Original Article

Development and Validation of Stability Indicating UV-Spectrophotometric Method for the Estimation of Hesperidin in Bulk Drugs, Plant Extract, Ayurveda Formulation and Nanoformulation

Supriya Suresh Chimagave, Sunil Satyappa Jalalpure*, Akshay Kalgonda Patil, Bhaskar Kallappa Kurangi

KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Nehru Nagar, Belagavi, Karnataka, INDIA.

ABSTRACT

Introduction: In present work attempt has been made to develop and validate a simple and sensitive UV spectrophotometric method for estimation of Hesperidin from bulk drugs, plant extract, Ayurveda formulation and novel nanoformulation. Hesperidin is a heteropolycyclic aromatic bioflavonoid found abundance in citrus plants that has been developed into a topical nanoformulation for the treatment of anti-inflammatory activity. It has numerous anti-inflammatory, anti-cancer, and anti-arthritis effects. **Objectives:** To develop simple, precise, robust, sensitive and accurate UV-Spectrophotometric method for the estimation of hesperidin in bulk drugs, Plant extract, Ayurveda formulation and nanoformulation. **Materials and Methods:** The optimum condition for the analysis of the drug was established with methanol and water as solvent (1:1). Maximum absorption wavelength was found to be 284 nm with line equation y = 0.0672x +0.003. It showed linear response between the concentration ranges of 2-10 μg/ml. The linear regression coefficient was found to be 0.999. The method was validated for linearity, precision, ruggedness, specificity, sensitivity as per ICH guidelines and all the values of validation was found to be within the acceptance. **Conclusion:** The developed method was proved to be new, simple, consistent, and accurate it can be used in the pharmaceutical industry as a routine quality control parameter for raw material and formulation.

Keywords: Hesperidin, Stability indicating, UV-Spectrophotometric, Ayurveda formulation, Nanoformulation.

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Correspondence:
Prof. (Dr.) Sunil S Jalalpure
Professor and Principal,
KLE College of Pharmacy,
Belagavi, KLE Academy
of Higher Education and
Research, Nehru Nagar,
Belagavi-590010 Karnataka,
INDIA.
E-mail: jalalpuresunil@
rediffmail.com

INTRODUCTION

Hesperedin (Figure 1), (7-O—rutinoside) and its aglycone hesperetin (4'-methoxy3',5,7-trihydroxyflavanone) are flavonoids plentiful in citrus fruits as grapefruits, lemons, and oranges.[1] It has anti-inflammatory, antiviral, anticancer, and antidepressant like activities in animal studies and has been shown to help treat many different types of central nervous system illnesses.[2] Hesperidin was used in a variety of Ayurvedic formulations marketed to health-care providers.[3] In Ayurveda formulations, quality monitoring of hesperidin-containing formulations is critical. Analytical methods for estimating hesperidin in various plant extracts have been published in the literature, including spectrophotometric,[4] HPLC,[5] and HPTLC[6] approaches. The listed methods have their own limitations, such as taking more time and requiring the use of expensive and complex chemicals. According to the literature review, there is no FDA-approved UV spectrophotometric method for evaluating hesperidin in its pure form, Ayurveda formulation, or novel topical nanoformulation. As a result, a UV spectrophotometric method is used to estimate hesperidin must be developed and validated using a suitable solvent system. The goal of this study is to create

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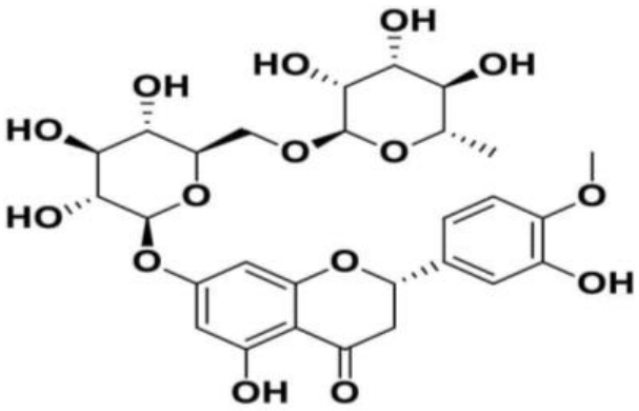

Figure 1: Structure of Hesperidin.

and validate a new UV-spectrophotometric method for estimating hesperidin in bulk powder, plant extracts, Ayurveda formulation, and a novel topical nanoformulation. For diosmin, hesperetin, rutin, and quercetin in conjunction with hesperidin drug, many assays have been published, including HPLC, HPTLC, GC-MS, and UV-Spectroscopy. To date, no UV-Spectrophotometric approach for single Hesperidin has been reported. As a result, the current study aimed to design and validate a simple, rapid, sensitive, precise, and accurate UV-Spectrophotometric process for assessing Hesperidin.

