



Development of HPTLC method for the simultaneous estimation of quercetin, curcumin, and ascorbic acid in herbal formulations

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Abstract

Antioxidants can scavenge free radicals in the human body and have been associated with a protective effect on the human body against a variety of diseases. As a part of normal cellular function, free radical production occurs continuously within the body at a cellular level. Excess-free radical production from endogenous or exogenous sources may result in a variety of diseases. Vitamins (A, C, and E), carotenoids, polyphenols, and flavonoids are some examples of common natural antioxidants. Among the different antioxidants quercetin, curcumin, and ascorbic acids have been extensively studied, and numerous reports on their antioxidant activity are available in the literature. Antioxidants are regarded as a key player in the current pandemic situation, and they play a key role as a preventive and controlling tool. There are a variety of herbal formulations and immune booster therapies available, many of which claim to contain various antioxidants and are effective against covid-19. Numerous herbal formulations and immune booster therapies are available and claimed to have different antioxidants and other constituents. In the present study simple, rapid, accurate, precise, and sensitive high-performance thin-layer chromatographic analytical method was developed for simultaneous estimation of quercetin, curcumin, and ascorbic acid. The developed method can effectively employ for different polyherbal formulations. The developed method was validated according to ICH guidelines. In the present study, an aluminum plate precoated with 60F 254 silica gel was used as a stationary phase whereas chloroform: ethyl acetate: formic acid (6:6:2.5 v/v/v) was used as a mobile phase. Chromatographic detection was performed with a Camag TLC scanner at 265 nm. The linearity range for the proposed method was found to be 500–1000 ng/band for all three analytes. The antioxidant potentiality of selected antioxidants was evaluated by an optimized UV visible spectroscopic-reducing power method. The reducing power method was optimized for the concentration and volume of reagent, incubation temperature, and time. The results of the study revealed that quercetin is having the best antioxidant potential compared to curcumin and ascorbic acid.

Keywords Natural antioxidants · Simultaneous determination · High-performance thin layer chromatography · ICH guideline · Antioxidant activity

Introduction

Many herbal drugs and supplements have been used for a long time and are now officially acknowledged as alternative health systems. Namely, Ayurveda, Yoga, Unani, Siddha, Homeopathy, and Naturopathy [1, 2]. These systems have rightfully existed side-by-side with allopathy. In day-to-day

food habits, Indians are consuming large numbers of spices and herbs for good health and to achieve better health benefits against different diseases [3, 4]. It is universally true that good nutrition always acts as a protective tool. Food and food habits are an important part of a healthy lifestyle as a diet with essential nutrients and natural protectors can help to reduce the risk of various diseases [5, 6]. In recent years, the role of nutrition in the prevention of different diseases has been explored. The current situation of COVID 19 (coronavirus disease 19) is the best example where many home-made remedies proved to deliver protective action against this virus [7, 8]. The severity of COVID-19 is tremendously increased by oxidative stress and inflammation, especially in the presence of chronic illnesses. Hence antioxidant

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supplementation is recommended as a helpful strategy against COVID-19. It is also observed that COVID-19 may cause disease by interacting with a widely distributed target at the different levels of several organs, in which oxidative stress and inflammatory processes play important roles. As antioxidants are effective against a variety of free radicals they can be used as a preventative or therapeutic agent to protect against the oxidative stress and hyper inflammation caused by COVID-19. COVID-19 is marked by an abnormal host immune response that leads to an overabundance of inflammatory reactions (or cytokine storm) as demonstrated by elevated blood levels of biomarkers such as cytokines, chemokines, and C-reactive protein—and is linked to severe respiratory impairment and multi-organ failure [9, 10]. Biomarkers such as white blood cell count, lymphocyte count, C-reactive protein, D-dimer, and ferritin have been demonstrated to have abnormal counts during COVID infection [11, 12]. Systemic oxidative stress during the COVID 19 is also measured with redox biomarkers namely advanced oxidation protein products and 8-hydroxy-2'-deoxyguanosine [13]. Monitoring the levels of these various biomarkers is an important tool for determining the severity of the infection and, as a result, different treatment approaches may be used to treat the infection.

