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Development and Validation of a UV-spectroscopic Method for the Analysis of Black Plum in Marketed Preparations Using Ethanol as a Solvent

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Herbal drugs are composed of single or several types of medicinal plants with additives. This type of preparations is gaining a wide range of popularity among a large a number of people throughout the world. With the increase of usage, it is necessary to maintain the quality of these drug preparations. Therefore, analytical approaches for their intended use in drug quality evaluation need to be validated. Our aim was to develop simple, rapid, selective, precise and economical UV-spectroscopic method for the quality control of black plum containing herbal preparation in different marketed formulation. Spectrometric analysis of black plum using ethanol shows the maximum absorbance at 279 nm. It has shown linear absorbance over the concentration range of 0.1-0.5 μg/ml with R2 value 0.9914. The developed method was validated as per ICH Q2 (R1) guidelines for various parameters namely linearity, precision, accuracy, robustness. The results were calculated statistically and it showed compliance with ICH guidelines. The relative standard deviation values and percent recovery has been found to be satisfactory, suggesting that the approach proposed is accurate. This proposed method can thus be used for the regular analytical study of black plum in bulk and pharmaceutical formulation.

Keywords: Herbal drugs; Method development; Method validation; UV spectroscopy.

Drugs which are made from botanicals, or plants and that are used to cure diseases or to maintain health are called herbal drugs. For thousands of years, herbal medicine has been used among the people for the treatment of various diseases.80 percent of the global population is estimated to rely on traditional herbal medicine for primary health care[1]. Many consider herbal medicine to offer an alternative treatment for different diseases, especially diseases requiring lifelong pharmaceutical medication. It is believed that phytoconstituents present in herbal preparations show better compatibility in human system. With the increasing usage of herbal drugs, maintaining the safety and efficacy of these drugs have become a major concern. Safety and efficacy of drugs must be ensured through standardization before the drug entering into the market. It is very difficult to maintain the quality of herbal drug formulation due to variation in chemical profile

of plant sources and these variations is affected by various factors like as growing, harvesting, storage and drying processes[2-4]. This variation can affect chemical constituents as well as pharmacological activity of drug. Therefore, it is necessary to develop methods for standardization of preparation available in the market[5,6].

Syzygium cumini or black plum is a member of Myrtaceae family and native to Indian subcontinent. It is widely distributed in tropical and subtropical region. It is found in India, Pakistan, Bangladesh, Myanmar, China, Indonesia, Ceylon. It is also found in Asia, South Africa and Nepal [7-9]. It is also cultivated in different parts of the world like as United states and Australia due to its great economic importance. It is grown as a fruit producer and as a source of timber.

S. cumini is rich in compounds containing phenolics, flavonoids, glucoside derivative. The leaf extracts contain flavonoids like as quercetin, kaempferol, myricetin, myricitrin and gallic acid, ellagic acid, ferulic acid, chlorogenic acid as phenol derivatives [10-12]. The stem bark of *S. cumini* contains gallic acid, ellagic acid, â-sitosterol, betulinic acid, myricetin, quercetin, friedelin, epi-friedelanol, eugenin, tyannin and flavonoids[13-16]. The most widely used parts of *S. cumini* is its seed. The seed contains hydrolysable tannins, phenolic contents, eugenol, terpenes. The fruits also contain glucose, gallic acid, citric acid, raffinose, anthocyanins, 7-hydroxycalamenene,oleanolic acid, â-sitosterol, methyl- â-orsellinate [17,18].

S. cumini seed is used to treat different types of diseases. It is also reported to have its usage in the treatment of diarrhea, stomach-ache, piles, dysentery, digestive problems[19].Different literature studies prove the usage of its seed has antidiabetic effects. Various parts of the Syzygium cuminiplantact as liver tonic, purifies blood, strengthen teeth and gums has a great role in the treatment of ringworm infection of head [20].

Spectroscopic analysis is a widely used technique in the analysis of herbal drugs. Some studies describe the uses of HPLC, HPTLC method in the analysis of different polyherbal formulations [21,22]. Single or multiple herbs containing preparation like as Ayurvedic, Unani or polyherbal formulations can be analysed using UV-spectroscopy. In this technique, it involves the measurement of ultra violet radiation absorption by the substance in solution. Both qualitative and quantitative analysis can be done through this technique. It is a simple, accurate, inexpensive technique for the analysis of small amount of sample. This technique is based on Beer-Lambert's law. In the present study, we aimed at development and validation of UV-spectroscopic technique according to ICH(Q2) guideline for the analysis of S. cumini seed in polyherbal formulation[23].