MATERIALS AND METHODS

Materials

Hesperidin was obtained as a generous gift sample from Shaanxi Yi an Biologicals, China, and Himalaya Wellness Company, Makali, Bengaluru. Phospholipid, (LIPOID 90G) was obtained as gift sample from Lipoid GmbH, Ludwigshafen, Germany. Dimethyl sulfoxide, Methanol was procured from Merck, Mumbai and Fisher Scientific Mumbai, India. Other chemicals used for the experiment were of Analytical grade.

Instruments

UV-Spectrophotometer of Shimadzu make and 1800 model having UV probe software were used for analysis.

Method development

The development of a new UV Spectrophotometric method was confirmed after selecting the suitable solvent combination and identifying the wavelengths in the literature. Different solvents were used to assess the solubility of the samples, including methanol, acetone, chloroform, dimethyl sulfoxide, 1N NAOH, and 0.1N HCL.[7] Methanol and water were selected as solvents after considering solubility conditions. Using methanol and water (1:1) as a solvent samples were scanned in the UV region between 200 and 400 nm. Hesperidin had the highest absorption at 284 nm.

Preparation of standard stock solution

10 mg of Hesperidin was accurately weighed and transferred to a volumetric flask of 10 ml, and the volume was made up to 10 ml with a solvent mixture of methanol and water, obtaining a concentration of 1000 µg/ml of analyte. 1 ml of Hesperidin solution has been pipette out from the volumetric flask and passed to a 10 ml volumetric flask, and the volume was made up to 10 ml with methanol and water to achieve a concentration of 100 µg/ml for Hesperidin.

Preparation of Plant extracts and Ayurveda formulation for estimation of hesperidin

The proposed UV spectrophotometric method was utilized to quantify the hesperidin concentration in Madhiphala rasayana, a commercial Ayurvedic preparation, and orange peel, bitter lime peel. To achieve a fine powder, orange and bitter lime peel were powdered with a mortar and pestle.[8] Weigh precisely 10 g of powdered drug and dissolve in 1 mg/ml stock solution of methanol and water. The volumetric flasks containing the above-mentioned solutions were sonicated for 15 min. The stock solutions were then filtered with a 0.45 m syringe filter, diluted with solvent phase, and the hesperidin content was estimated using a UV spectrophotometric method.[3] The same method was used for Ayurveda formulation. 2 mL Ayurveda formulation, dissolved in methanol and water to obtain 1 mg/mL stock solution. The procedure for assessing hesperidin content using the UV spectrophotometric method remains the same as outlined above.

Nanoformulation for hesperidin estimation[9-13]

Thin film hydration was used to create a hesperidin-entrapped topical nanoformulation. Briefly, Weigh the exact amount of drug (30mg) and dissolve in 5ml of methanol before sonication. In a suitable beaker, accurately weigh phospholipid choline 90G (800mg) and tween 80 (100mg) with 10 ml chloroform and vigorously mix with a sonicator for 5 min. This mixture was then added to a rotary flask for thin film for 60 min at 60 rpm. After hydrating the thin film with phosphate buffer under vacuum for an overnight period and reducing the particle size of the nanoformulation with a probe sonicator, the UV spectrophotometric method was used to estimate hesperidin.

Forced degradation studies[14]

To assess the stability of the developed UV-Spectroscopic method, samples were subjected to acid, base, oxidation, and photolytic degradation. Percentage degradation was calculated in all studies. The forced degradation study's limit is acceptable and within limits

Acid degradation study[15,16]

Weighing 10 mg of Hesperidin in 10 ml of volumetric flask resulted in acid degradation. 1000 µg/ml concentration obtained by dissolving in methanol and water. Secondary stock solutions containing 100 µg/ml for Hesperidin were prepared. This 10 µg/ml Hesperidin solution was stressed for 2 hr in 0.1 N HCl on a water bath at 80°C. Spectra were observed after scanning samples in the UV range of 200-400 nm.