It is well documented that the generation of free radicals in any healthy human body leads to the development of several diseases, like cancer [14–17], diabetes [18–22], neurodegenerative diseases [23–27], cardiovascular diseases [28–34], and aging [35–38]. The harmful effects produced by such diseases can be effectively controlled by antioxidants. These substances are very much important as they can prevent or reduce the possible damage, caused by oxidative stress [39]. Several natural antioxidants have been well characterized to date, belonging to different classes namely carotenoids, polyphenols, vitamins, flavonoids, etc. These naturally occurring antioxidants find their therapeutic application not only in the treatment of various diseases but also many a time used for the prevention of different physiological conditions [39, 40]. It was found that many human diseases were induced or exaggerated by free radicals. These free radicals are responsible for causing cellular damage by a chain of oxidative reactions within the body and that may lead to the development of many human diseases including kidney disease, age-related diseases, macular degeneration, arthritis, cardiovascular disease, and their risk factor like obesity, hypertension, diabetes, atherosclerosis, metabolic syndromes, dementia, cancer, osteoporosis, etc. [41–43]. Each class of antioxidants acts on a different level of biochemical pathways, and they can prevent, intercept, reduce, or repair damages erupt by free radicals within the body [44, 45]. A large number of herbal formulations are introduced into the market during this pandemic. These formulations contained distinct categories of herbal constituents like an

immune booster, respiratory stimulant, vitamins, minerals, antioxidants, etc., and claimed to be effective against the infection. Antioxidant class constitutes a major segment for the prevention of COVID 19 [46–50]. There is a pressing need to monitor the levels of these ingredients in various formulations and to determine their efficacy as a preventive tool for such infections. Hence, in the present study, three natural antioxidants, i.e., curcumin, quercetin, and ascorbic acid (Fig. 1) were selected.

Curcumin has antioxidant, anti-inflammatory, antifungal, antiviral, hepatoprotective properties [51–53]. Curcumin is the leading curcuminoid of the Indian spice turmeric, which is a member of the family Zingiberaceae. Along with curcumin, two other curcuminoids are present, namely

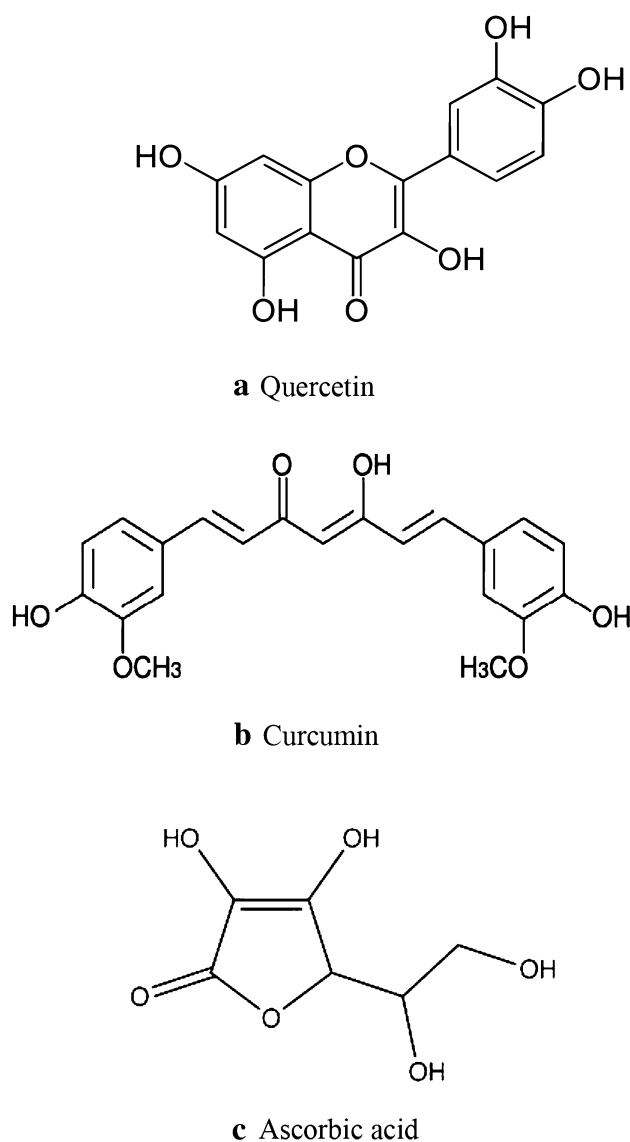


Fig. 1 Chemical structure of analytes (**a** Quercetin, **b** Curcumin, **c** Ascorbic acid)

