World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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Development and validation of UV-spectrophotometric method for estimation of pterostilbene in *Pterocarpus marsupium*

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World Journal of Advanced Research and Reviews, 2023, 17(01), 1123–1131

Publication history: Received on 04 December 2022; revised on 25 January 2023; accepted on 27 January 2023

Article DOI: https://doi.org/10.30574/wjarr.2023.17.1.0116

Abstract

Aim: To develop and validate a simple, precise and cost effective UV-visible spectrophotometric method for the estimation of pterostilbene in Pterocarpus marsupium extract. All the parameters of the analysis were chosen as per ICH Q2 (R1) guideline.

Methods: Pterostilbene solution was scanned over UV-visible range for its wavelength of maximum absorbance. Various calibration standards of pterostilbene were prepared. Calibration curve of concentration vs. absorbance was plotted and developed UV – visible spectrophotometry method was used for estimation of pterostilbene in standardized Soxhlet assisted extract (SAE)as well as ultrasound assisted extracts (UAE) of *Pterocarpus marsupium*.

Results: The maximum wavelength of pterostilbene was found to be 306 nm. The correlation coefficient of developed UV-Visible spectrophotometric method over the concentration range of 1-6 µg/ml was found to be 0.999. The validation study of the said method was carried out in terms of linearity, accuracy, precision, robustness, ruggedness, limit of detection and limit of quantitation studies. Developed UV – visible method was found to be precise for the intra- and inter- day studies and showed standard deviation in the range of 0.83 to 1.912&0.762 to 1.542respectively. The pterostilbene content in *Pterocarpus marsupium* extract was found to be 4.5% using the proposed UV – visible spectrophotometry method.

Conclusion: A simple, precise and cost-effective UV- visible spectrometry method was developed for the estimation of pterostilbene in standardized extract of *Pterocarpus marsupium*. The method was developed using solvent containing economical percentage of organic phase in aqueous media. Said validated UV- visible method can be efficiently used for the estimation of pterostilbene in extracts of *Pterocarpus marsupium*.

Keywords: UV- Visible Spectrometric Method; Pterostilbene; *Pterocarpus marsupium*; Validation

1. Introduction

Pterocarpus marsupium is the plant mainly found in central and peninsular India specifically in dry mixed deciduous tropical forests of Gujarat, Madhya Pradesh, Karnataka, Kerala, Maharashtra and Sub-Himalayan areas. *Pterocarpus marsupium* also known as Bijasar, Vijaya Saar and Asan belongs to family *Fabaceae*.[1-7]. Various parts of *Pterocarpus marsupium* viz. heartwood, fruits, bark and leaves are prominently used for the therapeutic benefits. Heartwood of *Pterocarpus marsupium* contains pterostilbene as vital phytochemical. Pterostilbene is chemically known as 3',5'-dimethoxy-4'-hydroxy-E-stilbene.

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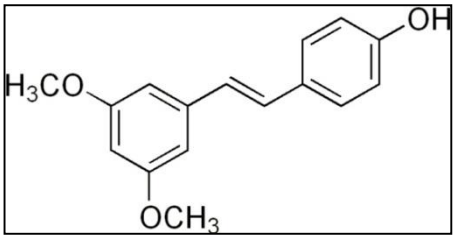

Figure 1 Chemical structure of Pterostilbene

Pterostilbene is widely used for the prevention and treatment of Alzheimer's disease, diabetes, high blood pressure, high cholesterol and supportive supplement during cancer treatment.[18-24]

Pterostilbene is soluble in organic solvent such as methanol, ethanol, dimethyl sulfoxide and sparingly soluble in water. Partition coefficient for Pterostilbeneis 3.69. The pKa values of pterostilbene are 9.5 and 4.5.

In spite of well-established physicochemical andtherapeutic importance, very few analytical techniques of pterostilbenehave been reported across the world. Till date, there is no single UV Visible Spectrophotometric method available for the accurate quantification of pterostilbene in the *Pterocarpus marsupium*, particularly in its Ultrasound Assisted (UAE) and Soxhlet Assisted (SAE) extracts.