Base degradation study[17-18]

For Base degradation study Hesperidin solution (10 µg/ml) was prepared from primary and secondary stock solutions and diluted with 0.1 NaOH. Both solutions were stressed for 2 hr at 80°C and their spectra were scanned.

Oxidation degradation study

Hesperidin solution (10 µg/ml) was prepared from primary and secondary stock solutions and diluted with 30% hydrogen peroxide. Both solutions were stressed at 80°C for 2 hr and their spectra were scanned.

Photo degradation study

The photo degradation study involved exposing 10 mg of Hesperidin drug to UV light at 284 nm for 24 hr. After that, solutions were prepared with methanol and water to achieve a final concentration of 10 µg/ml of Hesperidin and then scanned to obtain appropriate spectra.

Formula to calculate % degradation

$$\% \text{ Degradation} = \frac{(\text{Initial deg radation} - \text{Final deg radation})}{\text{adation}} \times 100$$

Method Validation

The method was developed and validated in accordance with ICH guidelines in order to determine Linearity, Specificity and selectivity, Precision, Ruggedness, Stability, LOD, and LOQ.[19-20] Hesperidin has a linearity range of 2-10 µg/ml. The calibration curve was created by plotting the area versus the concentration. The precision study was performed in system, intraday, and interday precision, and the results were expressed in percent RSD. Ruggedness was measured by changing the analyst and recording the instrument's percent RSD. The solution was stabilised in bench and freeze conditions for 72 hr. The linearity slope was used to calculate the LOD and LOQ.[21-22]

Table 1: Parameters of method development.

Parameters	Specifications
Analytes	Hesperidin
Solvents	Dimethyl sulfoxide, Methanol, Water
Lamda max of Hesperidin	284 nm

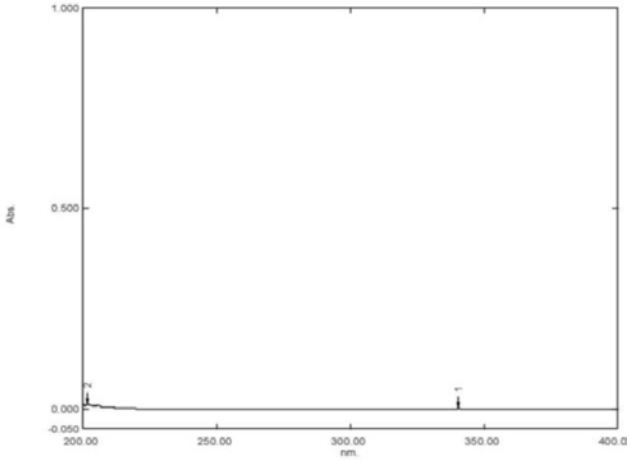

Figure 2: UV-Spectrum of methanol: water

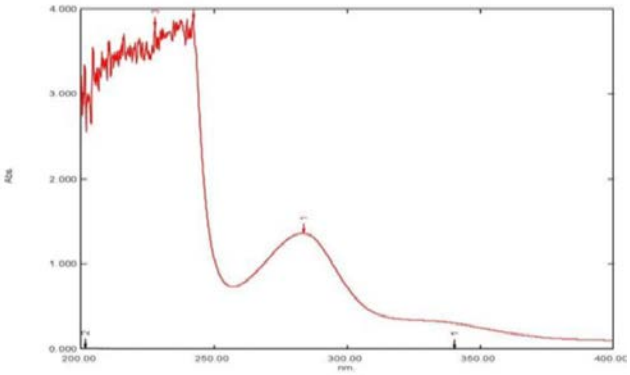

Figure 3: UV-Spectrum of Hesperidin

RESULTS AND DISCUSSION

The solvent development method utilizes methanol and water, and hesperidin demonstrated a spectrum with maximum absorbance at 284 nm. The parameters for method development and validation were presented in (Table 1).

