overlaid HPTLC chromatograms of different extracts of *Oroxylum indicum* and standard baicalin at λ_{max} 318 nm

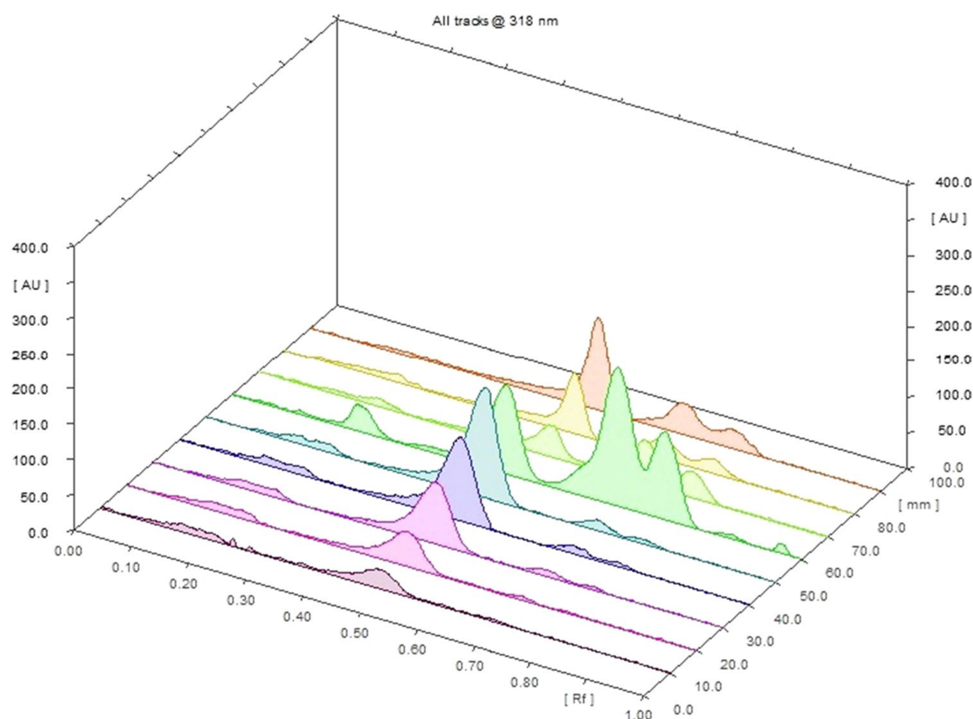

Fig. 6 Overlaid spectra of standard baicalin and sample between the ranges of 200−400 nm

