Research Paper

Ratio Derivative Spectrophotometric Method for Guggul Estimation from Polyherbal Formulation of Triphala-guggul Endorsed by High-performance Liquid Chromatography Method

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The aim of the study was to develop a simple and economic ultra violet-spectrophotometric technique for determination of guggul from a polyherbal formulation, following international council for harmonisation guidelines. Endorsement of this method was done by using the high-performance liquid chromatography technique. Ratio and ratio derivative spectroscopy were used and later these were validated by high-performance liquid chromatography method. Inter and intra-day precision, % recovery were done for accuracy of the method. Further the methods were employed for the determination of guggul concentration in a formulation. The maximum absorption (λ_{max}) of guggul in aqueous solution was found to be 280 nm. The linearity concentration range of 1-5 mg/ml with satisfactory R2 value of 0.998 was observed. % recovery at three different levels, 80 %, 100 % and 120 % found within the range of 90.90-93.93 %, indicating good accuracy. Furthermore, the low values of % RSD demonstrated the method's accuracy and reproducibility. The precision of the method was evaluated through intraday and inter-day studies, as well as repeatability. The % RSD value, which was found to be less than 2, indicated the method was precise. The results obtained from the ratio derivative spectroscopy analysis were further analysed using the high-performance liquid chromatography method. The % recovery data of high-performance liquid chromatography method was in good agreement with the % recovery data of the ratio derivative spectroscopy analysis, confirming the reliability of the method. Based on the results it can be concluded that ratio derivative spectroscopy method was comparable with high-performance liquid chromatography method. Ratio derivative spectroscopy method was reliable and precise technique for determining guggul from polyherbal formulations. Hence ratio derivative spectroscopy might be the used as a method for the quality control of guggul from polyherbal formulations in industry as a simple, rapid and economic method compared to high-performance liquid chromatography method.

Key words: Ultra violet-spectrophotometric method, guggul, high-performance liquid chromatography, polyherbal formulation, method validation

Since ancient time, in accordance with information, it is estimated that about 80% of total world population were dependent on various herbs and their many kind of medicinal formulation for basic primary health care[1]. Market of polyherbal medicine is fast growing industry[2]. Hence, World Health Organisation (WHO) guidelines for various polyherbal products are being standardized to evaluate their quality[3]. Since there is lack of information regarding estimation of active phyto-compound by using a standard protocol, it is very challenging in analytical research field.

The herbal medicine is prepared following the

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Ayurvedic system and those are categorized into two types of formulation such as single herb formulation and polyherbal formulation[4]. *Commiphora wightii* (*C. wightii*) is one of the most useful and valuable medicinal plant mentioned in literature and is commonly known as 'guggul' in India. Oleo-gum-resin is secreted from the incision part of stem and main branches which are active against arthritis, rheumatism, hypercholesterolemia and hyperlipidemiaetc and various other inflammatory diseases. Many varieties of guggul polyherbal formulations are marketed commercially but no analytical method is still available for quantification of guggul from polyherbal formulations. "Triphalaguggul" is an important polyherbal formulation which is composed by dried fruit part of three plants such as *Terminalia chebula* (Haritaki), *Terminalia belerica* (Bibhitaki), *Emblica officinalis* (amlaki) along with *Commiphora wightii* and *Piper longum*. It is used as blood purifier and for its anti-inflammatory, anti-arthritic effect[5,6].

The Ratio Derivative Spectroscopy (RDS) is very useful method for quantification of analytes from the unresolved spectral band of a mixture[7,8]. It is already used to determine β-carotene and astaxanthin simultaneously secreted from Phaffiarhodozyma[9]. In this method divisor spectra of individual analyte is needed to divide the zero order spectrum of a mixture formulation through the derivatization method[10,11]. The divisor spectrum is selected on the basis of minimal background noise and experimental error. Ratio derivative spectra show lot of maxima or minima peaks at various wavelength, which is advantageous for this method. Therefore, these wavelengths help to determine the particular compound from a mixture of other compounds[12,13]. Thus RDS method is fast and economic.

High-Performance Liquid Chromatography (HPLC) is a reliable method for the estimation of phyto compounds from polyherbal formulations. Bacoside A3 and piperine were estimated by HPLC method from the ayurvedic formulation BramhiVati[14]. Gallic acid was quantified from the traditional ayurvedic formulation Pathyashadangam kwath using by HPLC method[15]. Curcumin and quercetin were also estimated from polyherbal formulation Divyamadhunashini Vati by the HPLC method[16]. Ellagic and gallic acid were quantified from the polyherbal formulation-Triphala Churna using by HPLC method[17]. The outcome from HPLC is precise and accurate but the process is costly and time consuming.

In our study, we tried to perform a comparison between the results of the two methods and to establish the results of RDS. As RDS method was found to be economic, simple, rapid, and accurate method, it could be widely used to estimate a guggul from a polyherbal formulation-Triphala guggul. The proposed method was developed and validated in accordance with International Council for Harmonisation (ICH) Q2 (R1) guideline. This was a novel approach for qualitative and quantitative estimation of guggul as bio-active compound in polyherbal formulation like Triphala-guggul.

