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Article

HPLC and HPTLC Methods by Design for Quantitative Characterization and *in vitro* Anti-oxidant Activity of Polyherbal Formulation Containing *Rheum emodi*

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Safoof-e-Pathar phori (SPP) is a traditional polyherbal formulation and has been used since long time for its anti-urolithiatic activity. It contains three plant constituents *Didymocarpous pedicellata*, *Dolichous biflorus* and *Rheum emodi*. Emodin and chrysophanic acid were selected as chemical markers for SPP and quantified using simultaneous HPTLC and RP-HPLC methods in *R. emodi* and in SPP. The simultaneous methods were found linear $r^2 = 0.991$ in a wide range (10–800 ng spot^{-1} with HPTLC, 5–500 μg mL^{-1} with HPLC) precise, accurate and robust for both the drugs. Anti-oxidant activity of SPP, *R. emodi* as well as standard emodin and chrysophanic acid were determined by using DPPH (2,2-diphenyl-1-picryl hydrazyl radical), which showed better activity of *R. emodi* ($IC_{50} = 12.27$) extract when compared with SPP ($IC_{50} = 32.99$) and standard drugs ($IC_{50} = 66.81$). The robustness of methods were proved by applying the Box–Behnken response surface design software and other validation parameters evaluated were satisfactorily met; hence, the developed method found suitable for application in the quality control of several formulations containing emodin and chrysophanic acid.

Introduction

Plant-derived products supported by scientific evidence have been gaining user assurance as complementary and alternative medicine (CAM) for prevention and cure of disease. Traditional medicines have been used from ancient times, but they have not been standardized or validated for their efficacy. Trade of herbal products is rising rapidly as a result of popularization of CAM, but they lag behind in the case of the knowledge on active principles, validation etc. Herbs and herbal medicines are most prone to variation in their phytochemical profile due to variability in climatic conditions, maturity, post-harvest processing, storage, stability etc. (1).

It is extremely important to standardize the formulation and its ingredients, based on marker compounds specific to each plant followed by validation for their efficacy. Rhizomes of *Rheum emodi* have been used in Unani system of medicine either alone or as an ingredient of many polyherbal formulations for treating various ailments.

Safoof-e-Pathar phori (SPP) is an Unani polyherbo-mineral formulation and has been used in Unani system of medicine for its anti-urolithiatic activity (2). It is a powdered formulation, which contains six different plant/mineral constituents: Pathar phori (*Didymocarpous pedicellata*) (3), Kulthi (*Dolichous biflorus*) (4), Revand chini (*Rheum emodi*) (5), Namak turb (*Raphanus sativus*), Jawakhar (Potassium carbonate) and Shora qalmi (Potassium nitrate).

Rheum emodi contains a large number of hydroxyanthraquinone derivatives such as chrysophanic acid, emodin, physcion, aloe emodin, emodin glycoside, rhein etc. (6), which are reportedly known for various biological activities such as anti-oxidant (7), anti-microbial (8), anti-fungal (9), cytotoxicity (10), nephroprotective (11) and anti-viral activities (12). This has also been used as a purgative, stomachic, astringent and for the treatment of chronic bronchitis, asthma and in certain skin diseases (13).

The *D. pedicellata* contains chalcones, flavones and didymocarpene (14), whereas *D. biflorus* seeds contain a dipeptide pyroglutamyl glutamine as active principal which are rare and not available in pure form for analysis purpose (15). *Rheum emodi* is a vegetable, however often thought as fruit. It is used in desserts, jams, jellies as well as sauces due to its tart flavor. Its stalks have also been consumed with sugar; however, it may cause poisoning in people with susceptibility to oxalic acid (16).

Anti-oxidants from natural sources play a paramount role in helping endogenous anti-oxidants to neutralize oxidative stress. Several epidemiological, clinical and experimental data suggested that plant-based anti-oxidants effects on prevention of chronic diseases (17). Studies have shown that treatment with anti-oxidants prevents calcium oxalate (CaOx) deposition in the kidney and reduce oxalate (Ox) excretion (18). Improved renal anti-oxidant status leads to cell membrane integrity and thus prevents the recurrence of renal stone (19). The emodin had shown to be a potent inhibitor of superoxide radicals and probably depends on scavenging hydroxyl radicals (20).