dimethoxy curcumin and bis-dimethoxy curcumin. Curcuminoids are polyphenols and are responsible for the yellow color of turmeric. Another naturally occurring antioxidant is quercetin which is dietary flavonol belonging to a polyphenolic flavonoid substance. Citrus fruits, apples, onions, parsley, sage, tea, and red wine are important dietary sources of quercetin. Quercetin has antioxidant, anti-carcinogenic, anti-inflammatory, and cardioprotective properties [54, 55]. Whereas ascorbic acid is a naturally occurring water-soluble vitamin found in citrus fruits and vegetables. It is well established for its antioxidant and anti-inflammatory properties [56].

The study was conducted to develop and validate a new high-performance thin-layer chromatographic (HPTLC) analytical method. The developed method was applied to marketed formulations for the quantification of these selected antioxidants. The study extended with the evaluation of the antioxidant potential of the selected analytes by an optimized colorimetric method. The developed HPTLC method was validated according to ICH guidelines [46]. Numerous reports are available in the literature that revealed the use of different analytical techniques for the estimation of selected antioxidants. Reported methods fundamentally covered the chromatographic [57–70] and spectroscopic techniques [71, 72] of analysis. These available methods are either used for the estimation of an individual antioxidant or antioxidant present along with other antioxidant(s) or constituent(s). However, no method is available for the simultaneous estimation of selected antioxidants. Hence the study was conducted to develop an accurate, precise, specific, and reproducible HPTLC method for simultaneous estimation of curcumin, quercetin, and ascorbic acid.

Experimental

Instrumentation and reagents

The standard of quercetin, curcumin, and ascorbic acid were procured from Sigma Aldrich Pvt, Ltd. Bengaluru, India. All other chemicals and reagents (potassium dihydrogen phosphate, sodium hydroxide, potassium ferricyanide, trichloroacetic acid, ferric chloride) were of analytical grade and procured from Merck India Pvt, Ltd. Mumbai, India. Solvents used during the study were of HPLC grade and purchased from Merck India Pvt, Ltd. Mumbai, India. Polyherbal tablets containing all three antioxidants were purchased from a commercial source.

Instrumentation and chromatographic conditions

Camag, Muttenz, Switzerland- HPTLC system with the applicator, scanner, and visualizer employed for the study.

The samples were applied using applicator Linomat 5 using a Hamilton syringe having a capacity of 100 μ L. TLC plates used for the study were prewashed with methanol. These plates were then activated at 110 $^{\circ}$ C in a dry heat oven for 10 min before use. Samples were applied on aluminum plates precoated with silica gel 60F254 (20 \times 10 cm and 10 \times 10 cm) and kept in the TLC chamber for 15 min to attain complete saturation of the chamber. Scanning was performed using the TLC scanner at 265 nm. The mixture of chloroform: ethyl acetate: formic acid (6:6:2.5 v/v/v) was used as a mobile phase.

Preparation of standard and sample solution

Accurately weighed 10 mg of each ascorbic acid, quercetin and curcumin were transferred to 10 ml of volumetric flask. Added with 7 ml of methanol and sonicated for 5 min to dissolve it completely. Finally, volume was made up to the mark with methanol and appropriate dilutions were made to obtain a concentration of 100 μ g/ml for each of the selected analytes. UV overlaying spectra of ascorbic acid, quercetin, and curcumin are presented in Fig. 2. Sample solutions were prepared by extracting the constituents from the commercially available formulation. About 50 g of powder was taken and extracted for 24 h with 100 ml of methanol by the Soxhlet extraction technique and the methanolic extract was collected. Finally, the extract was dried in a vacuum oven at 30 $^{\circ}$ C at for 3 h and stored in a tightly closed light-resistant container in a cool place.