Specificity and selectivity

By obtaining solvent spectrum there was no interference of absorbance at 284 nm hitch highlighted the specificity

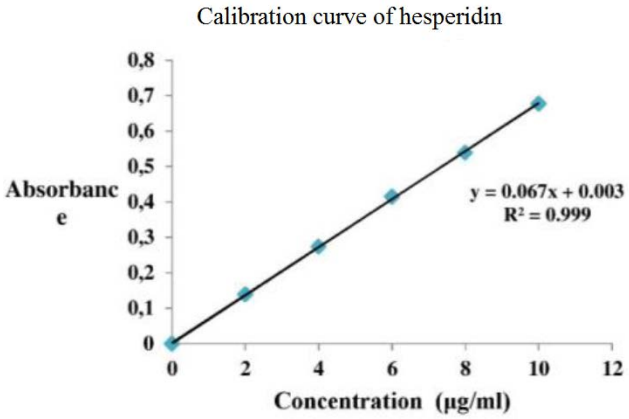

Figure 4: Linearity curve for Hesperidin.

Table 2: Linearity and range data of Hesperidin.

Sl. No	Concentration (µg/ml)	Absorbance of Hesperidin at 284 nm
1	2	0.139
2	4	0.274
3	6	0.415
4	8	0.539
5	10	0.677
R^2		0.999
Slope		0.067
LOD		0.214 µg/ml
LOQ		0.648 µg/ml

and method selectivity. The UV spectrum of solvent and hesperidin is represented in Figure 2, and the spectrum of Hesperidin was absorbed at 284 nm it was showed in Figure 3.

Specificity and selectivity

The lack of interference of absorbance at 284 nm in the solvent spectrum highlighted the specificity and method selectivity. Figure 2 depicts the UV spectrum of the solvent and hesperidin, while Figure 3 depicts the spectrum of Hesperidin absorbed at 284 nm.

Linearity range Response

Linearity and range for Hesperidin were determined by analysing 2-10 µg/ml for Hesperidin in triplicate, which were estimated at 284 nm with a correlation coefficient $R^2 > 0.999$. Figure 4 shows graphs of absorbance and calibration curves.

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

LOD and LOQ are the lowest detectable and quantifiable concentrations of analytes, resulting in signal-to-noise ratios of 3:1 and 10:1, respectively. The detection and quantification limits for hesperidin were found to be 0.214 and 0.648 µg/ml at 284 nm, respectively, indicating that the developed UV spectrometric method was sensitive to determining hesperidin concentration in bulk drugs, plant extract, Ayurveda formulation, and topical nanoformulation (Table 2).

Table 3: System Precision of Hesperidin.

Sl. No	Concentration (µg/ml)	Hesperidin	% RSD
1	2	0.144	**1.40845**
2	2	0.142	
3	2	0.14	
4	6	0.419	**0.60447**
5	6	0.416	
6	6	0.414	
7	10	0.736	**0.91168**
8	10	0.723	
9	10	0.732	

Table 4: Data for Intraday Precision of Hesperidin.

Sl.no	Concentration (µg/ml)	Hesperidin (Morning)	Hesperidin (Afternoon)	Hesperidin (Evening)
1	2	0.162	0.157	0.159
2	2	0.157	0.159	0.16
3	2	0.16	0.155	0.156
%RSD		**1.57616**	**1.27388**	**1.31473**
4	4	0.291	0.319	0.32
5	4	0.295	0.312	0.316
6	4	0.303	0.309	0.31
%RSD		**1.81063**	**1.6079**	**1.58714**
7	6	0.454	0.452	0.457
8	6	0.441	0.466	0.449
9	6	0.447	0.464	0.451
%RSD		1.45448	1.64367	0.92041

Precision

Precision was investigated by performing system, intraday, and interday precision tests. The system precision was determined by examining six replicates of each drug solution. The solution was analysed on the same day at three different intervals and on three different days to determine intraday and interday precision. The absorbance was measured, and the percent RSD was calculated (Table 3-5).

Stability

The solution's stability was tested by exiting it in unstable conditions for 72 hr and afterwards checking it once again (Table 6).

Table 5: Interday Precision data for Hesperidin.

