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A single robust stability-indicating RP-HPLC analytical tool for apigenin quantification in bulk powder and in nanoliposomes: a novel approach

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Abstract

Background: Apigenin (4′, 5, 7-trihydroxyflavone), a flavonoid, is present usually in fruits and vegetables possessing numerous biological properties like antioxidant, anti-viral, antibacterial, anti-inflammatory, and chemoprevention activity. So present study was aimed to prepare and characterize nanoliposomes of apigenin and estimate its encapsulation efficiency by stability-assisted reverse-phase (RP)-HPLC method.

Results: The stability indication of the RP-HPLC method developed for apigenin-loaded nanoliposomes was successfully demonstrated and parameters were mainly the retention time which was 4.21 min, limit of detection (LOD) 0.49 μg/mL, limit of quantification (LOQ) 1.48 μg/mL, and %relative standard deviation (RSD) less than 2%. Therefore, the stability indication of the developed reverse-phase HPLC method for apigenin-loaded nanoliposomes was demonstrated successfully and parameters like accuracy, linearity, LOD, LOQ, precision, and %RSD were within the limit range and found to be satisfactory.

Conclusion: The developed RP-HPLC method was found to be suitable for the quantification or estimation of apigenin with its stability in apigenin-loaded nanoliposomes, and this method will be a powerful tool in the future for the estimation of apigenin present in any pharmaceutical preparations.

Keywords: Apigenin, Nanoliposomes, Stability-indicating method, RP-HPLC, Validation

Background

Apigenin (4,5,7-trihydroxyflavone) (Fig. 1) is one of the most novel flavonoids in plants and formally belongs to the flavone sub-class. Apigenin possess moderate antioxidant property, anti-hyperglycemic [1], anti-inflammatory [2], myocardial ischemia, and anti-apoptotic effects [3]. A review by Zhou et al. summarized various biological effects of apigenin being effective against cytostatic and cytotoxic activities of a number of cancer cells and anti-atherogenic effects and also protective effects on hypertension, cardiac hypertrophy, and autoimmune myocarditis [4]. Novel approach has led to the development of apigenin in the pharmaceutical era, and quantification and estimation of apigenin in plant extracts has need raised. Nanoliposomes are similar to nano-metric kind of liposomes which tend to be the further most applied in encapsulation and controlled release systems [5]. These deliver more surface area, compared to liposomes, hence enhancing the bioavailability and controlled release, facilitating accurate target of the encapsulated material to a larger amount [6]. Apigenin having potential to increase the performance of the bioactive agent helps in enhancing solubility, bioavailability, and stability reported in vitro and in vivo. Due to prevention in unnecessary interactions with other molecules with this support, these nanoliposomes have diverse applications; current study was

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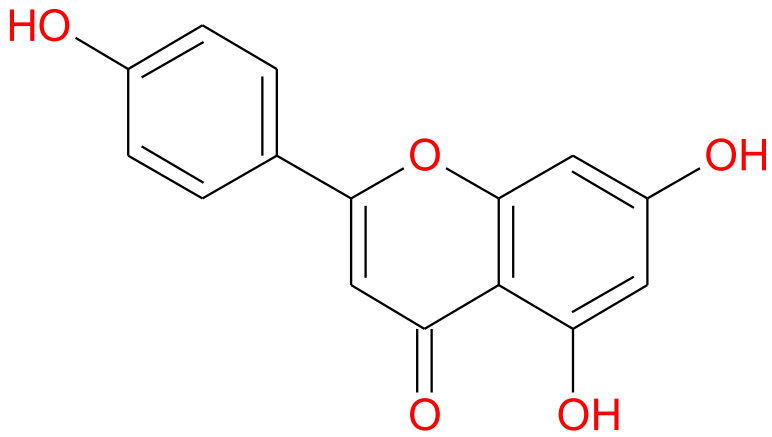

Fig. 1 Chemical structure of apigenin

commenced for the preparation, characterization, and confirmation of apigenin-loaded nanoliposomes for its encapsulation stability by RP-HPLC method.

Methods

Chemicals and reagents

Apigenin was a gift sample from Aktin Chemicals Ltd., China. Methanol, acetonitrile, and formic acid (HPLC grade) were purchased from Merck Limited (Mumbai, India). Other common laboratory reagents were of analytical grade.

Instrumentation

Shimadzu HPLC (LC- 2010) (Kyoto, Japan), pump system (LC-20 AD), degasser (DGU-20A5), a column oven (CTO-10ASVP), an auto-injector (SIL-20ACHT), PDA Detector (SPD-M20A), and a computer software (lab solutions, version 1.25) were used.