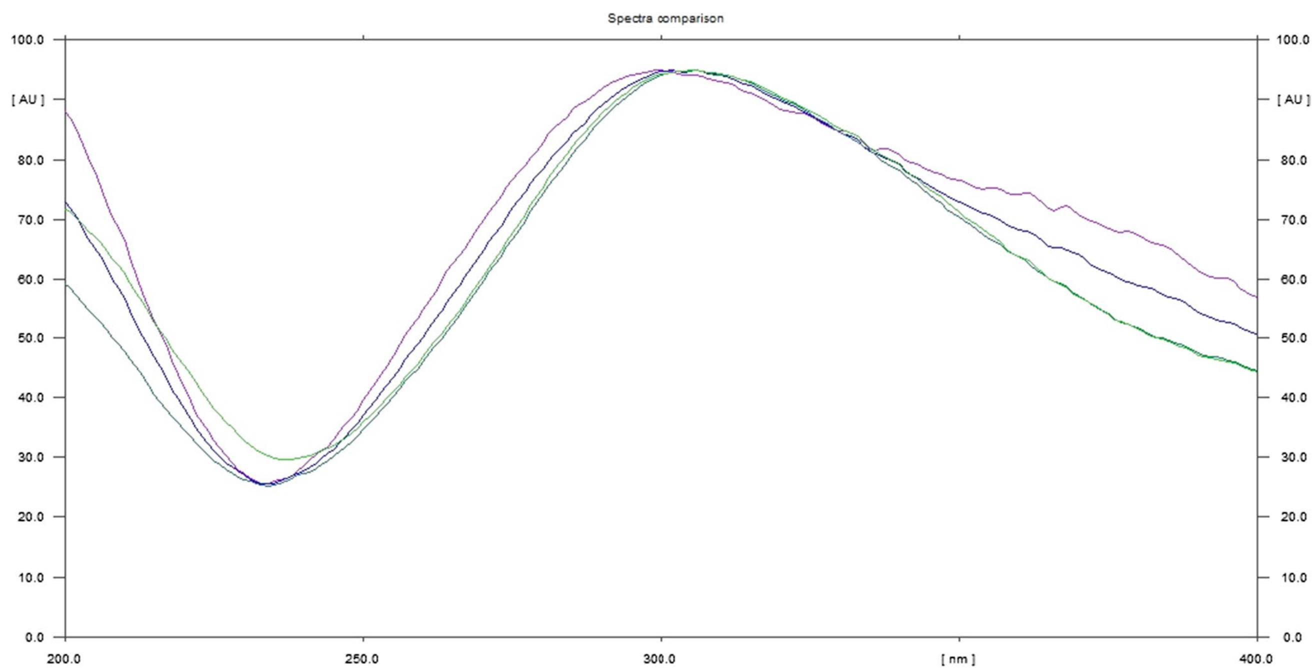

Fig. 7 Calibration plot for baicalin at λ_{max} 318 nm

Table 5 Recovery studies

Standard	Amount added	Amount recovered (% mean)	SD	*%RSD*
Baicalin	50	98.96	0.17	0.18
	75	99.05	0.24	0.25
Mean	100	99.52	0.27	0.28
		99.17		

3.2.6 Robustness

The SD of peak areas was determined for every parameter, and *%RSD* was reckoned to be < 2%, indicating the method's robustness. This also shows that the propound method was reproducible and precise.

3.3 Antioxidant activity

The antioxidant potential of the different extracts of *O. indicum* was scrutinized by employing DPPH radical scavenging activity. Test samples were estimated by utilizing DPPH free radicals in the form of purple color in test samples. When the DPPH solution is exposed to tested samples, it donates a hydrogen atom and the resultant gets converted into its reduced form, i.e., diphenylpicrylhydrazine (yellow color non-radical). The antioxidant potential of the different extracts were collated with vitamin C (standard antioxidant compound). The IC_{50} value was quantified graphically for determining the DPPH scavenging activity of different extracts of *O. indicum*.

The ethanolic extract of *O. indicum* showed maximum DPPH scavenging activity at a concentration of 100, i.e., 67.34% compared to *O. indicum* extracts in acetone, DMSO, and DMF, which were 53.27%, 54.28%, and 50.67%. The standard vitamin C showed 88.15% activity.

From Fig. 8, it is intuited that the DPPH scavenging activity of the extracts increases as the concentration of extracts increases and ethanolic extract showed maximum activity compared to other extracts; it can be divulged that the antioxidant potential of the ethanolic extract of *O. indicum* may be accredited with the presence of phenolic compounds and flavonoids.

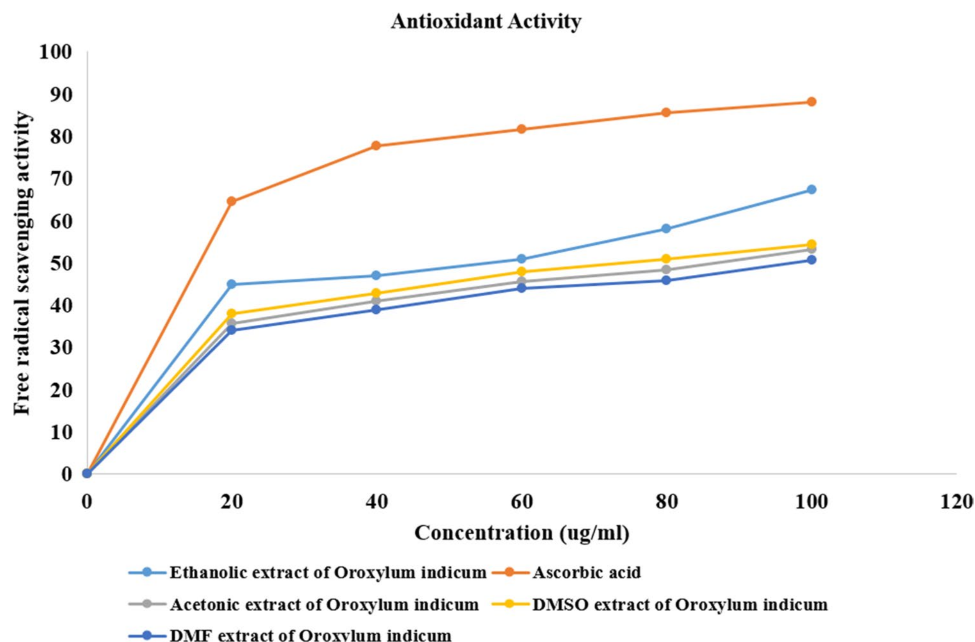

Fig. 8 Dose-dependent scavenging of DPPH radicals by the different extracts of *Oroxylum indicum* compared with standard drug ascorbic acid. Each value represent mean ± SD ($n = 3$)

4 Conclusion

A simple validated HPTLC method for the quantification of baicalin in *O. indicum* has been developed. The established method is precise, specific, simple, time-saving, and cost-effective. The proposed HPTLC method can be used as a quality control tool for the quantification of bioactive baicalin in different plant extracts and polyherbal formulations. From DPPH scavenging activity, it was found that the plant exhibits significant antioxidant activity and further research on characterization, separation, and pharmacological evaluation of other antioxidant compounds in different extracts of *O. indicum* may help the researchers to find new chemical entities with therapeutical potential.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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