Method validation

The developed method was validated according to the International Council for Harmonization (ICH) guideline [51] for linearity, precision, accuracy, specificity, robustness, LOD, and LOQ. The calibration plot was recorded, and the range

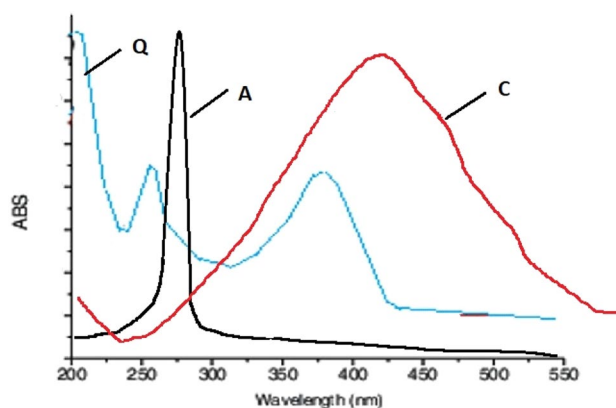


Fig. 2 UV overlaying spectra for A ascorbic acid, Q quercetin, and C curcumin

of the developed method was found to be 500–1000 ng/band for quercetin, curcumin, and ascorbic acid (Fig. 3). The standard addition method was used to establish the accuracy of the developed method. At three different concentration levels, i.e., 80%, 100%, and 120%, percentage recovery was calculated. The precision of the developed method was checked by recording chromatograms of six replicates of the same concentration and % RSD was calculated from the peak area. Similarly, intraday and interday precisions were determined by the calculation of % RSD. The specificity of the developed method was checked by recording chromatograms of standard in the presence of excipients and chromatograms of standard and test were compared (Fig. 4). For the determination of the method, robustness was determined by deliberate changes in detection wavelength and saturation time. The limit of detection (LOD) and limit of

quantification (LOQ) were calculated from the calibration curve at a lower concentration. The equation is as follows.

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

where σ = standard deviation of the response, S = slope of the calibration curve.

Optimization of the method

Concentration and volume of reagent: Antioxidant potentiality was decided by measuring the absorbance of a blue-colored chromogen at 700 nm. Investigations were made to prove the most favorable conditions for the formation of this colored product. Hence the influence of the concentration as well as the volume of reagents on the reaction has been

Fig. 3 Photograph of HPTLC plate for standards quercetin (Q), curcumin (C) and ascorbic acid (A), quercetin formulations—QF1, QF2, QF3, curcumin formulations—CF1, CF2, CF3, ascorbic acid formulations—AF1, AF2, AF3, Synthetic mixture (quercetin, curcumin, and ascorbic acid)

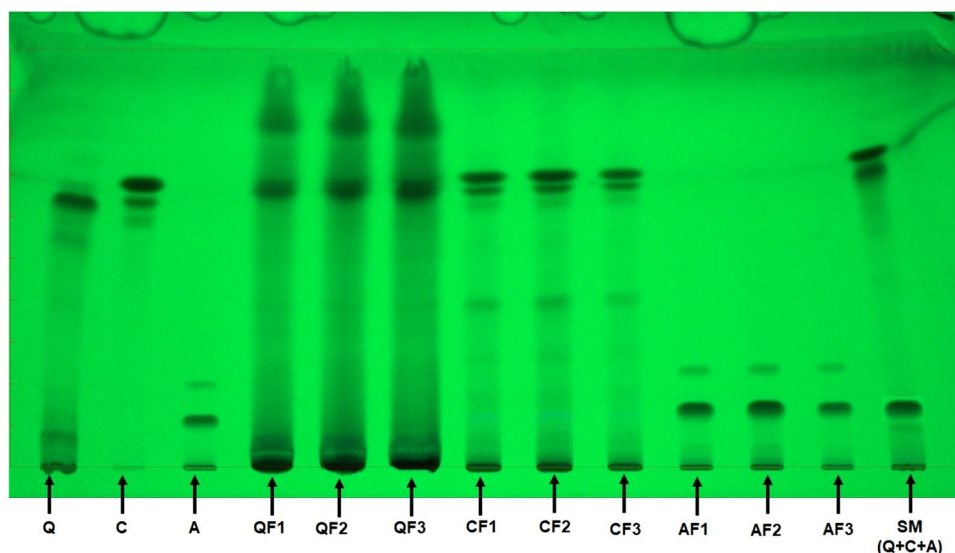
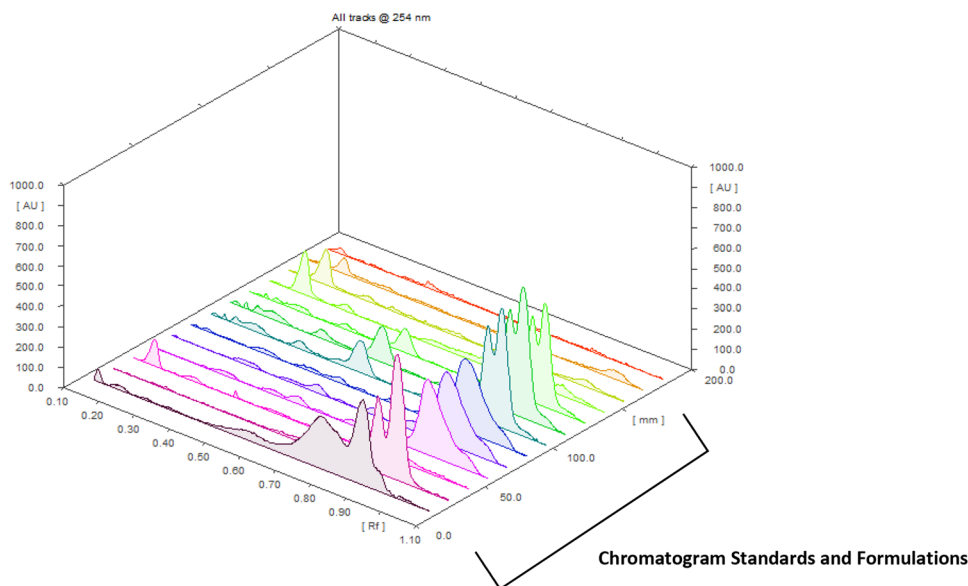