Chromatographic conditions

The chromatographic separation was carried out by injecting (10 μL) a sample to the HPLC system connected to a C-18 analytical column (Phenomenex Luna 5 μm, 250 mm × 4.6 mm) (set at 35 °C) operating at 1 ml/min flow rate, and detection was done at 269 nm. Acetonitrile and 0.1% formic acid, 55:45 (v/v) at pH = 7.4, was used as a mobile phase.

Table 1 Results of system suitability studies of quality control samples of apigenin

Parameter	Mean ± SD	% RSD
Retention time (min)	4.21 ± 0.00[a]	0.13
Peak area	127512 ± 273[a]	0.21
Theoretical plates	7010 ± 38.23[a]	0.54
Tailing factor	1.35 ± 0.00[a]	0.22

RSD relative standard deviation
[a]*n* = 6

Standard solution preparation

Stock solution was prepared (100 μg/mL) in the mobile phase. Further dilutions were carried out to get the calibration curve ranging from 0.5 to 16 μg/mL.

Preparation of stealth apigenin-nanoliposomes

Formulating the stealth apigenin-nanoliposomes, an ethanol injection technique was used in the preparation of nanoliposomes as described by Sudhakar et al. [7]. Briefly, apigenin (10 mg) and stearic acid (10 mg) were dissolved in 2 ml of absolute ethanol with gentle heating (< 50 °C) on a hot plate. Then, lipid di-stearoyl-sn-glycero-phosphoethanolamine–N (polyethyleneglycol) (DSPE-PEG) (0.003%) and cholesterol (0.0015%) were added into the same mixture with stirring. Phosphate-buffered saline (PBS) (pH 7.4) (10 ml) was added to mixture dropwise with constant stirring for 90–120 min to remove ethanol. The nanoliposomes were optimized for various concentrations of lipid. The optimized batch was homogenized at 10,000×*g* for 10 min followed by sonication for 15 min, which were further filtered through 0.45-μm and 0.2-μm sterile syringe filters and were allowed to equilibrate for 1 day and then stored at 4 °C. In the time, apigenin-loaded nanoliposomes were also assessed for their average particle size, poly dispersity index (PDI) according to Sharma et al. [8]. Moreover, encapsulation efficiency of nanoliposome was also assessed by centrifugation at 10,000×*g* for 30 min, and supernatant solution obtained after centrifugation was mixed with mobile phase and analyzed by using HPLC [9, 10].

Method development

The chromatographic conditions were optimized and steady baseline was obtained. Chromatogram was assessed by injecting standard apigenin solution, and analyses were repeated in a similar way for six times.