There are various analytical methods reported for determination of chrysophanic acid and emodin (the major hydroxyanthraquinones of *R. emodi*) in plant extracts such as high-speed counter-current chromatography (21), capillary zone electrophoresis (22), high-performance thin layer chromatography (23, 24) and most commonly used high-performance liquid chromatography (25, 26). However, there has been no report till date for simultaneous determination of emodin and chrysophanic acid content in traditional Unani/Ayurvedic formulations by HPTLC or by using HPLC. Therefore, an attempt has been made for quantitative characterization of traditional Unani formulation (SPP) for their emodin and chrysophanic acid content using developed and validated HPTLC as well as RP-HPLC methods, as per the ICH guidelines with improved range of linearity when compared with the methods reported for extracts due to large variation in contents of multi-component traditional formulations. The anti-oxidant potential of traditional polyherbal

formulation and its constituents *R. emodi*, emodin and chrysophanic acid was also investigated by using DPPH (96-well plate) method to validate its anti-urolithiatic activity.

Experimental

Materials and chemicals

The rhizomes of *R. emodi* were collected from Khari Baoli local drug market New Delhi and were authenticated by Dr. H.B. Singh, Ref. NISCAIR/RHMD/1327/129, New Delhi. It was dried and powdered in an electric grinder. The powdered rhizomes of *R. emodi* and SPP were used for the extraction of emodin and chrysophanic acid. Standard emodin, chrysophanic acid and DPPH were procured from Sigma Aldrich (USA). All solvents used were of analytical and HPLC grade (Merck, Mumbai, India). Membrane filters (0.45 μm) used for filtration were obtained from Millipore, Germany.

Sample preparation

The powdered SPP and *R. emodi* powder (1.0 g each) were extracted separately with 15 mL of methanol by sonication for 30 min at 45°C. The process was repeated twice to ensure complete extraction. The extracts obtained were pooled and dried under reduced pressure. The residue obtained for each extract was redissolved separately in 25 mL of HPLC grade methanol and subjected to RP-HPLC and HPTLC analysis for quantitative determination of emodin and chrysophanic acid as well as for determination of anti-oxidant potential in different dilution. Prior to use, all samples were filtered through a 0.45 μm nylon membrane filter.

HPTLC instrumentation

Chromatography was performed on 20 × 10 cm aluminum HPTLC plates coated with 0.2 μm layers of silica gel 60F-254. Samples were applied as bands of 4.0 mm wide, 8.3 mm apart by the use of a CAMAG (Switzerland) Linomat V sample applicator fitted with a microliter syringe. A constant application rate of 120 nL s^{-1} was used. Linear ascending development, to a distance of 80 mm, with toluene:ethyl acetate:formic acid (10:10:1.0, v/v/v) as mobile phase, was performed in a 20 × 10 cm twin-trough glass chamber. Before chromatography, the chamber was saturated with mobile phase vapors for 15 min; 15 mL mobile phase was used for each development. After the development, plates were dried in a current of air by means of an air dryer. Densitometric scanning at 430 nm was performed with a CAMAG TLC scanner III operated by winCats software. The source of radiation was a tungsten lamp, the slit dimensions were kept at 4.0 × 0.30 mm and the scanning speed was 10 mm s^{-1}.

HPLC instrumentation

The HPLC-PDA method for the determination of emodin and chrysophanic acid was carried out on a Waters Alliance e2695 separating module (Waters Co., MA, USA) using photodiode array detector (Waters 2998) with autosampler and column oven. The instrument was controlled by the use of Empower software installed with equipment for data collection and acquisition. Compounds were separated on a C18 reverse phase column (25×4.6 mm, particle size 5.0 μm, Merck, Germany) maintained at room temperature. The mobile phase consisted of methanol and 0.1% ortho-phosphoric acid in the ratio of 80:20 (v/v). The flow rate was 1.0 mL min^{-1}; and column was maintained at room temperature. Analysis was performed at a wavelength of 430 nm using 10 μL of injection volume.

Method validation

The developed HPTLC and RP-HPLC methods were validated as per the ICH guidelines (27). The specificity of the methods was ascertained by comparing the retention data and spectra of the peaks with those of reference emodin and chrysophanic acid. The peak purity was assessed by comparing the spectra at three different peak levels, i.e. start, apex and end positions of the peak corresponding to emodin and chrysophanic acid.

The linearity of HPTLC and RP-HPLC methods was obtained by determining the detector responses against a series of varying concentrations of reference emodin and chrysophanic acid. Five analyses per concentration were conducted and calibration curves were plotted.

Limits of detection (LOD) and limit of quantification (LOQ) of the methods were calculated using the equations:

$$\mathrm{LOD} = \frac{3.3\sigma}{S},$$

$$\mathrm{LOQ} = \frac{10\sigma}{S},$$

where σ is the standard deviation of response and S the slope of the calibration curve.

The precision of the methods was validated in terms of repeatability and intermediate precision. Inter- and intra-day precisions were done by preparing and applying three different concentrations of samples (in triplicate) in the same day and in three different days, respectively. The results of repeatability are expressed as % RSD.