Fig. 4 Overlay chromatogram of standards with the formulations



studied. Different concentrations and different volumes of $K_3Fe(CN)_6$ and $FeCl_3$ were tried for optimal experimental conditions.

Incubation temperature and incubation time: To obtain a maximum color intensity of a developed chromogen, the study was performed under four different incubation temperatures i.e., room temperature, 40 °C, 50 °C, and 60 °C, to select the most appropriate temperature for the reaction. For this study, samples were kept in glass vials at room temperature and elevated (40, 50, and 60 ± 2 °C). Similarly, for identification of the most favorable incubation time study was conducted at a different time, and samples were analyzed at 10, 20, 30, and 40 min.

In-vitro antioxidant potential measurement

Reducing power method: The reducing power of each of the standard antioxidants was determined according to the reported method [73, 74]. An aliquot of 0.5 ml extract was added to 0.1 ml of 1% potassium ferricyanide. After incubating the mixture at 50 °C for 30 min, after the reduction (ferricyanide to ferrocyanide), it was supplemented with 0.1 ml of 1% trichloroacetic acid and 0.1% $FeCl_3$ and left for 20 min. Absorbance was read at 700 nm to determine the amount of ferric ferrocyanide (Prussian blue) formed. The higher absorbance of the reaction mixture shows a higher reducing power of the sample.

Results and discussion

Different drug regulatory authorities including the World Health Organization (WHO) have always demanded data on the scientific validity of synthetic and herbal drugs and drug formulations. The purpose is to control, monitor, and ensure drug quality and safety. Especially in the current pandemic situation many substandard and poor-quality drugs have mushroomed in the market. Hence it is very vital to monitor and control the quality criteria of such drug formulations. Numerous analytical techniques can be employed for analytical purposes, such commonly used techniques are high-performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS) gas chromatography (GC), gas chromatography-mass spectroscopy (GC-MS), nuclear magnetic resonance (NMR), near-infrared (NIR), UV visible spectroscopy and High-performance thin-layer chromatography (HPTLC).

HPTLC is the method of choice whenever the analyte sample is complex and seeks good selectivity and sensitivity with a reduced amount of solvent usage. One added advantage of simultaneous multi-residue analysis eventually leads to saving of time, money, and overall cost of analysis.

HPTLC method development

For the HPTLC chromatographic method development, several preliminary tests were performed for critical parameters including different stationary phases, mobile phases, and saturation time of the solvent chamber, to attain the best separation and resolution for selected analytes. The optimized mobile phase, i.e., chloroform: ethyl acetate: formic acid (6:6:2.5 v/v/v) was selected for well resolved and satisfactory separation of selected analytes. Overlay spectra are shown in Fig. 5. Silica gel was used as a stationary phase and chamber saturation time was 15 min. With the above-established parameters well-resolved peaks of ascorbic acid, quercetin and curcumin were obtained with the R_f value of 0.10 ± 0.02 , 0.72 ± 0.02 , and 0.83 ± 0.02 , respectively.