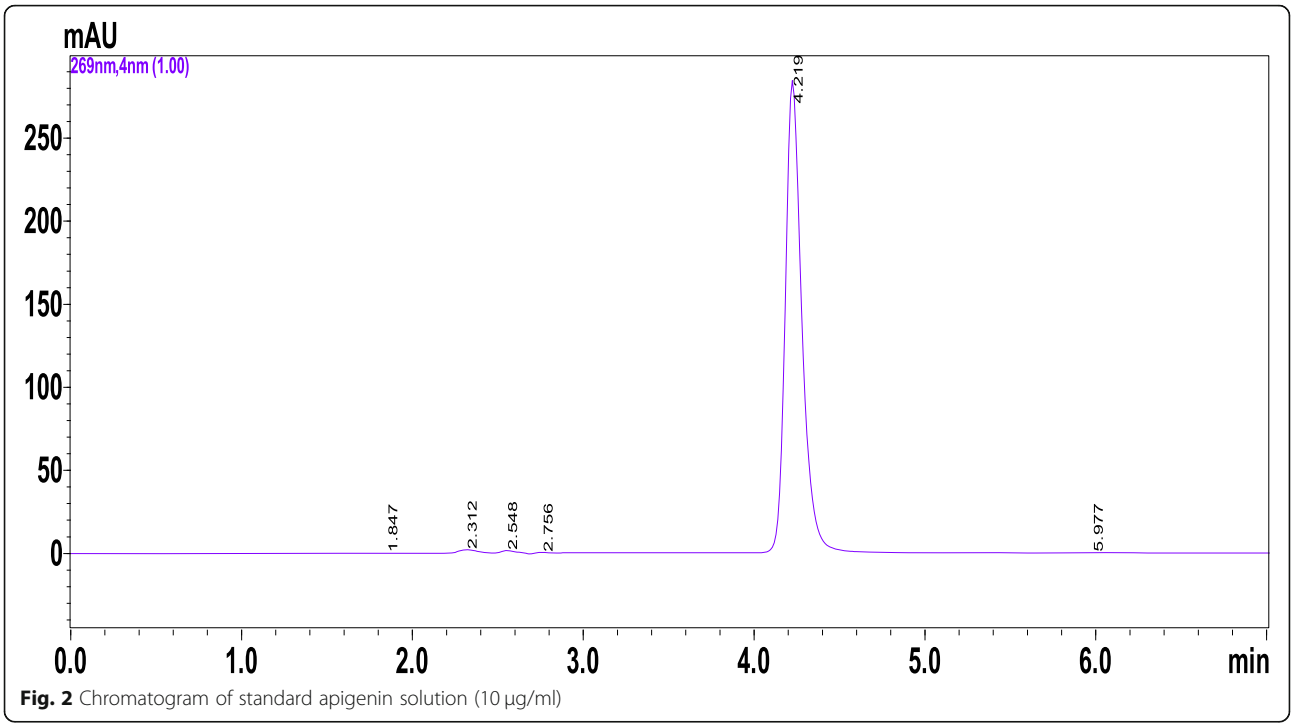

Fig. 2 Chromatogram of standard apigenin solution (10 μg/ml)

Method validation

Linearity

Linearity studies reveal the standard calibration curve was plotted from 0.5 to 16 μg/mL concentrations. Standard calibration curves were determined for concentrations versus peak area. Sample run in triplicate of all the sample solutions was assessed, and a chromatogram was recorded.

Precision and accuracy

The apigenin samples were subjected to three levels of quality control; namely low, medium, and high respective concentrations of 0.5, 4, and 16 μg/mL were prepared and used in determining the precision and accuracy. For intra-day precision and accuracy, triplicates of standard solutions were injected on the same day and also for interday; triplicates of standard solutions (50%, 100%, and 150%) were injected over three consecutive days. %relative standard deviation (RSD) and % RE were calculated respectively.

Table 2 Results of linearity and regression analysis of apigenin

Analyte	Apigenin at 269 nm
Concentration range (μg/ml)	0.5–16
Slope	34,367
Intercept	5495
R^2	0.999
LOD(μg/ml)	0.49
LOQ (μg/ml)	1.48

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

The LOD was determined by a series of dilutions of apigenin stock solutions to find a signal to noise (S/N) ratio of at least 3.3:1 for LOD and 10:1 for LOQ. Both parameters obliged the quantity of analyte that can be quantified.

System suitability

To validate the chromatographic system, suitability test is carried out, and various factors such as tailing factor, theoretical plates, and peak area and resolution data were also considered.

Robustness

The robustness of the method was evaluated by deliberately making a slight change in the optimized value. The evaluated parameters were as follows: detector wavelength (± 1 nm), flow rate (± 0.2 ml/min), and oven temperature (± 2 °C). Apigenin peak areas and the %RSD of robustness testing were statistically measured.

Specificity

The specificity study chance of interferences from PBS and apigenin-loaded nanoliposomes at the retention time of apigenin was analyzed by comparing the chromatograms obtained from the standard apigenin solution and PBS.