The accuracy of the methods was determined by standard addition techniques. Known amounts of reference emodin and chrysophanic acid in a range of low, medium and high concentrations were added to pre-analyzed samples of SPP and further analyzed under the optimized conditions. Additional experiments for each concentration were performed in triplicate and the accuracy was calculated as the percentage of analyte recovered. Three analyses per concentration were performed and mean ± SD were determined.

The robustness of the method was performed by introducing very small changes in the analytical methodology at a single concentration level (100 μg mL^{-1}) in two different ways, i.e. by making deliberate changes in the flow rate and detection wavelength of the analysis in HPLC, whereas mobile phase composition and detection wavelength in HPTLC. In the present study, the robustness was evaluated by using the Box–Behnken response surface design (28). The design simultaneously evaluated the effects of the three important parameters on the peak area: flow rate of the mobile phase, detection wavelength and temperature of the column oven in HPLC, whereas mobile phase composition, detection wavelength and temperature of the

analysis in HPTLC. Design Expert version 7.1.6 (Stat-Ease, Minneapolis, MN, USA) was used to evaluate the results. Three-dimensional graphs represented the peak area dependence on the flow rate of the mobile phase, detection wavelength and temperature of the column. Effects of the selected factors were evaluated over a range of conditions by determining the maximum area response of the emodin and chrysophanic acid peaks.

DPPH radical scavenging activity

The free radical scavenging activity of the extracts of samples was estimated by the method reported by Blois (29), with some modifications. In brief, the samples were dissolved in methanol. One hundred microliter of 0.5 mM 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH) in methanol was mixed with 100 µL of samples in 96-well plate at various concentrations (0.781, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0 and 100.0 µg, for SPP and *R. emodi*: 2.34, 4.68, 9.37, 18.75, 37.5, 75.0, 150.0 and 300.0 µg for emodin and chrysophanic acid: 0.781, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µg for ascorbic acid) in triplicate. The 96-well plate was allowed to stand at room temperature for 30 min in dark. The control was prepared as described above without sample or standards, whereas blank was prepared without DPPH containing sample and methanol. The changes in absorbance of all the samples and standards were measured at 540 nm in Elisa plate reader (Bio Rad 680). A lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was used as standards.

Radical scavenging activity was calculated using the corrected ODs (COD) of control and samples as per the below mentioned formula:

$$\text{COD control} = \text{OD control} - \text{OD control blank}$$

$$\text{Radical scavenging activity } (\%) = \frac{\text{COD control} - \text{COD sample}}{\text{COD control} \times 100}$$

Results

Optimization of HPTLC densitometric method

For the optimization of HPTLC densitometric method, it was necessary to select first a mobile phase, which could be used for routine HPTLC analysis of formulations containing emodin and chrysophanic acid. Initially, mixtures of toluene and ethyl acetate in different proportions have produced good resolution with tailing, addition of formic acid improved the resolution between bands and finally, the mobile phase consisting of toluene:ethyl acetate:formic acid (10:10:1.0, v/v/v) was found to produce a sharp and well-defined peaks of emodin and chrysophanic acid at Rf 0.72 and 0.84, respectively (Figure 1).

RP-HPLC-PDA analysis of emodin and chrysophanic acid

The mobile phase consisted of methanol and 0.1% orthophosphoric acid 80:20 found better for resolution between emodin and chrysophanic acid at retention times of 11.1 and 16.1 min, respectively (Figure 1). The same conditions were followed for the separation of emodin and chrysophanic acid present in the crude methanolic extract of rhizomes of *R. emodi* and SPP.

The obtained peak was calculated for theoretical plates and found 3269, tailing factor 1.48, resolution 5.18 and asymmetry was 2.231. The values obtained ($N>2000$, $T\leq 2$, $Af\leq 2$) showed chromatographic conditions are appropriate for separation and quantification of these compounds.

Validation of HPTLC and RP-HPLC methods

The developed HPTLC and RP-HPLC methods were validated as per the ICH guidelines in terms of their linearity, LOD and quantification, precision, accuracy and robustness as detailed below.

Linearity

Linearity between the detector response and concentration of emodin and chrysophanic acid in HPTLC analysis was evaluated and found to be linear over a range of 10–800 ng spot^{-1} with a correlation coefficient $r = 0.9995$ and 0.9996, respectively. The LOD and LOQ of the methods were 3.63, 3.85 and 10.0, 10.0 ng spot^{-1}, respectively.

The concentration of emodin and chrysophanic acid with respect to the peak area at 430 nm was linear in the range of 5–500 µg mL^{-1}, having the correlation coefficient of 0.9998 and 0.9978, respectively by the RP-HPLC method. The LOD and LOQ were found as 1.78, 1.96 and 5.0, 5.0 µg mL^{-1} for emodin and chrysophanic acid, respectively.