Considering the future potential of pterostilbene, an accurate, precise and cost-effective UV-visible spectrophotometric method was developed and validated. Developed method was successfully used for the estimation of pterostilbene in the various extracts of *Pterocarpus marsupium*.

2. Materials and method

2.1. Materials

Pterostilbene was purchased from TCI Chemicals (India) Pvt.Ltd, Chennai. Methanol was purchased from Merck. All the chemicals of analytical grade were used for the proposed study.

2.2. Instruments Used

A double beam UV-visible spectrometer (UV-530, Jasco) connected to a computer loaded with spectra manager software was used for the analysis. Quartz cells having 3 cm length with 1 cm path length were used for spectral measurement. Electronic Balance (Essae, Vibra HT) with internal calibration mode was used for the accurate weighing purpose.

2.3. Preparation of standard stock solution

Accurately weighed 10 mg of Pterostilbene was transferred in to the calibrated volumetric flask and dissolved using 10 ml co-solvent system consisting of methanol and water (40:60 v/v) to achieve a stock solution of 1000 µg/ml (Stock-I) i.e., Primary Stock. Stock- I solution was suitably diluted with co-solvent system to achieve solution of 100 µg/ml working stock.

2.4. Determination of wavelength of maximum absorbance (λ_{max})

Stock-II solution was scanned using full scan mode for the entire range of UV and visible i.e. 200 to 800 nm with co-solvent system as a blank. After obtaining the spectrum, λ_{max} was identified with the help of software the spectrum is shown in (figure.2). In order to achieve reproducible results, above method was repeated five times.

2.5. Preparation of calibration curve

Calibration curve was prepared by diluting the stock-I solution to achieve the six different calibration standards representing CAL STD – 1µg/ml, CAL STD – 2µg/ml, CAL STD – 3µg/ml, CAL STD – 4µg/ml, CAL STD – 5µg/ml & CAL STD - 6µg/ml strength. Absorbance of each calibration standard was measured at pre-identified λ_{max};306nm using fixed wavelength measurement mode. The calibration curve representing concentration vs. absorbance was plotted. Above mentioned procedure was repeated five times so that reproducible results can be obtained.

2.6. Method Validation

Developed UV method for the estimation of pterostilbene was validated as per the ICH guideline. Different parameters like linearity range, accuracy, precision, robustness, and ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were evaluated [19-23].

2.7. Linearity and Range

Linearity of the proposed UV method was established using six different calibration standards. Based on analysis of calibration standards, calibration curves in terms of absorbance vs. concentration plots were developed and subjected to linear least square regression analysis. R^2value was considered to be important factor for establishing linearity of the proposed method. The interval between upper and lower concentration limit with acceptable linearity was reported to be the range of the proposed UV method.

2.8. Accuracy

The accuracy of the proposed UV method was evaluated using recovery studies after standard addition of analyte of interest. Three different solutions of pterostilbene were prepared in triplicate at the level of 80%, 100% and 120% of its predefined concentration. To the predefined concentrations, different amounts of pterostilbene were added (standard addition method) and the accuracy was calculated on the basis of percent recovery. For calculating the percent recovery following formula was used.

$$\% RC = (SPS-S/SP) \times 100$$

Where,

SPS = Amount found in the spiked sample
S = Amount found in the sample
SP = Amount added to the sample
% RC = Percent recovery

2.9. Precision

The precision of the proposed UV method was established by performing intra- and inter-day UV analysis of predefined samples. The study was performed at three concentration levels. Preparing nine different solutions of 1.5µg/ml, 3µg/ml, and 5.5µg/mlstrength of pterostilbene (3 solutions of each concentration) and evaluating them at morning, afternoon, and evening times on the same day was used to perform an intra-day precision study. The percent relative standard deviation was used to calculate the Percent Relative Standard Deviation (%RSD). Similarly, an inter-day precision analysis was carried by examining the above-mentioned solutions on 3 consecutive days.