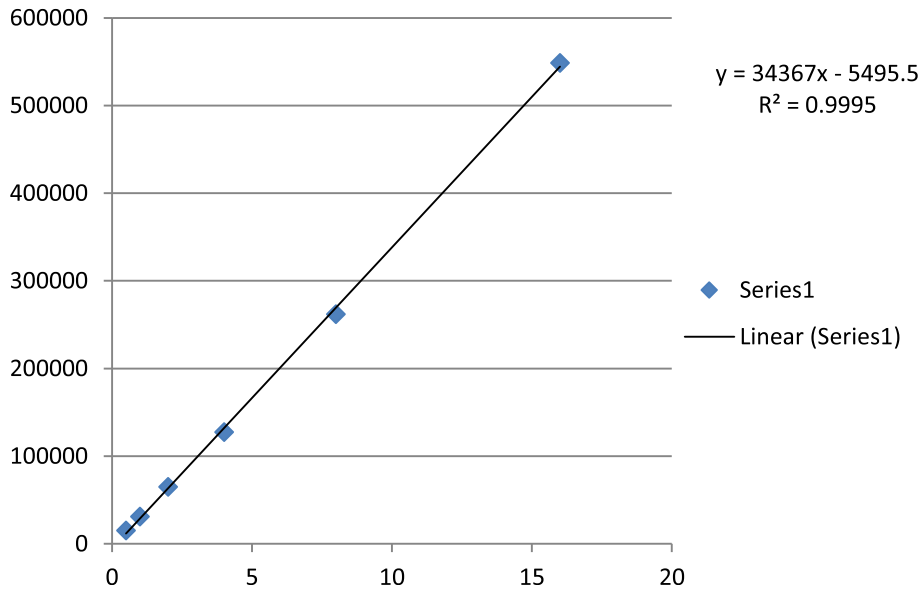

Fig. 3 Calibration curve for apigenin 269 nm

Force degradation studies

The stability-indicating property of the HPLC method which was developed was carried out as per the International Conference on Harmonization (ICH) guidelines [11]. Forced degradation studies of apigenin were conducted by exposing the working sample to oxidation, photolytic, acidic, and alkaline and heat conditions.

Results

Optimization of sample preparation and chromatographic conditions

Various trials were performed by changing the composition of the mobile phase. Finally, composition of ACN, 0.1 % FA (55:45 v/v, pH = 7.4), gave the better separation. During these studies, the 10 µl injected volume and the flow rate (1 ml/min) found to be constant.

Analytical method validation suitability of system

The system suitability results are tabulated in Table 1. The peak retention time was found to be 4.21 min (Fig. 2), the average theoretical plate was > 7000, and the tailing factor was < 2. Presence of higher theoretical plates with a lower tailing number indicates the efficiency of the system. Additionally, the % RSD values for all the parameters including retention time, peak area, and theoretical plates and tailing number were less than 1% justifying the better suitability of the system.

Table 3 Results of accuracy study of apigenin

Sl. No.	Conc. (µg/ml)	Level in (%)	Added qty (µg/ml)	Found qty (µg/ml)	Recovery (%)	RSD (%)
1	2	50	1	1.02	99.96	0.76
2	2	100	2	2.00	100.20	0.28
3	2	150	3	3.15	101.40	0.12

RSD relative standard deviation

Linearity and range

The linearity and regression are tabulated in Table 2 and Fig. 3. None of the constituents from the nanoliposomes or the derivative products of stress treatment interfered with apigenin peaks. A calibration curve was generated by plotting concentration of apigenin (x) against the peak area. A linearity (R^2 = 0.9995; equation y = 34367x–5495.5) was achieved for the concentration range of 0.5–16 µg/mL. Apigenin was detected employing C_{18} column with methanol to 0.2% phosphoric acid mobile phase and had an accuracy and recovery of 93.82 and 88.35% respectively.

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

The LOD and LOQ of apigenin were found to be 0.49 and 1.48 µg/ml, respectively. A higher LOD (1.94 µg/ml) and LOQ (6.26 µg/ml) of apigenin was reported.

Accuracy and precision

The obtained results are expressed in Tables 3, 4, and 5. The intra-day precision for apigenin was between 0.11 and 1.38%, while the accuracy ranged between 0.12 and 0.76%. The interday precision was 0.27% and 1.69%, respectively. Three separate samples injected showed a best recovery of 99.96–101.4% indicating a higher accuracy of the method.

Table 4 Results of intra-day precision of apigenin solution

Experiment time for day 1	Concentration (μg/ml)	Peak area	Retention time (min)	(%)RSD of peak area
9:00 am	0.5	19,404.17	4.28	1.38
	4	129,483	4.29	0.39
	16	673,361.8	4.30	0.78
1:00 pm	0.5	19,404.17	4.31	1.38
	4	129,707.2	4.29	0.27
	16	673,361.8	4.28	1.05
6:00 pm	0.5	19,497.17	4.27	0.34
	4	129,882.5	4.27	0.32
	16	682,828	4.28	0.11

RSD relative standard deviation

Robustness and ruggedness

The results of the robustness study are presented in Table 6. Parameters including mobile phase, flow rate, detection wavelength, column oven temperature, and the type of column did not hinder the resolution in terms of similar retention time and lower RSD values.

Stability-indicating study

The results of the stress degradation studies (acid, alkali, oxidation, heat, and light) for the standard apigenin solution are shown in Table 7. The chromatograms of the stressed standard apigenin solution after 2 h under various stress conditions are shown in Figs. 4 and 5. In the current study, apigenin showed a degree of degradation ranging from as low as 5.87% for alkaline hydrolysis to 20% by photolytic damage. The degradation of apigenin was perfectly estimated by the developed method. HPLC analysis of aqueous and ethanolic chamomile extracts exposed to different temperature and pH showed that apigenin-7-O-glucosidedegraded by 6% at pH 8, 9% at pH 9, and 11–12% at pH 10–12; 48% at pH 13.0, with no loss below pH 5.0. Long-term storage of extracts at room temperature revealed conversion of glycoside to aglycone as compared to storage at − 20 °C.