Precision

The results of precision of the methods are presented in Table I for HPTLC and RP-HPLC methods, which were found in acceptable range.

Accuracy as recovery

The accuracy of the methods was determined by standard addition method as percentage recovery by reanalyzing the samples after adding 50, 100 and 150% of emodin and chrysophanic acid (Table II). The recovery results showed 95–105% using HPTLC, whereas 97–103% using RP-HPLC for both the drugs.

Robustness

The robustness of HPTLC and HPLC methods was evaluated by using the Box–Behnken response surface design. The Design Expert software proposed the following polynomial equations for HPTLC and HPLC emodin and chrysophanic acid peak area:

Peak area emodin by HPTLC = +5447.67 + 66.50 A + 52.38 B + 181.13 C + 25.50 AB + 2.00 AC − 33.25 BC − 25.21 A2 + 27.04 B2 + 27.54 C2

Peak area chrysophanic acid by HPTLC = +4342.33 + 43.25 A + 41.12 B + 6.88 C − 9.25 AB − 3.25 AC − 20.00 BC + 1.33 A2 + 12.58 B2 + 3.58 C2

Peak area emodin by HPLC = +2.570E + 005 − 1581.88 A + 977 .50 B + 809.63 C + 814.50 AB + 793.75 AC − 1811.50 BC + 2051.71 A2 + 1599.46 B2 + 1459.71 C2