2.10. Robustness

Robustness of the developed UV method was established using different percentage of methanol in co-solvent system. Methanol concentrations in the co-solvent system were kept at 41:59 and 39:61 percent, respectively, and pterostilbene was dissolved separately in the same co-solvent system. The absorbance of three samples was measured at 306 nm. The levels of pterostilbene in each sample were calculated usingthepre-defined calibration curve. The percentage RSD was used to calculate the results.

2.11. Ruggedness

Ruggedness study of the method was carried out by analyzing triplicate samples of pterostilbene solution (3µg/ml) on two different Instruments (V-530, Jasco and BA-UV-2600, BIOAGE)and absorbance were noted in terms of % RSD.

2.12. Limit of Detection (LOD)

The LOD of the developed UV method was calculated by using following formula

$$LOD=3.3\times SD/S$$

Where, SD= Standard deviation of Y-intercepts

S= Slope

2.13. Limit of Quantitation (LOQ)

The LOQ of the developed UV method was calculated by using following formula

$$LOQ= 10\times SD/S$$

Where, SD= Standard deviation of Y-intercepts

S= Slope

2.14. Estimation of Pterostilbene in Pterocarpus marsupium extract

2.14.1. Drying treatment for preparation of Pterocarpus marsupium powder extract

Pterocarpusmarsupium dried at 50 °C using a Microtray drier (S.B. Panchal and company, Mumbai, India) and powdered using a twin-blade mixer (Bajaj electrical ltd., Mumbai, India). To select uniform particle size, the powder was sifted in a sieve shaker (CIP Machineries, Ahmedabad, India) with sieves of different sizes (12, 24, 45, 85 and 120 mesh, Swastika electric and scientific works, Ambala, India) for 15 min. Powder passed through 120 mesh sieve was collected and used for further extraction.

Soxhlet assisted extraction (SAE)

Pterocarpus marsupium powder were dried at 50^{o}C using a Micro tray drier (S.B. Paschal and company, Mumbai, India) and powdered using twin blade mixer (Bajaj electrical ltd, Mumbai, India). Powder was sifted in a sieve shaker (CIP Machineries, Ahmedabad, India) for 15 minutes with sieves of various sizes (12, 24, 45, 85, and 120 mesh), Swastika electric and scientific works, Ambala, India) to select uniform particle size. Powder was collected and used for extraction after passing through a 120 mesh sieve. The extraction was done using the Soxhlet assisted extraction (SAE) technique. A thimble (Borosil, Mumbai, India) containing 10g of powdered Pterocarpus marsupium Powder was inserted into a Soxhlet apparatus. Methanol was used to extract the material completely. SAE was carried out for 2 hours. The solvent was distilled off under reduced pressure using a rotary vacuum evaporator (Heidolph instruments GmbH & co. Germany) to obtain the dry extract after a predetermined extraction period. 1 was precisely weighed.1 mg of Pterocarpus marsupium Powder dry extract was transferred to a calibrated volumetric flask and dissolved in 10 ml of methanol to make a 1000 g/ml stock solution (Stock-III). The stock-III solution was diluted with a co-solvent system and the pterostilbene content was determined using the proposed UV method.

Ultrasound Assisted Extraction (UAE)

The extraction of *Pterocarpus marsupium* was conducted using a tunable ultrasonic bath (PCi^{Tm}Analytics, 230V AC, 50 Hz, Mumbai, Maharashtra, India). 10 gm of powder was weighed and placed in 100 ml of conical flask. The extraction of Pterocarpus marsupium powderwas carried out by placing the beaker in an ultrasonic bath with the fixed power of 150W. The conical flask was immersed in the ultrasonic bath and extracted for 20 min. After extraction process, extract was cooled to room temperature and kept for centrifugation by using micro centrifuge at 25^{0}C using 10,000 rpm for 10 min. Lastly, the supernatant was collected and filtered by using 0.45 µm fitted with syringe filter. The filtrate was suitably diluted with a co-solvent system and analyzed for the pterostilbene content using the proposed UV- visible spectrophotometry method.