Table 5 Results of interday precision data of apigenin samples

Day / time	Concentration (μg/ml)	Peak area	Retention time (min)	(%)RSD of peak area
Day 1 10:00 am	0.5	18,728.5	4.28	0.93
	4	129,084.2	4.29	0.28
	16	647,448.3	4.30	1.16
Day 2 10:00 am	0.5	19,404.17	4.31	1.38
	4	129,707.2	4.29	0.27
	16	673,361.8	4.28	1.05
Day 3 10:00 am	0.5	20,680	4.30	1.69
	4	130,179	4.29	0.27
	16	721,931.5	4.28	1.02

RSD relative standard deviation

Table 6 Analysis of robustness and ruggedness using apigenin solution

Parameters	Variations	Time (min)	%RSD
Mobile phase(± 2 v/v)	55:45	4.30	0.08
	53:47	4.51	0.10
	57:43	4.00	0.03
Flow rate(± 0.1 ml)	1 ml/min	4.33	0.08
	0.9 ml/min	4.71	0.10
	1.1 ml/min	3.86	0.09
Detection wavelength (± 2 nm)	267	4.24	0.13
	269	4.23	0.11
Column oven temp(±1 °C)	35 °C	4.21	0.13
	34 °C	4.22	0.15
	36 °C	4.20	0.17
Column	Luna 5u(250X4.6)	4.33	0.08
	Luna 5u(150X4.6)	3.84	0.09

RSD relative standard deviation

Preparation and characterization of stealth liposomes

The apigenin-loaded nanoliposomes were successfully prepared by the ethanol injection technique; the nanoliposomes was 124 nm of the blank liposome and apigenin-loaded liposome was 151 nm. The average polydispersity index for blank and drug-loaded liposome was 0.23 and 0.16, respectively (Table 8). These results summarized that the PDI value is < 0.3 and indicated narrow homogeneous presence of particle size distribution. Meanwhile, these apigenin-loaded liposomes were also subjected to the encapsulation efficiency (EE) of apigenin in the formulation. The chromatogram showed an intense characteristic apigenin peak in nanoliposomes, and t 80.18% of encapsulation efficiency was achieved by this method (Fig. 6, Table 8).

The average particle size of apigenin-loaded lecithin liposomes was in the range of 158.9 ± 6.1 to 260.3 ± 11.1 nm. The PDI ranged from 0.205 ± 0.02 to 0.331 ± 0.063 with a remarkable encapsulation efficiency of more than 92% and possessing high potential for nutraceutical formulations. In another study, apigenin-loaded distearoylphophatidylcholine (DSPC) liposomes showed a particle

Table 7 Results of the stress degradation studies of apigenin solution at 2 h

Stress degradation studies	Conc. used (μg/ml)	Conc. left after degradation (μg/ml)	% Degradation
Acid hydrolysis	2.0	1.62	18.88
Alkaline hydrolysis	2.0	1.88	5.87
Oxidation	2.0	1.70	14.61
Thermal	2.0	1.66	16.55
Photolytic	2.0	1.60	20.00