Method validation

The developed method was validated according to ICH guidelines [51]. Analysis of herbal formulation with a validated analytical method is a major public health concern as in the market a large number of herbal formulations are available which are either substandard or many a time adulterated with some adulterant. Method validation assures method performance which is to be used for its quality control tests. According to ICH guidelines following method parameters were evaluated for their quality performance.

Specificity

The specificity of the analytical method is its ability to assess specifically the analyte of interest without the interference of other components which may be expected to be present. The developed HPTLC method was found to be specific, as each peak is well resolved and separated from each other without interference from other components as shown in Fig. 5.

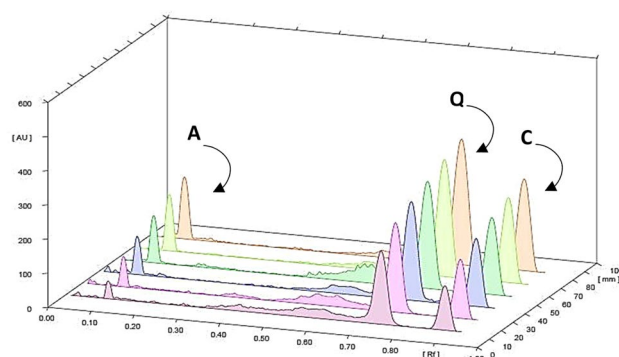


Fig. 5 Overlay chromatogram of linearity for A ascorbic acid, Q quercetin, and C curcumin

Linearity and range

The linearity of the analytical method is its ability to give a linear and proportionate concentration of an analyte. The linearity of the developed method was determined by recording the analyte response in triplicate results are shown in Table 3. The calibration curve method was used for the determination of linearity and range of quercetin, ascorbic acid, and curcumin. Linearity was statistically calculated for ascorbic acid, quercetin, and curcumin by the regression equation.

Accuracy

The accuracy of an analytical method is the closeness of agreement between the test results and true value. The accuracy of the developed method was evaluated by the standard addition method where pre-analyzed samples were added

with a known amount of the standards at three different concentrations levels, i.e., 80%, 100%, and 120%. Recovery was found to be 99.04–99.80%, 99.10–98.85%, and 99.32–100.76% for quercetin, curcumin, and ascorbic acid, respectively. The results are presented in Table 1.

Precision

The precision of an analytical method is expressed as the degree of agreement between a series of multiple measurements of a given homogeneous sampling under the prescribed experimental conditions. According to the ICH guidelines, the repeatability of the developed method was performed to check the possible difference that may be observed between the same subject test results under identical chromatographic conditions. Data of repeatability is given in Table 3 which indicated the %RSD of ascorbic acid, quercetin, and curcumin were within the acceptable

Table 1 Accuracy of ascorbic acid, curcumin, and quercetin by standard addition method ($n=6$)

Analyte	Level of standard addition (%)	Mean area	% Recovery	SD	% RSD
Ascorbic acid	80	2735.77	100.49	0.305 ± 5.60	0.30
	100	3268.95	100.76	1.468 ± 9.27	1.47
	120	3714.42	99.32	0.322 ± 8.75	0.35
Quercetin	80	7903.04	99.80	0.227 ± 2.54	0.23
	100	8845.09	99.41	0.375 ± 9.97	0.38
	120	9778.81	99.04	0.278 ± 1.42	0.28
Curcumin	80	12,767.10	99.85	1.119 ± 2.43	1.12
	100	13,979.56	99.10	0.530 ± 7.55	0.54
	120	15,357.73	99.70	0.977 ± 9.65	0.98

Table 2 Precision of ascorbic acid, curcumin, and quercetin ($n=6$)

Analyte	Intra-day precision				Inter-day precision			
	Concentration (ng/band)	Mean Area	SD	%RSD	Concentration (ng/band)	Mean Area	SD	%RSD
Ascorbic acid	700	1473.45	22.67 ± 2.44	1.53	700	1471.05	24.46 ± 3.75	1.65
Quercetin	700	4505.28	19.89 ± 1.26	0.44	700	4507.46	23.31 ± 4.54	0.53
Curcumin	700	7500.74	13.16 ± 3.21	0.17	700	7498.26	14.92 ± 3.88	0.20