MATERIALS AND METHODS

Instruments used

Shimadzu double-beam UV-Vis spectrophotometer (UV-1800 Model) having 1.5 nm spectral bandwidth with 10 mm quartz cuvette cell was employed in the assay purpose. UV-probe software version 2.43 was used for data acquisition for the studied sample. For weighing the standard and sample, analytical balance (Model- Ht224R, Shinkodens-hi Co. Ltd, Japan) was used.

Materials

S. cumini fruits were purchased from local market and seeds were collected from these. Then the seed was identified in the national herbarium of Bangladesh having accession no. 50,443. Laboratory grade ethanol was used in analysis and it was bought from Merck Chemicals, India. Three formulations containing *S. cumini* seed were collected available in local market. All the other chemicals, kits and reagents employed were of laboratory grade.

S. cumini extract preparation

Through proper washing of *S. cumini* seeds, the dust attached to it was eliminated properly and was air dried until it is crispy. Then it was ground to powder. About 350 gm of powdered fruit materials were taken in an amber-colored reagent bottle and kept for soaking in 1.5 litres of ethanol. The soaked materials-containing container was properly sealed and held for a period of about 14 days with occasional shaking and stirring. The whole mixture was filtered using cotton and then Whatman No. 1 filter paper successively to get the clear filtrate. Then concentrated crude seed extract was collected using rotary evaporator through evaporation of solvent. Next the concentrated extract was further taken in water bath and air dried for complete drying. The obtained extract was preserved for further analysis.

Solvent selection

The solubility of *S. cumini* seeds was checked using methanol, ethanol, acetone and ethyl acetate. The extract showed better solubility in ethanol in compare to other solvents.

Preparation of standard solution

*10 mg S. cumini*seeds extract was accurately weighed and taken in a 100 mL volumetric flask, volume was adjusted up to the mark with ethanol. A stock solution having concentration of 100 ìg/mL was found. From the stock solution, standard solution of different concentration (0.1-0.5 ìg/mL) was prepared by dilution with ethanol.

Selection of the wavelength

From these prepared solutions, 0.03µg/mL solution was scanned in 200-800 nm uv-visible range in 1.0 cm cell using ethanol as blank.The maximum absorbance for this solution was found at 279 nm.

Standard calibration curve preparation

At 279 nm wavelength, the absorbance of *S. cumini*seeds extract was measured at different concentrations (0.1-0.5 ìg/mL). A calibration curve was plotted between concentrations versus absorbance of the extracts and a regression equation was found. From the calibration curve, linearity was observed utilizing a regression equation.

Validation of method

Validation is an integral part of quality assurance. A good analytical practice cannot be achieved without validation. Method validation is defined as the process that is used to ensure that the analytical technique used for a particular test is sufficient for its intended use. Method validation results can be used to assess the efficiency, reliability and accuracy of analytical findings. Validation of the developed analytical method was carried out following the guidelines set by International Conference on Harmonization (ICH) which is known as ICH Q2 (R1) guidelines [23]. The major validation parameters described in the guidelines like as specificity, linearity, range, precision, accuracy and robustness were studied for validation of the method.

Specificity

Standard solution of *S.cumini*seeds extract at the concentration of 0.03 ìg/mL was analyzed by the proposed method and specificity of the method was studied.

Linearity

Linearity of the method was studied through measuring the absorbance of five standard concentrations of*S. cumini*seedsextract(0.1-0.5 ìg/mL) at 279 nm using ethanol as blank. A calibration curve was plotted between concentrations versus measured absorbance of the extracts and regression analysis was done from the calibration curve.

Precision

Precision was established by evaluation of repeatability, intra-day precision and intermediate precision. To evaluate precision, the absorbance of standard which is *S. cumini* seeds extract was determined at a concentration of 0.1 ìg/mL for 6 (six) times in the same day known as repeatability. The absorbance of 3 (three) concentrations of *S. cumini*seeds extract standard at a concentration of 0.1 ìg/mL, 0.3 ìg/mL and 0.5 ìg/mL was measured in six letat same day and later on three consecutive days which are known as intra-day precision and inter-day precision respectively.