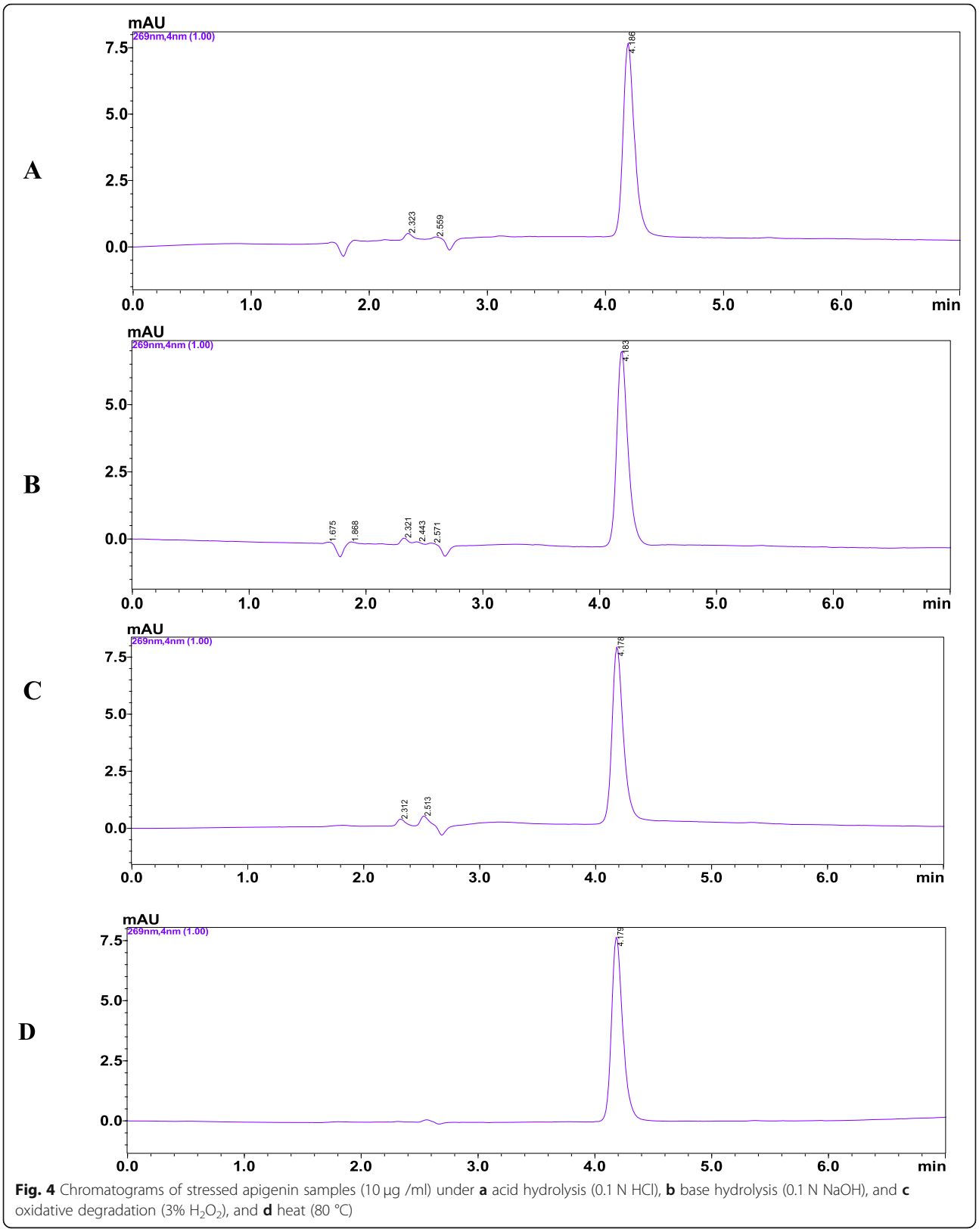

Fig. 4 Chromatograms of stressed apigenin samples (10 µg /ml) under **a** acid hydrolysis (0.1 N HCl), **b** base hydrolysis (0.1 N NaOH), and **c** oxidative degradation (3% H_2O_2), and **d** heat (80 °C)