Table 3 Summarized method validation parameters ($n=6$)

Validation parameters	Ascorbic acid	Quercetin	Curcumin
Linearity range (ng/spot)	500–1000	500–1000	500–1000
Regression equation	$Y=4.755x-1864$	$Y=7.594x-909.5$	$Y=9.373x+1012$
Coefficient of correlation	0.997	0.984	0.993
Limit of detection (LOD) (ng/spot)	12	6.0	4.0
Limit of quantitation (LOQ) (ng/spot)	36	18	13
Robustness (% RSD)	< 1	< 1	< 1
Repeatability (% RSD)	0.94	0.31	0.16

criteria. In all tested conditions %, the RSD value was found to be less than 2% indicating that the developed method is precise enough for the analytical estimation of the selected analyte. Intraday precision was measured by recording chromatograms of six replicates of the same concentration, three times on the same day, and %RSD was calculated. Data on intraday precision is given in Table 2. In all tested conditions, the %RSD value was found to be less than 2%, indicating that the method is precise. Similarly, interday precision was checked by recording chromatograms of six replicates of the same concentration three times on three different days. Data on interday and intraday precision revealed that the developed method is precise.

Robustness

The robustness of an analytical method is very much important. The robust analytical method indicated that the method remains unaffected by small and willingly variations in method parameters. It indicates its reliability during normal usage of the analytical method. The robustness of the developed method was evaluated by the change in scanning wavelength and chamber saturation time. In all tested conditions, it was found that variations in selected experimental parameters do not cause any significant variation in experimental results and it was found that in each case the results were within the acceptable limits showing that the method is robust. Hence, it was concluded that the proposed method is robust. Data on robustness are presented in Table 3. The limit of detection is the smallest amount of an analyte effectively detected (not necessary to quantitated) is known as the limit of detection of the method. Whereas the smallest amount of an analyte is effectively quantitated with considerable accuracy and precision then it is known as the limit of quantification for the method. The limit of detection and limit of quantitation are calculated by the equation method by using the calibration curve. LOD and LOQ data of ascorbic acid, quercetin, and curcumin are given in Table 3.

Optimization of the method

Optimization was performed for different concentrations of reference standard solutions and test solutions. In all tested conditions absorbance of each tested solution was measured with a double beam UV–visible spectrophotometer against a solvent blank at 700 nm. The influence of the concentration and volume of reagent on the reaction has been studied. Different concentrations and different volumes were tried for both the reagents, by varying one parameter at a time. It was found that the optimum concentration and volume of $K_3Fe(CN)_6$ were 1.5% w/v and 2 ml,

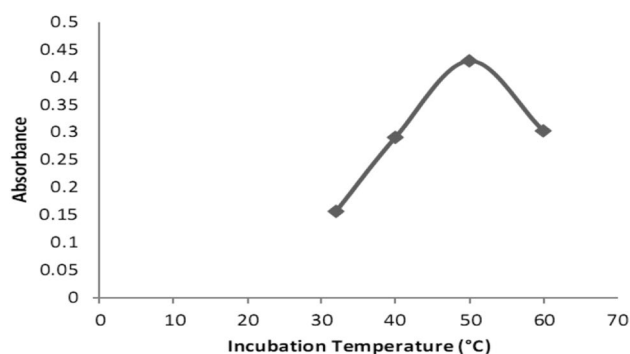


Fig. 6 Optimization of incubation temperature

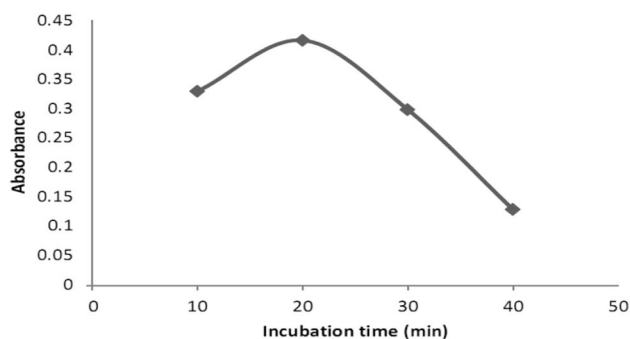


Fig. 7 Optimization of incubation time

respectively. Whereas optimum concentration and volume of $FeCl_3$ were found to be 0.2% w/v and 1 ml, respectively. It was found that the maximum color intensity of the chromogen was observed when the incubation temperature was fixed at 50 °C (Fig. 6). Similarly, the optimum time for the reaction to complete was found to be 20 min, as during this time duration maximum absorbance was found (Fig. 7).