Accuracy

The accuracy of the proposed method was established by recovery investigation with the addition of known amounts to a pre-analyzed sample solution. For this purpose, known concentrations of *S. cumini* fruits extract standard solution at a concentration of 0.1, 0.2 and 0.3 ìg/mL were added to three pre-analyzed sample solutions respectively which concentration was 0.2 µg/mL. The observed result was used to assess the percentage recovery of the standard.Same process is repeated for three times for each concentration[24]. The percent recovery was calculated using following equation.

$\%\ \text{Recovery} = [C_t/C_a] \times 100$

Where,

C_a = Conc. of the *S. cumini* after standard addition;

C_t = Conc. *S. cumini* in the test sample

Range

Five different concentration of *S. cumini*seeds (0.1 ìg/mL,0.2 ìg/mL,0.3 ìg/mL,0.4 ìg/mL and 0.5ìg/mL) were used for the assessment of the range of the method.

Robustness

Robustness of the method was studied by analyst to analyst variation and instrument to instrument variation studies. For this purpose, the value of absorbance 0.5 ìg/mL standard *S. cumini* seeds was estimated and % RSD (percentage of relative standard deviation) values were calculated

between them (first analyst and second analyst; UV-spectrophotometer of model UV-1800 and UV-1240V) were calculated.

Use of proposed method for estimation of *S. cumini* seeds in marketed preparation

0.263 ml *S. cumini* seed containing market preparation, equivalent to 10 mg *S. cumini*seed extract was measured and transferred in a separating funnel. 10 ml water was added to it and extracted with 15 ml of Chloroform. Then the aliquot was filtered using Whatman No. 1 filter paper and the filtrate was collected. For better extraction, addition of water and chloroform was repeated three times. In order to get the dry material, the acquired filtrate was subjected to evaporation in a water bath. The residue was dissolved well in ethanol and volume was adjusted up to 100 ml with ethanol. This solution was used as stock for further analysis. A test solution was prepared having concentration of 0.5 ìg/mL using ethanol as diluent. The absorbance of the solution was measured at 279 nm wavelength against ethanol as blank.

Statistical analysis

All the observed results have been shown as Mean ± Standard deviation and % RSD.

RESULTS AND DISCUSSION

Method development

Different solvents like methanol, ethanol and ethyl acetate were used to determine the solubility, peak quality and peak shape of the studied drug. Among these, ethanol fulfilled all criteria in showing better solubility and giving better peak quality. The sample showed maximum absorbance at 279 nm which is shown in Figure 1.

Method validation

The developed method was validated according to the rules of ICH Q2 (R1) guidelines and the found results were summarized in Table 1.

Specificity

The ability of an analytical method to reliably measure an analyte in the presence of interferences that may be present in the sample

Table 1. Obtained results of validation parameters by developed UV method

Validation parameters	Obtained results
Absorption maxima (λ_{max})	279 nm
Limit of Beer's Law (ìg/mL)	0.1-0.5
Equation for Regression (y=mx +c)	y = 1.3273x + 0.0441
	R^2 = 0.9914
Slope	1.3273
Intercept	0.0441
Correlation coefficient	0.9914
% RSD of Repeatability, (n=6)	0.7950
Accuracy	99.647-101.767%
Precision (% RSD)	Intra-day = 0.924; 0.571; 0.467
	Inter-day= 0.892; 0.835;0.342
Robustness (% RSD)	0.825; 0.361

Table 2. Linearity and range study of the developed UV method

S. cumini seed standard conc. (ìg/mL)	Absorbance (279 nm) (Mean±SD)	% RSD
0.1	0.201±0.002	1.034
0.2	0.283±0.003	0.935
0.3	0.432±0.003	0.694
0.4	0.577±0.005	0.794
0.5	0.718±0.003	0.418

matrix is referred to as specificity. It signifies that the presence of excipients in formulation does not interfere with the drug peak. The result of specificity is represented at figure 1 and the result indicates the proposed method was found specific and selective for the drug.

Table 3. Results of precision (intermediate precision, intra-day precision and repeatability) of the developed method

S. cumini seed standard conc. (µg/ml)	Absorbance (279 nm)	% RSD
Intra-day precision of *S. cumini* seed standard (n=6)		
0.1	0.234±0.002	0.924
0.3	0.432±0.002	0.575
0.5	0.735±0.003	0.467
Inter-day precision of S. cumini seed standard (n=3)		
0.1	0.233±0.002	0.892
0.3	0.432±0.004	0.835
0.5	0.736±0.002	0.004
Repeatability (n=3)		
0.1	0.2250±0.002	0.342

Linearity and range

Linearity refers to a method's ability to achieve test results that are proportional to the analyte concentration in the sample. The range of an analytical method is the distance between the upper and lower levels that have been demonstrated to be calculated with precision, accuracy, and linearity using the set method.