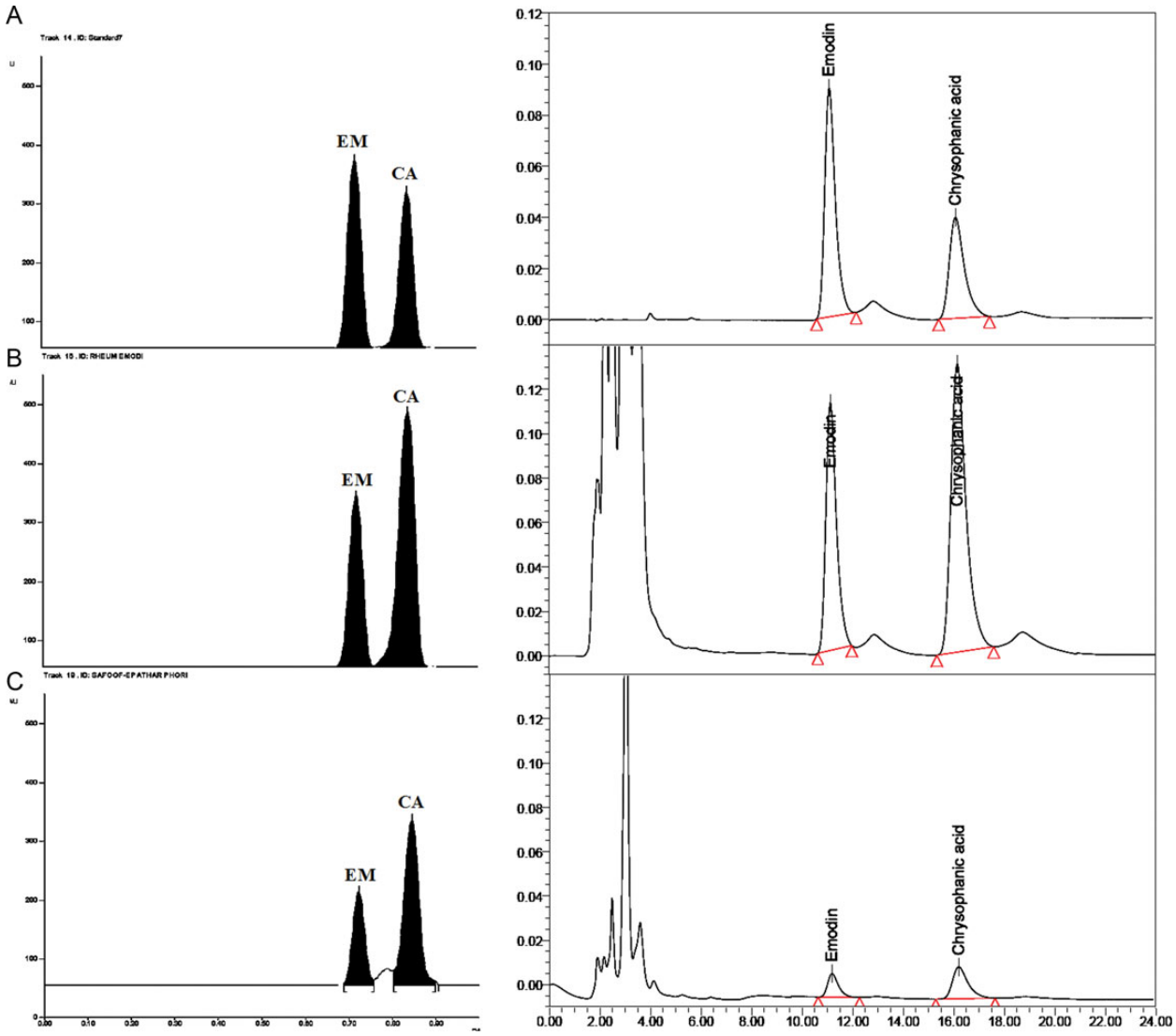

Figure 1. HPTLC and HPLC chromatograms at 430 nm standard emodin and chrysophanic acid (A), *Rheum emodi* rhizome extract (B) and SPP extract (C).

Table I
Precision (% RSD) of emodin and chrysophanic acid

Precision	Emodin (% RSD)		Chrysophanic acid (% RSD)	
	HPTLC	HPLC	HPTLC	HPLC
Intra-day ($n = 5$)	2.03	1.11	1.39	1.00
	0.65	0.50	1.89	1.50
	1.09	1.13	0.84	0.79
Inter-day ($n = 5$)	1.67	1.82	2.09	1.46
	0.53	1.22	0.93	1.84
	1.98	1.99	1.89	1.33

Table II
Accuracy of emodin and chrysophanic acid

Excess spike concentration added (%)	% recovery of Emodin		% recovery of Chrysophanic acid	
	HPTLC	HPLC	HPTLC	HPLC
50	95.28 ± 1.04	97.64 ± 0.16	97.13 ± 1.04	98.4 ± 0.45
100	102.39 ± 0.78	101.09 ± 0.23	101.82 ± 0.93	100.88 ± 0.29
150	105.46 ± 1.87	102.24 ± 0.24	104.17 ± 2.47	102.96 ± 0.93

Peak area chrysophanic acid by HPLC = +1.687E + 005 + 1828.37A−835.88B+1764.75C+1085.25AB−1136.00AC−1583.00 BC+209.63A2−1346.87B2−1792.13C2.

In HPTLC, A is the analysis temperature (K), B the mobile phase composition and C the detection wave length (nm). According to the equations, mobile phase appeared to have more effect on the peak area than detection wavelength and temperature of analysis. Mobile phase composition is directly proportional to the peak area. The detection wavelength and temperature were also found to affect the peak area but less than the mobile phase composition.

In HPLC, A is the column temperature (K), B the flow rate and C the detection wave length (nm). According to the equations,

flow rate appeared to have more effect on the peak area than detection wavelength and temperature. The flow rate is directly proportional to the peak area, as the flow rate increases, the peak area also increases. The detection wavelength and column temperature were also found to increase the peak area but less than the mobile phase effect on the peak area (Figures 2 and 3).