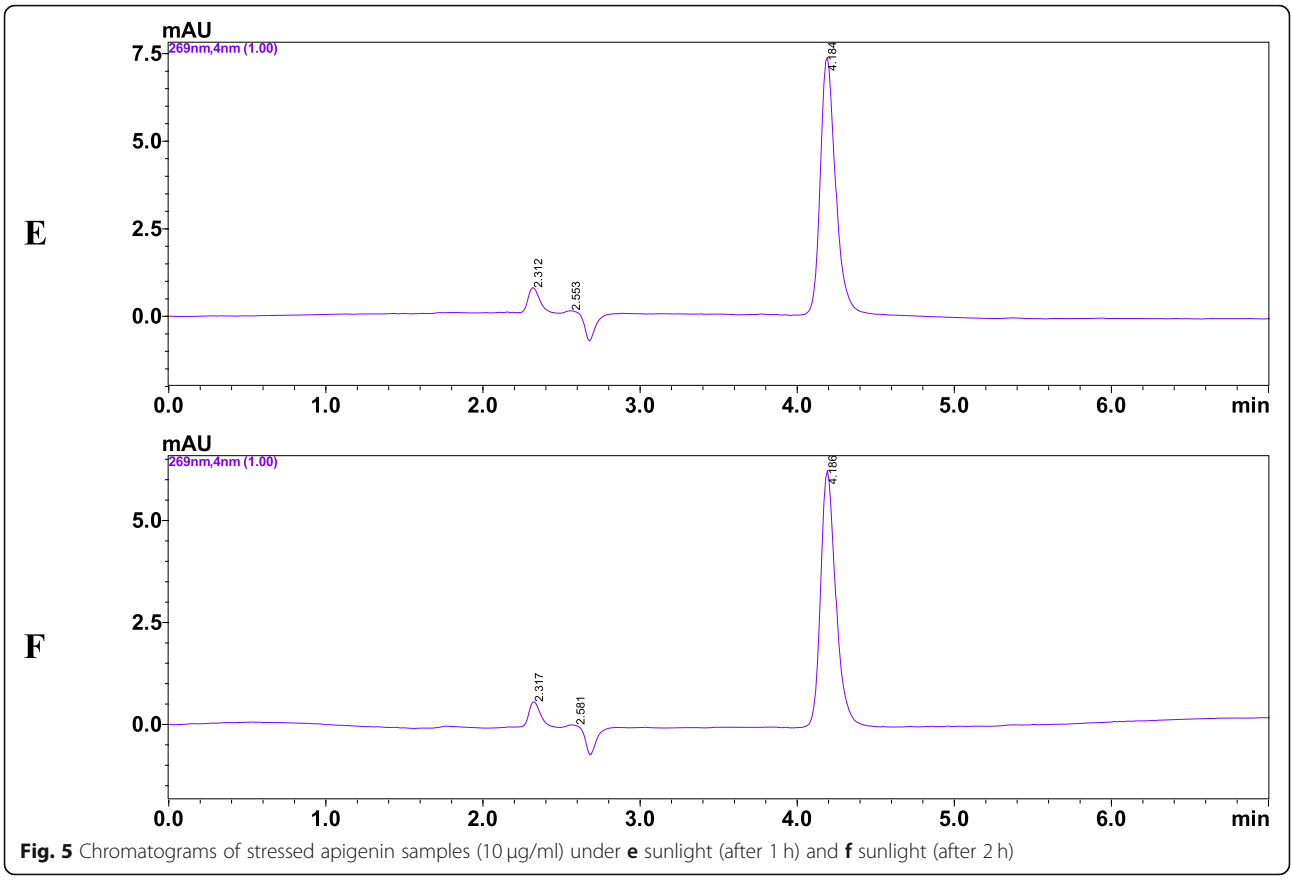

Fig. 5 Chromatograms of stressed apigenin samples (10 μg/ml) under **e** sunlight (after 1 h) and **f** sunlight (after 2 h)

size of 104.3 ± 1.8 nm, PDI of 0.204, and a high EE of 89.9%. The apigenin-loaded DSPC liposomes were effective in delivering the drug within bacterial cells and thus were antibacterial in nature.

Discussion

Presence of higher theoretical plates with a lower tailing number indicates the efficiency of the system. Additionally, the % RSD values for all the parameters including retention time, peak area, and theoretical plates and tailing number were less than 1% justifying the better suitability of the system [12].

None of the constituents from the nanoliposomes or the derivative products of stress treatment interfered with apigenin peaks. A calibration curve was generated by plotting concentration of apigenin (x) against the peak area. A linearity of r = 0.9995 with equation y = 34367x−5495.5 was achieved for the concentration range of 0.5–16 μg/ml. Apigenin was detected utilizing C_{18} column with methanol to 0.2% phosphoric acid mobile [13]. A higher LOD (1.94 μg/ml) and LOQ (6.26 μg/ml) of apigenin was reported, which could be due to variance in the mobile phase [13] and depending on the type of analysis and analytical conditions used [12]. Three separate samples injected showed a best recovery of 99.96–101.4% indicating a higher accuracy of the method. The RSD values of intra- and interday precision of the method was less than 2% [14–16].

Parameters including mobile phase, flow rate, detection wavelength, column oven temperature, and the type of column did not hinder the resolution in terms of similar retention time and lower RSD values. Triplicate analysis of the samples showed the better robustness of the developed method which was similar to those reported [17].