3. Results and discussion

3.1. Determination of wavelength of maximum absorbance

Identification of wavelength of maximum absorbance is prerequisite for quantitative UV analysis. In general, a solution with an absorbance value less than 1 is considered suitable for determining the wavelength of maximum absorbance. The maximum wavelength for pterostilbene solution was determined using the full scan mode of a UV-Visible spectrophotometer, taking into account the prerequisites and suitability. UV software was used to process the entire scan, and software was used to identify the maximum. The wavelength of pterostilbene was discovered to be 306 nm. (Fig.2).

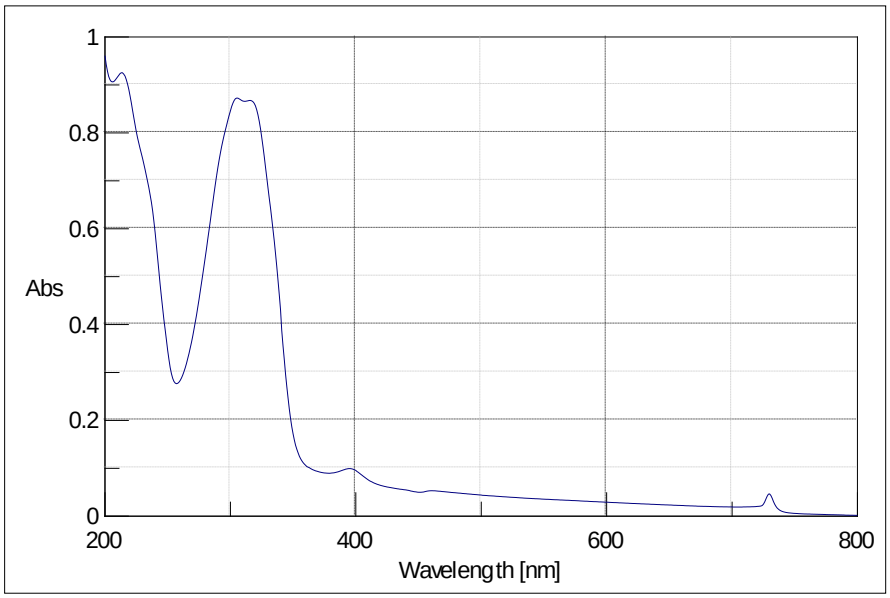

Figure 2 UV-visible spectra of Pterostilbene

3.2. Preparation of calibration curve

Quantification of unknown samples by UV-Visible spectrophotometer or any other instrumental method of analysis needs a reproducible calibration curve and an equation stating correlation between concentration and the response. In comparison to the graphical technique, the above-mentioned method is widely recognised and repeatable. The calibration curve for pterostilbene was built using six different calibration standards, taking into account the utility of quantitative analysis of pterostilbene. Using the fixed wavelength mode of a UV-Visible spectrophotometer, the absorbance of several calibration standards at 306nm was measured. The calibration curve was performed five times, with the mean values and deviations presented in Table 1.

Table 1 Calibration standard data for Pterostilbene

Concentration (µg/ml)	Absorbance
1	0.2569 ±0.0015
2	0.4156±0.0019
3	0.5582 ±0.0014
4	0.7109 ±0.0016
5	08589. ±0.0012
6	0.9892±0.0011

3.3. Method validation

3.3.1. Linearity and Range

Linearity and range are the key parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. A six-point calibration curve for pterostilbene was developed, encompassing a range of 1-6µg/ml, taking into account the relevance of linearity and range. Table 1 shows the concentrations and their corresponding mean absorbance values. When the calibration curve was submitted to least square regression analysis, the equation y = 0.0147x+0.117 was discovered, with a correlation value of 0.999, as shown in Figure 3.