Analysis of the formulation

The developed method was applied to the marketed formulation containing selected analytes. For each selected antioxidant, three different formulations were estimated with the developed method. The formulation powder was extracted with methanol and after the extraction 50 mg of extract was transferred to a 10 ml volumetric flask and the final volume was adjusted to the mark using methanol. By taking reference to R_f values of standard ascorbic acid, curcumin, and quercetin, chromatographic spots of samples were detected (Fig. 4). Some peaks were also observed due to the presence of other phytoconstituents, but these did not interfere with the peak of interest hence they are omitted from the measurement. The results of the study are presented in Table 4.

Table 4 Analysis of herbal tablet/capsule formulation by HPTLC

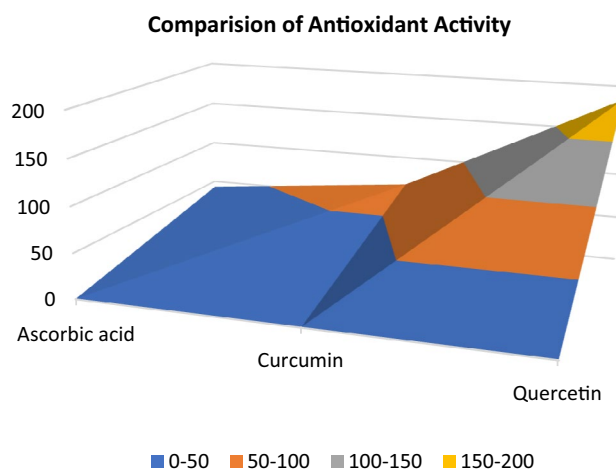
Analyte	Formulation type	Label claim (mg)	Amount found (%)	% RSD
Ascorbic acid	Formulation A1	500	98.12	0.12
	Formulation A2	500	97.58	0.18
	Formulation A3	500	97.62	0.09
Quercetin	Formulation Q1	100	96.92	1.01
	Formulation Q2	100	97.12	0.91
	Formulation Q3	100	95.25	0.89
Curcumin	Formulation C1	500	96.88	0.65
	Formulation C2	500	97.25	0.52
	Formulation C3	500	97.37	0.57

In- vitro antioxidant potential measurement

The antioxidant activity of all three antioxidants from commercially available formulations was evaluated by reducing the power method. In this assay, the ability of antioxidant extracts to reduce Fe^{3+} to Fe^{2+} was determined. Chemically, the presence of antioxidants in the extracts resulted in a reduction of the ferric cyanide complex to the ferrous cyanide form. In this method, antioxidants cause the reduction of the Fe^{3+} into Fe^{2+} , thereby changing the solution into various shades of color, from green to blue, depending on the reducing power of the antioxidant. The higher the absorbance of the reaction mixture, the greater would be the reducing power. Strong reduction property presented by the characteristic development of Prussian blue color and showed absorbance at 700 nm. It is observed that the absorbance intensity was increased with enhanced antioxidant potentiality. An antioxidant constituent may function as an electron donor and could react with free radicals to convert them into more stable products and terminate the free radical chain reaction. Quercetin showed maximum intensity followed by curcumin and ascorbic acid. The comparative antioxidant activity of the analytes is presented in Fig. 8.

Conclusion

A high-performance thin-layer chromatographic method for simultaneous estimation of curcumin, quercetin, and ascorbic acid has been developed and validated according to ICH guidelines. The antioxidant potential of selected analytes was evaluated by an optimized reducing power method and according to the method, quercetin has higher antioxidant potentiality. The developed method can effectively be employed for the estimation of selected natural antioxidants and to evaluate their quality. As the developed method is simple, rapid, accurate, precise, and reproducible

**Fig. 8** Comparative antioxidant potential of the selected analytes

can effectively be employed for the simultaneous estimation of curcumin, quercetin, and ascorbic acid for routine quality testing of different herbal formulations. The developed method is efficiently applied to foods supplement or nutraceuticals for the determination of selected antioxidant constituents.

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Declarations

Conflict of interest The authors have declared that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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