Sl. no	Concentration (µg/ml)	Hesperidin (DAY 1)	Hesperidin (DAY 2)	Hesperidin (DAY 3)
1	2	0.147	0.141	0.144
2	2	0.143	0.143	0.149
3	2	0.146	0.145	0.147
%RSD		**1.43233**	**1.38860**	**1.71587**
4	4	0.26	0.263	0.252
5	4	0.265	0.261	0.26
6	4	0.257	0.255	0.257
%RSD		**1.55042**	**1.60333**	**1.57663**
7	6	0.393	0.387	0.39
8	6	0.396	0.382	0.383
9	6	0.399	0.379	0.378
%RSD		**0.75757**	**1.05612**	**1.57108**

Table 6: Solution stability data of hesperidin.

Solution stability		Fresh stock dilutions	Old stock dilutions
Replicates	Concentartion	Hesperidin	Hesperidin
1	2µg/ml	0.15	0.149
2	2µg/ml	0.151	0.152
3	2µg/ml	0.147	0.148
4	2µg/ml	0.149	0.151
5	2µg/ml	0.15	0.147
6	2µg/ml	0.154	0.15
RSD %		**1.542690375**	**1.25139043**

Table 7: Data for Ruggedness of Hesperidin.

Sl. no	Concentration (µg/ml)	Change in analyst	Change in instrument
		Hesperidin	Hesperidin
1	2	0.15	0.149
2	2	0.152	0.152
3	2	0.147	0.147
% RSD		**1.681477603**	**1.685230901**
4	4	0.282	0.272
5	4	0.279	0.267
6	4	0.272	0.276
% RSD		**1.848115765**	**1.659846535**
7	6	0.384	0.395
8	6	0.376	0.386
9	6	0.384	0.382
% RSD		**1.211224341**	**1.717539497**

Ruggedness

The ruggedness parameter was tested by changing in an analyst and changing in an instrument, and the percent RSD was calculated (Table 7).

Table 8: Accuracy data for Hesperidin.

Drug	Level	Absorbance	Recovery Amount (µg/ml)	%Mean Recovery±SD (n=3)
Hesperidin	50%	0.141	1.015	**101.1±0.4**
		0.139	0.986	
		0.136	0.987	
	100%	0.278	3.029	**101±0.6**
		0.275	3.014	
		0.281	3.058	
	150%	0.401	4.95	**99.9±0.4**
		0.406	4.92	
		0.403	5.01	

Table 9: Robustness data for Hesperidin.

Sl. no	Concentration (µg/ml)	Change in Wavelength		Change in solvent make (methanol: distilled water)
		Hesperidin		Hesperidin
		282 nm	286 nm	(8:2)
1	0.141	0.155	0.151	0.153
2	0.142	0.157	0.154	0.155
3	0.145	0.152	0.157	0.156
%RSD		**1.45911**	**1.62711**	**1.94805**
4	4	0.265	0.287	0.282
5	4	0.257	0.279	0.286
6	4	0.264	0.281	0.29
%RSD		**1.66370**	**1.47461**	**1.39860**
7	6	0.397	0.422	0.423
8	6	0.384	0.432	0.432
9	6	0.392	0.42	0.437
%RSD		**1.67709**	**1.51391**	**1.64735**

Accuracy

Accuracy data was collected at three different levels: 50 percent, 100 percent, and 150 percent, and the percentage recovery were found to be within the acceptable limit, indicating a good recovery value and the method's accuracy (Table 8).

Robustness

The robustness Method was established to be Robust because the change in analyst and instrument made little difference and the percent RSD for the drug was within limits. The Robustness data for hesperidin is shown in (Table 9).

Analysis in orange, bitter lime, Ayurveda formulation and topical nanoformulation[23-24]

The newly developed analytical method was used to estimate the content of hesperidin in bulk, plant extract, Ayurveda formulation, and novel topical nanoformulation. The hesperidin content in orange peel, bitter lime peel, Ayurveda formulation, and nanoformulation was found to be 98.15, 98.25, 98.32, and 97.19 percent, respectively, which was within the appropriate range as stated on the label. The lack of other peaks observed for the UV spectrum of orange peel, bitter lime peel, Ayurveda formulation, and nanoformulation indicated that other drugs and active ingredients used in the marketed formulation and new topical nanoformulation can't interfere with the parenting spectrum of hesperidin, denoting that this established analytical method is applicable for routine hesperidin evaluation in quality control industries Figure 5. (Figure A summarises the UV spectrum of a standard drug; Figure B depicts the UV spectrum of orange peel; Figure C depicts the UV spectrum of bitter lime peel; Figure D depicts the UV spectrum of an Ayurveda formulation; and Figure E depicts the UV spectrum of a topical nanoformulation.)