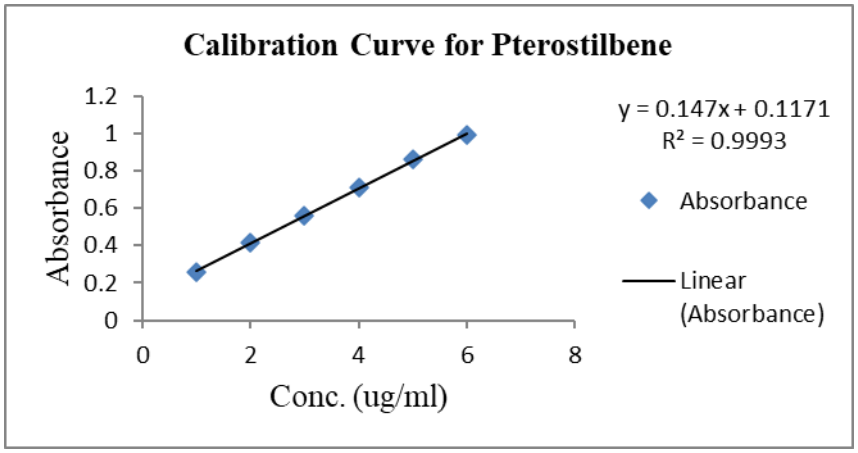

Figure 3 Calibration curve for Pterostilbene

According to linearity analysis, the developed UV method was found to be linear over the pre-defined concentration range of calibration standards.

3.4. Accuracy

Accuracy is a measure of the closeness of the experimental value to the actual amount of the substance in the matrix. Accuracy is to be established over the entire calibration range of the analytical method so that at any point of determination, results obtained would be reliable. In case of UV method for pterostilbene, accuracy was established using recovery studies. At 80 % standard addition, mean recovery of pterostilbene was found to be 100.10% whereas at 100 and 120 % standard addition, it was found to be 100.61 and 100.42% respectively. Percent RSD was found to be less than 2% for the pterostilbene recovery studies as shown in Table 2.

Table 2 Accuracy data of UV method for Pterostilbene

Concentration (%)	Origin level (µg/ml)	Amount added (µg/ml)	% Recovery	Mean % Recovery	% RSD
80	1.5	1.2	99.66	100.10	0.457
80	1.5	1.2	100.57		
80	1.5	1.2	100.07		
100	3	3	99.94	100.61	0.869
100	3	3	101.59		
100	3	3	100.30		
120	5.5	6.6	99.32	100.42	1.128
120	5.5	6.6	100.37		
120	5.5	6.6	101.58		

According to the observations of accuracy experiments, the proposed UV methodwas found to be accurate, with a percent recovery of 98 to 102%.

3.5. Precision

Precision is a measure of degree of scatter. It expresses the reproducibility of the measurements. It is anticipated that an analytical procedure would produce repeatable results. Accurate results are the outcome of a precise analytical process. Intra- and inter-day precision of the devised UV technique were established at 1.5µg/ml , 3µg/ml and 5.5 µg/ml levels of pterostilbene, taking into account the necessity of reproducible but precise findings. Table 3 and Table 4 show

the findings in terms of mean absorbance values, percent assay, and percent RSD for the intra- and inter-day precision studies, respectively.

Table 3 Intra-day precision data of UV method for Pterostilbene

	Morning			**Afternoon**			**Evening**		
Concentration Range (µg/ml)	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**
1.5	1.51	100.74	0.930	1.516	101.1	1.019	1.523	101.55	1.912
3	2.98	99.48	1.81	3.033	101.1	1.209	3.048	101.61	1.312
5.5	5.50	100.12	1.65	5.54	100.8	0.83	5.518	100.33	0.911

Table 4 Inter-day precision data of UV method for Pterostilbene

	Day 1			**Day 2**			**Day 3**		
Concentration Range (µg/ml)	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**
1.5	1.517	99.14	1.287	1.519	98.74	1.542	1.528	98.09	1.224
3	3.022	99.84	1.446	3.039	99.90	0.726	3.042	100.1	1.209
5.5	5.523	98.46	1.134	5.511	98.54	1.061	5.508	98.32	1.120

Intra-day precision of proposed UV-Visible spectrophotometric method was found to be in the range of 0.83 to 1.912 whereas inter-day precision of the proposed method was found to be in between 0.762 to 1.542.