A **g**ood linear correlationwas achieved between absorbance and concentration in the range of 0.1 – 0.5 µg/ml. A linear regression equation was found to having a slope of 1.327, intercept of 0.0441 and the coefficient of correlation value is 0.9914 (Figure 2). The result of linearity is represented in Table 2.

Precision

The degree of agreement among individual test results, when a procedure is replicated on multiple samples of the same homogeneous sample

Table 4. Accuracy study data of the developed UV method

Starting amount of *S. cumini* seed (µg/mL)	Added amount of *S. cumini*seed (µg/mL)	Expected conc. (µg/mL)	Obtained conc. (µg/mL)	Residual conc. (µg/mL)	Mean recovery percentage	% RSD
0.2	0.1	0.3	0.305	0.005	101.767	0.694
0.2	0.2	0.4	0.408	0.008	101.943	0.794
0.2	0.3	0.5	0.498	0.002	99.647	0.425

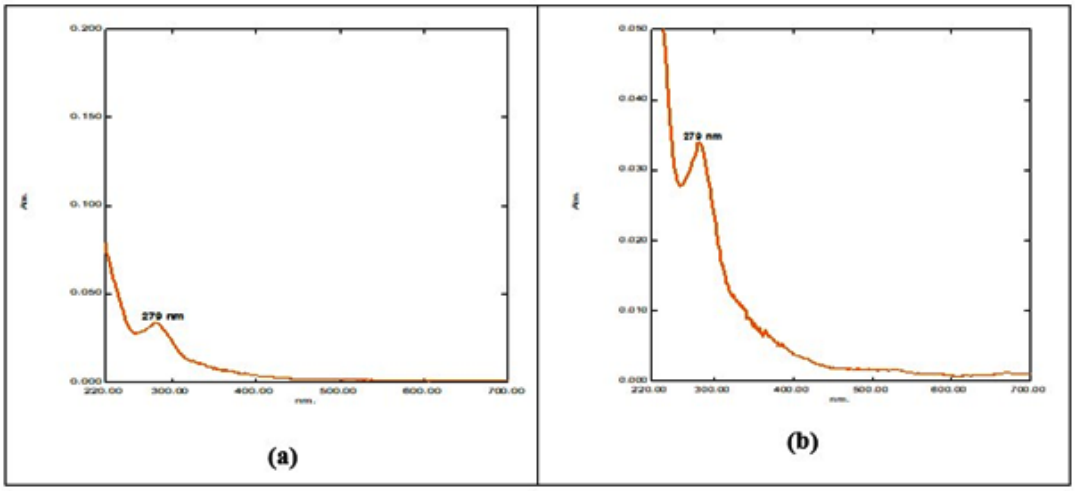

Fig. 1. UV spectrum of S. cumini seed in ethanol (a). Whole spectrum of *S. cumini* seed and (b) Zoomed spectrum of *S. cumini* seed

is called precision. Precision is calculated by injecting a set of standards or examining several samples from a homogeneous lot. Precision as relative standard deviation (percent RSD) is determined using the observed standard deviation (SD) and Mean values. Precision can be established through determination of intraday precision, inter-day precision and repeatability. Intraday precision refers to the use of an analytical technique in a laboratory over a short period of time by the same operator with the same equipment, while inter-day precision refers to the calculation of differences in analysis when a process is used in a laboratory on various days by different analysts. A selected concentration 0.1 µg/ml, 0.3 µg/ml and 0.5 µg/ml were analysed in six-let for intra-day and inter-day precision and 0.1 µg/ml was analysed for repeatability.