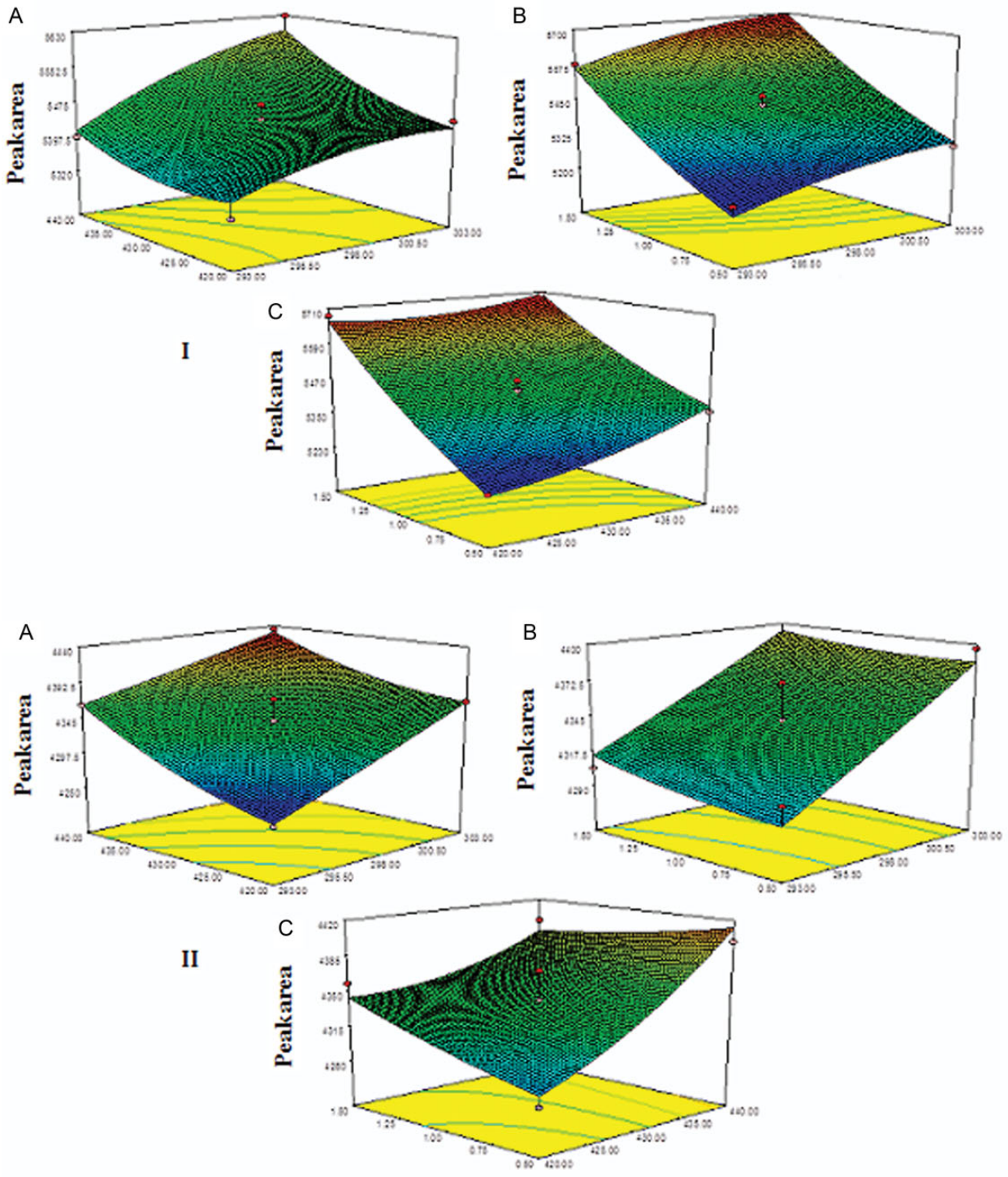

Figure 2. (I) Three-dimensional graphs for emodin by HPTLC: peak area = *f*(detection wave length, temperature of the analysis) (A); peak area = *f*(mobile phase composition, temperature of the analysis) (B); peak area = *f*(mobile phase composition, detection wave length) (C). (II) Three-dimensional graphs for chrysophanic acid by HPTLC: peak area = *f*(detection wave length, temperature of the analysis) (A); peak area = *f*(mobile phase composition, temperature of the analysis) (B); peak area = *f*(mobile phase composition, detection wave length) (C).