3.6. Robustness

Robustness of analytical method is the ability of a method to resist the change in its performance in spite of small, deliberate change in method parameters. It is a vital parameter of an analytical technique since a slight, unintentional change in method parameters such as solvent composition, pH, and others can occur during normal usage and impede the method's performance. It is expected that such a modification should have no effect on the analytical method's performance. As a result, a reliable analytical procedure can be chosen. Modifying the composition of the co-solvent solution showed the robustness of the suggested UV method. The method performance was unaffected by changing the methanol content in the co-solvent solution during slight change from 39-41%. Table 5 shows that the percent RSD values ranged between 0.664 and 1.347. The suggested UV technique is robust in nature, with percent RSD values below 2%.

Table 5 Robustness data of UV method for Pterostilbene

Concentration (µg/ml)	**% MeOH: Water**	**Absorbance**	**% RSD**
3	40-60	0.4351	1.313
3	39-61	0.5979	0.664
3	41-59	0.5165	1.347

3.7. Ruggedness

Ruggedness of analytical method is the ability of a method to resist the change in its performance in spite of influential environmental factors like change in labs; equipments and analyst. Rugged analytical method is selected since they are not affected by environmental or external variables. Pterostilbene solution was examined using two separate UV-Visible spectrophotometers from two different labs to determine the robustness of the proposed UV - visiblemethod. The

percent RSD values were found in between 0.2248 to 0.9415 after sample analysis and data processing. The suggested UV Visible spectrophotometric method was found to be robust; with percent RSD values less than 2%, as shown in Table 6.

Table 6 Ruggedness data of UV method for Pterostilbene

Concentration (µg/ml)	Instruments	Absorbance	% RSD
3	Jasco	0.6113	0.2248
3	Bioage	0.6255	0.7199
3	Analyst -I	0.6325	0.8421
3	Analyst -II	0.6415	0.9415

3.8. Limit of Quantification (LOQ) and Limit of Detection (LOD)

LOQ represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. LOD and LOQ of proposed UV method was found to be 0.2075 and 0.6288 µg/ml respectively as shown in Table 7.

Table 7 LOD & LOQ data for UV method for Pterostilbene

LOD	0.2075µg/ml
LOQ	0.6288µg/ml

Lower LOQ value indicated that proposed method would be suitable for analyzing the samples containing even small quantities of Pterostilbene.

3.9. Estimation of Pterostilbene in Pterocarpus marsupium powder extracts

Pterostilbene content in *Pterocarpus marsupium*extracts was successfully estimated using the developed UV-Visible spectrophotometric method. In Soxhlet assisted extracts of *Pterocarpus marsupium*, concentration of Pterostilbene was found to be 0.020g /10gwhereas in ultrasound assisted extracts, the Pterostilbene content was found to be 0.205 g/10g using the suggested UV – visible spectrophotometry method.

4. Conclusion

A sensitive, accurate and precise UV-Visible spectrophotometric method for the estimation of Pterostilbene was successfully developed. The developedmethod was found to be robust and rugged and capable of estimating pterostilbene content in variety of *Pterocarpus marsupium* extracts.

Compliance with ethical standards

Acknowledgments

The extramural grant funding from the DST-DPRP, Government of India (Ref:-VI-D&P/626/2018-19/TDT) to P.I. Dr. Sachin S. Bhusari for the suggested research effort is warmly welcomed.

Disclosure of conflict of interest

No conflict of interest.

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