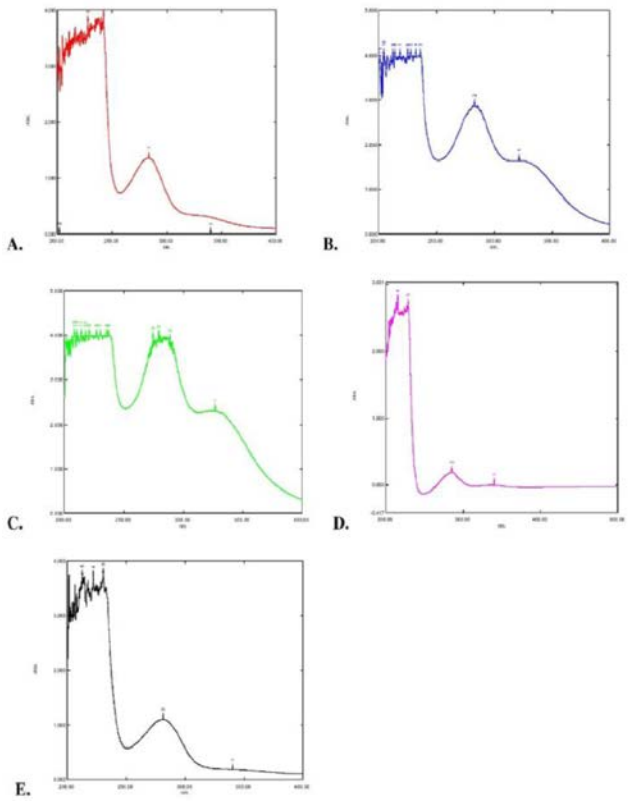

Figure 5: UV spectrophotometric spectrum for Normal (A), orange (B), bitter lime (C), Ayurveda formulation (D), topical nanoformulation (E).

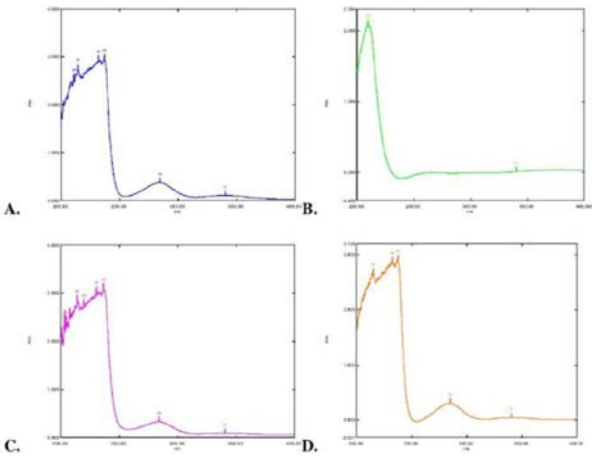

Figure 6: UV spectra of hesperidin (10 µg/ml) obtained in the stress degradation assays using Acidic (A), Basic (B), Oxidative (C), Photolytic (D)

Forced degradation study

The results of forced degradation study using methanol as a solvent were summarized in Figure 6.

Acid degradation study

The drug was found extreme acid degradation. 0.1 N HCl heating at 80°C for 2 hr showed more than 10% degradation and slightly change in spectra were found Figure 6 A.

Base degradation study

In base degradation heating at 80°C by using 0.1 N NaOH shows degradation of Hesperidin is more as compare to other Figure 6 B.

Oxidation Degradation study

Oxidation degradation by using 30% Hydrogen peroxide showed very less degradation compared to the other degradation Figure 6 C.

Photolytic degradation study

Photolytic degradation showed very slight changes in the spectra and level of the degradation was very less compared to other degradations Figure 6 D.