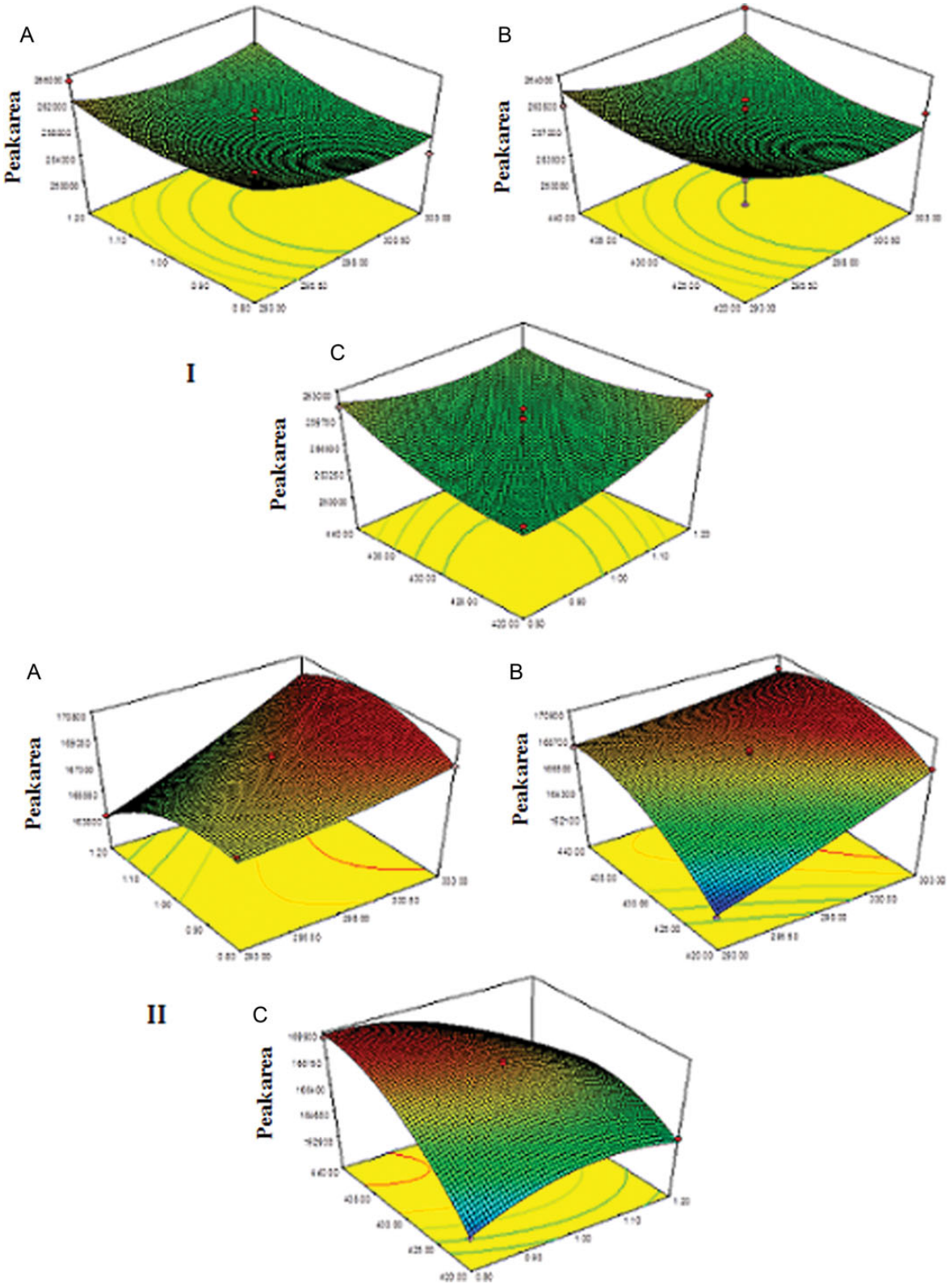

Figure 3. (I) Three-dimensional graphs for emodin by HPLC: peak area = *f*(flow rate of the mobile phase, temperature of the column) (A); peak area = *f*(detection wave length, temperature of the column) (B); peak area = *f*(detection wave length, flow rate of the mobile phase) (C). (II) Three-dimensional graphs for chrysophanic acid by HPLC: peak area = *f*(flow rate of the mobile phase, temperature of the column) (A); peak area = *f*(detection wave length, temperature of the column) (B); peak area = *f*(detection wave length, flow rate of the mobile phase) (C).

Analysis of emodin and chrysophanic acid in SPP and rhizome of R. emodi

The analysis of emodin and chrysophanic acid in SPP using HPTLC showed 45 and 85 mg kg^{-1} whereas HPLC showed 48 and 77 mg kg^{-1}, respectively.

Similarly, analysis of emodin and chrysophanic acid in *R. emodi* samples using HPTLC showed 3350 and 7060 mg kg^{-1} whereas HPLC 3380 and 7800 mg kg^{-1}, respectively. HPTLC and RP-HPLC chromatograms of samples are given in Figure 1.

Anti-oxidant activity of R. emodi and its polyherbal formulation

The effect of anti-oxidants on DPPH is thought to be due to their hydrogen donating ability. The 96-well DPPH anti-oxidant potential of traditional formulation, *Rheum emodi*, chrysophanic acid and emodin were carried out in different concentration. The IC_{50} values were determined by the dose–response curve (% inhibition vs log dose) (Figure 4) which proved anti-oxidant potential of standard ascorbic acid ($IC_{50} = 3.60$ μg), *R. emodi* ($IC_{50} = 12.27$ μg) and SPP ($IC_{50} = 32.99$ μg). The anti-oxidant potential of emodin ($IC_{50} = 87.65$ μg) and chrysophanic acid ($IC_{50} = 66.81$ μg) was found poor when compared with the extract proving synergistic effect of different other compounds and better anti-urolithiatic activity of extract when compared with pure compounds.

Discussion

HPTLC is a widely used technique for herbal drugs because of its high sample throughput at low operating cost and analytical assurance by means of multi-level calibration, which yielded précised results in measurement of number of analysis.