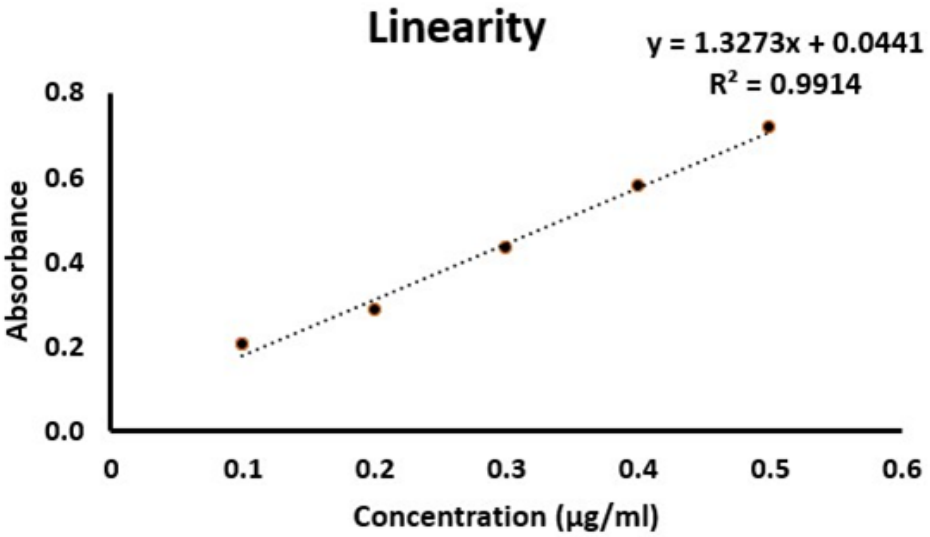

Fig. 2. Linearity and range study of the developed UV method

Table 5. Robustness study of the developed UV method by analyst and instrument

Studied parameters	Absorbance at 279nm (Mean±SD)	% RSD between sample	Mean Recovery percentage	% RSD between two analysts
Analyst 1	0.718±0.003	0.425	101.531	0.825
Analyst 2	0.710±0.002	0.282	100.353	
Instrument 1	0.717±0.005	0.688	101.296	0.361
Instrument 2	0.720±0.002	0.289	101.814	

Table 6. Assay results for *S. cumini* seed estimation in *S. cumini* seed market preparation

Formulation	Tested conc. of *S. cumini* seed in market preparation	Absorbance at 279 nm (Mean ± SD)	% RSD	Observed conc. of *S. cumini* seed in market preparation	Mean recovery percentage
A	0.5 µg/mL	0.614±0.0035	0.574	0.416 µg/mL	83.152
B	0.5 µg/mL	0.634±0.0050	0.973	0.434 µg/mL	86.821
C	0.5 µg/mL	0.659±0.0040	0.607	0.045 µg/mL	90.082

The obtained results of precision (intraday precision, inter-day precision and repeatability are represented in Tables 3 The value of Standard Deviation, % RSD for the intra-assay precision, intermediate precision and reproducibility for all the three concentration showed an excellent intraday precision, intermediate precision and reproducibility of the proposed method.

Accuracy

The accuracy of an analytical method is defined as the degree of closeness of the test results obtained by that method to the true value. It is also known as trueness. Accuracy was determined by injecting a known concentration of standard to a pre-analysed sample using the "method being validated." The % recovery for the standard addition and reference analysis method for all the three concentration levels found are 101.767% with % RSD 0.644, 101.943% with % RSD 0.7942 and 99.647% with % RSD 0.426. From the obtained result, this high degree of confidence interval signifies that any small change in the sample concentration can be accurately determined with high level of accuracy. The results found from the standard addition and reference analysis method were also found signifies the accuracy of the proposed method.

Robustness

The terms robustness is an analytical method's ability to remain unaffected by minor changes in its parameters. The results of robustness are shown in Tables 5.

To study robustness, the same sample of same at a same concentration (0.5 ìg/mL) was analyzed in triplicate by two different analysts (first and second analysts) and in two different instruments (UV-Jasco V-630 and UV-Secom am XTD6). The values of % RSD in both the parameters appeared to be <2 %. This result confirms robustness of the method.

Estimation of *S. cumini* seed in market preparation

The result of the assay of *S. cumini* seed in market preparation is shown in Table 6.

The developed method has been successfully used to estimate *S. cumini* seed in market preparations. A, B, C three market preparations were collected from the market and the percentage of S. cumini seed was determined. It was found to be 83.152 %, 86.821 % and 90.082 % with % RSD value 0.574, 0.973 and 0.607 respectively as shown in Table 6.

CONCLUSIONS

In conclusion, a simple, reliable, accurate and reproducible method has been developed and validated for analysis of *S. cumini* seed containing formulation. The developed method needs low cost, have faster speed with satisfactory precision. The method also has good specificity. The developed method was successfully validated following the guidelines of ICH and it could be employed for quality control analysis of *S. cumini* seed in market preparation.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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