CONCLUSION

A simple, specific, selective, linear, precise, rugged, and stability indicating UV Spectrophotometric method for hesperidin estimation in orange peel, bitter lime peel, Ayurvedic formulation, and nanoformulation was successfully developed and evaluated. The developed method made good results in less time. As an outcome, it was illustrated that the method was simple, precise, rugged, and stable for the analysis of hesperidin in

bulk, plant extract, Ayurvedic formulation, and novel nanoformulation can be used for quality control analysis. According to the results of the forced degradation study, hesperidin was stable in acidic, oxidative, and photolytic conditions. As a result, hesperidin was susceptible to degradation in the existence of basic stress conditions. As a result, this straightforward UV spectrophotometric method for indicating stability is useful for routine quality control analysis. This method could thus have been used to estimate hesperidin *in vitro* and *in vivo*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Author Contribution

All the authors have equally contributed for the development and validation of Hesperidin. Supriya S. Chimagave contributed to develop and validate and method for Hesperidin in its pure form. Dr. Sunil S. Jalalpure guided for the present research work also in the reviving literature and writing the paper work. Akshay K. Patil and Bhaskar K. Kurangi guided for the writing of manuscript and framing of the research paper.

ABBREVIATIONS

UV: Ultra violet; **µl:** Micro liter; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **RSD:** Relative standard deviation; **SD:** Standard deviation; **ICH:** International conference of harmonization; **HCL:** Hydrochloric acid; **NaOH:** Sodium hydroxide.

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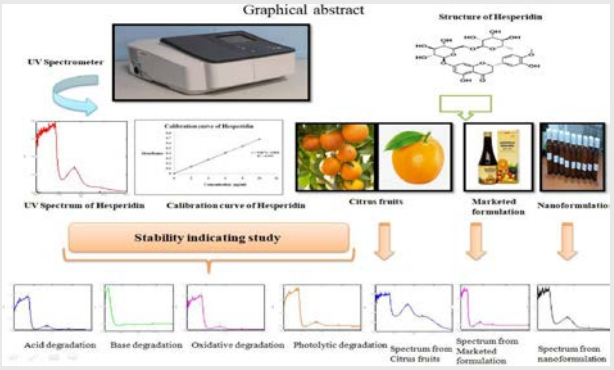
PICTORIAL ABSTRACT



SUMMARY

The UV-Spectrophotometric method was developed and validated for the estimation of Hesperidin in bulk drug, plant extracts, Ayurveda formulation, as well as nanoformulation. The method was validated according to the ICH Q2 guidelines. Hesperidin has low solubility and is soluble in DMSO. Many studies on HPLC and HPTLC analysis for Hesperidin have been reported, but to date, a UV Spectrophotometric method for Hesperidin isolated compound and nanoformulation has not been reported. The solvent used for the detection was methanol and water (1:1), and the detection wavelength was found at 284 nm. The forced degradation study was carried out in acidic, basic, oxidative, and photolytic conditions, and all the values were within the limit. Estimation of hesperidin from bulk, plant extract, Ayurveda formulation as well as nanoformulation was found to have a good percentage recovery. All the values of the validation parameters were found to be within the acceptance limit as per the ICH guidelines. Hence, the developed and validated UV-Spectrophotometric method can be used for routine quality control of Hesperidin.

About Authors

Ms. Supriya S. Chimagave is a PhD Scholar at KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research Belagavi. Current research focuses on the design and evaluation of nanoformulations for arthritis and inflammation treatment, as well as the development of analytical methods for herbal phytoconstituents using chromatography techniques.

Prof. (Dr.) Sunil Jalalpure is presently working as a Professor and Principal, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi. His areas of research include isolation/ characterization of active principles from medicinal plants and their pharmacological screening for various biological activities and training the research students in Pharmacognosy, Phytochemistry and Biotechnological aspects with modern tools and techniques. He is actively involved in nanoparticles drug delivery system of herbal phytoconstituents and green nanotechnology.

Mr. Akshay K. Patil is presently working as a Assistant professor, Department of Pharmacognosy, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi. His research areas include the development and evaluation of innovative topical drug - delivery systems based on herbal phytoconstituents for arthritis and inflammation research, as well as the development of analytical methods based on chromatographic techniques.

Dr. Bhaskar K. Kurangi is presently working as a Assistant professor, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, Belagavi. His research areas include the design and evaluation of novel drug delivery systems, development of analytical and bioanalytical techniques using HPLC, HPTLC and the application of nanoparticulate drug delivery in prophylactic and therapeutic treatment of cancer.

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