There are only two HPTLC methods reported for these anthraquinone glycosides in plant extracts, Kumar *et al.* (9) reported HPTLC of chrysophanic acid, whereas Singh *et al.* (24) reported HPTLC of physcion, chrysophanic acid, emodin and chrysophanic acid but methods were lacking proper validation in terms of precision, robustness, specificity range etc, and also analysis in polyherbal formulation (30). Therefore, we proposed a well-validated analytical methods to quantify the content of emodin and chrysophanic acid in traditional multi-component polyherbal formulation, which have not taken up by any researcher till date. The literature survey revealed no HPLC method for the simultaneous analysis of emodin and chrysophanic acid in polyherbal formulations. The present study was aimed at developing a

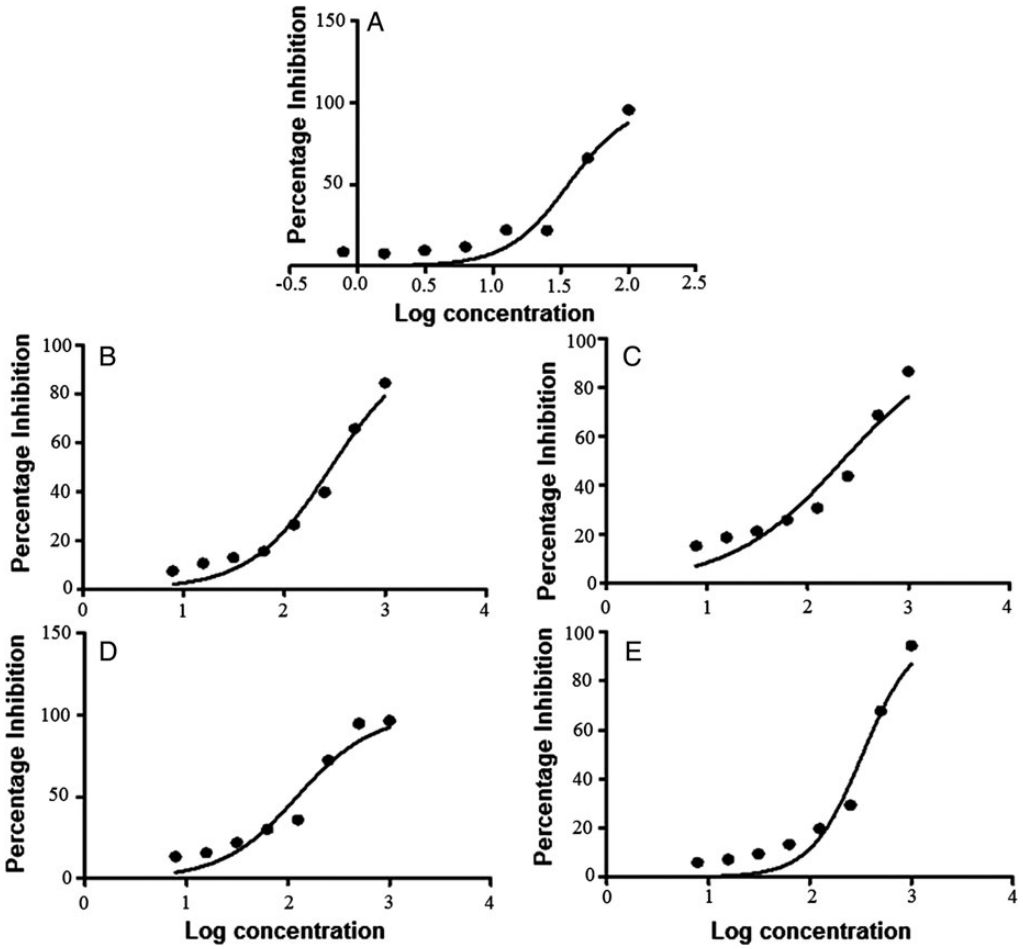

Figure 4. Scavenging effect on DPPH evident from the dose–response curve: standard ascorbic acid (A), emodin (B), chrysophanic acid (C), *Rheum emodi* rhizome extract (D) and SPP extract (E).

simple and reliable RP-HPLC and HPTLC method for better separation of emodin and chrysophanic acid from crude drug as well as from excipients of polyherbal formulation.

Methanol extract of SPP and rhizome of *R. emodi* were analyzed by HPTLC and RP-HPLC methods for amount of emodin and chrysophanic acid in crude drug and polyherbal formulation. The peaks corresponding to emodin and chrysophanic acid in samples were identified by comparing its retention data and UV spectra. Emodin and chrysophanic acid are hydroxyanthraquinone derivatives with potential biological significance and considered as a chemical marker for the standardization of polyherbal formulations containing *R. emodi* rhizomes. Unani formulations often contain a large number of herbs with varying proportions and are prepared by traditional methods. In this study, one frequently used polyherbal formulation, namely 'SPP' containing *R. emodi* rhizome was selected and the amount of emodin and chrysophanic acid was evaluated.

Conclusion

It is evident from present investigation that proposed analytical HPTLC and RP-HPLC methods can be used for quality control of several traditional Unani and Ayurvedic formulation containing *R. emodi* and other *Cassia* sp. containing emodin and chrysophanic acid as the principal component. The present investigations have also provided evidence for anti-oxidant potential of SPP and its constituents, which further suggest that anti-urolithiatic activity may be attributed due to anti-oxidant potential.

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