In the current study, apigenin showed a degree of degradation ranging from as low as 5.87% for alkaline

Table 8 Results of characterization of apigenin-entrapped liposomes

Liposome formulation	Particle size diameter (nm)	PDI	% Entrapment efficiency
Blank liposome	124	0.23	NA
Apigenin-loaded liposomes	151	0.16	80.18%

PDI polydispersity index

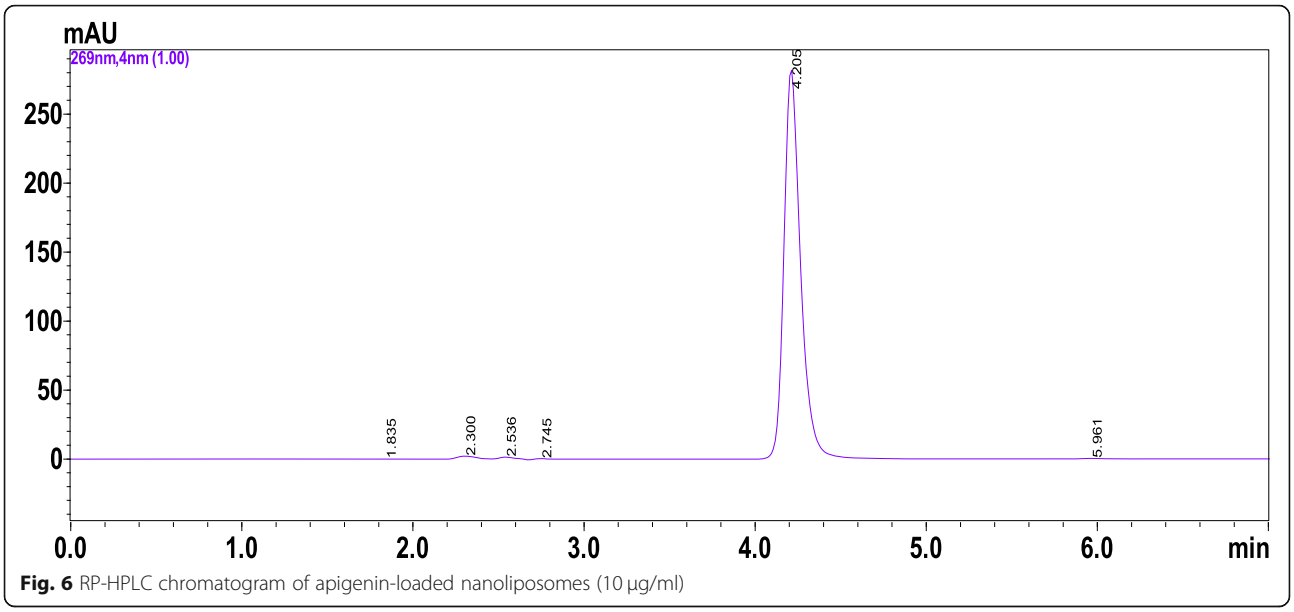

Fig. 6 RP-HPLC chromatogram of apigenin-loaded nanoliposomes (10 μg/ml)

hydrolysis to 20% by photolytic damage. The degradation of apigenin was perfectly estimated by the developed method [18]. Apigenin was also found to be structurally unstable when subjected to temperatures of 37 °C [19, 20].

A multiple emulsion solvent evaporation technique was employed to encapsulate apigenin in poly(lactide-co-glycoside), PLGA, which showed a drug loading efficacy of 19.14% [21]. The EE of apigenin-loaded liposomes was better than that reported and similar to encapsulation of apigenin in lecithin liposomes [22]. The average particle size of apigenin-loaded lecithin liposomes was in the range of 158.9 to 260.3 nm. The PDI ranged from 0.205 to 0.331 with a remarkable encapsulation efficiency of more than 92%. In another study, apigenin-loaded distearoylphophatidylcholine (DSPC) liposomes showed a particle size of 104.3 ± 1.8 nm, PDI of 0.204, and a high EE of 89.9%. Apigenin-loaded nanoliposome from the current study would thus function as an efficient drug delivery system [23–25].

Conclusion

A sensitive, simple, specific, and stability-indicating RP-HPLC tool for the quantification of apigenin bulk and apigenin-loaded nanoliposomes were developed effectively. Interferences of peaks with apigenin by the encapsulation substances were not detected. The method could be used in herbal drug industries for quantification of apigenin.

Abbreviations

RP-HPLC: Reverse-phase high-performance liquid chromatography; PDI: Polydispersity index; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation; PBS: Phosphate-buffered saline; EE: Encapsulation efficiency; DSPC: Distearoylphophatidylcholine; ICH: International Conference on Harmonization; DSPE-PEG: Di-stearoyl-sn-glycero-phosphoethanolamine (polyethyleneglycol)

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Authors' contributions

PS contributed for experimental work and manuscript preparation. SSJ contributed in hypothesis and finalization of manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

Authors declare to produce the data and material on demand/request.

Declarations

Ethics approval and consent to participant

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors do not have any conflict of interest.

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