MATERIALS AND METHODS

Chemicals and apparatus:

Raw guggul, triphala and triphala-guggul were supplied by the pharmacy department of I.P.G.A.E. and R, Kolkata. HPLC grade methanol and acetic acid were supplied by the Merck. Ultra Violet Visible (UV-Vis) spectrophotometer (Double beam UV-Vis spectrophotometer instrument; model/no. UV-2450 makes SHIMADZU) and HPLC (515 HPLC pumps and 2489 UV/VIS detectors of Waters Company, United States of America (USA), and Empower software using Reverse phase C-18 column) were used.

Preparation of standard solutions:

Guggul from market was powdered and 0.1 g was taken. 10 ml of water was added followed by vortexing for 5-10 min. The resulting mixture was kept for overnight and then it was filtered. The filtrate was again filtered by the 0.2 um, 25 mm nylon membrane filter to obtain the clear solution. The strength of the stock solution of guggul was adjusted to 10 mg/ml. The final concentrations were made in the range of 1-5 mg/ml which was used for standard curve preparation[18,19].

Determination of wavelength of maximum absorbance (λ_{max}) of guggul and preparation of standard calibration curve

The wavelength of maxima (λ_{max}) was determined with 5 mg/ml solution of guggul by scanning from 200 nm to 800 nm using distilled water as a blank solution[18]. The scan was done in triplicate set and a standard calibration curve was prepared. Linearity was checked by investigating the regression equation[18].

Procedure for RDS:

The standard spectrum divisor concentration, points of smoothing function and Δλ are needed to determine for first order derivative ratio spectroscopy for the optimization of signal with good selectivity and higher sensitivity[20]. 0.04 mg/ml of triphala was the divisor concentration to estimate the guggul from a polyherbal formulation. The divisor concentration was chosen on the basis of minimal background noise. The guggul zero order spectrum was divided by the spectra of triphala divisor concentration to obtain the ratio spectra of guggul. A Δλ=5 was the optimum for the minimum background noise. The first order derivation of guggul ratio spectra was performed at Δλ=5.

HPLC analysis:

Primarily in HPLC, column conditioning was done by an isocratic method with 1 ml/min flow rate for 20 min before injecting of sample. 20 µl of sample solution was injected using hamilton microliter syringe and the outcome was analysed by the Empower 2 software. The selected wavelength from UV scan was used for the HPLC experiment. The solvent system was-Methanol (solvent system A), 1 % acetic acid (solvent system B).The gradient method was followed by 60 % of solvent A (10 min), 35 % of A (5 min) then 90 % of A (5 min) then last 5 min was run at initial condition. The standard curve of guggul was also prepared by HPLC to estimate the guggul from polyherbal guggul formulation. The concentration range of calibration curve was remained same as RDS method for HPLC[4].

Method validation:

The method was validated by the ICH Q2 (R1) guideline. According to the guideline, specificity, linearity, range, precision, accuracy were evaluated for validation. These parameters are most important for the validation of analytical methods[4,18,19].

Specificity: UV-spectrometric scanning of each standard solution was performed to check specificity in the range of 200 nm to 800 nm against water as blank.

Linearity: The absorbance of standard guggul concentration (1-5 mg/ml) at determined wavelength (λ_{max}) was plotted against water as a blank to determine the linearity. The calibration curve was generated by plotting the absorbance *vs*. concentration and regression equation help to signify the linearity of standard curve of guggul.

Range: The range of this method was determined by the data which was generated from the linearity and accuracy study.

Precision: Repeatability and intermediate precision were followed to evaluate the precision of this method. Repeatability was performed by recording the absorbance of one concentration of guggul at three time in a same day which implies the intra-day precision. The intermediate precision was determined by recording the absorbance of three guggul concentration (1, 3, 5 mg/ml) for the three times execution on three consecutive days, i.e. inter-day precision.

Accuracy: The percentage recovery signifies the accuracy of the method by adding the known different concentration of guggul standard to one fixed pre-analysed particular concentration of guggul. Expected concentration and observed concentration both were equally important for the calculation of percentage recovery and this protocol was repeated for the three times. The calculation was followed by the equation: % of recovery=Ct/Ci×100

Where, Ct=Total guggul concentration after addition of standard known concentration. Ci=the initial concentration of guggul.

Limit of Quantification (LOQ) and Limit of Detection (LOD) determination: The LOQ and LOD were estimated by following the equations: LOD=3.3×σ/S LOQ=10×σ/S

Where S indicates the slope of linearity curve and σ implies the standard deviation of Y-intercepts.

RESULT AND DISCUSSION

The absorption (zero order) spectra of guggul and triphala over the range 220-310 nm is shown in fig. 1. Simultaneous determination of guggul and triphala from the polyherbal formulation exhibited overlapping region with low sensitivity and presence of interference of components of the mixture (fig. 1 and fig. 2). To address the issue of spectral overlapping, the utilization of ratio and ratio derivative spectrophotometry is imperative. This technique is intricately connected to the zero-crossing point.

RDS method is more suitable for detection of single compound from polyherbal formulation from

interference of excipient. So, this study was focused on detection of guggul concentration using RDS method.

Divisor concentration (Δλ) and smoothing function are needed to optimize for simultaneous detection of both by using RDS method. For Guggul, standard spectrum of triphala at a concentration of 0.04 mg/ml was used as divisor concentration with R2 0.9978 (fig. 3). Further the ratio spectra were derivative (1st order) to get the more accurate concentration of Guggul in presence of Triphala. It was observed that when the divisor concentration was raised or lowered, the resulting derivative values were correspondingly diminished or augmented respectively. However the maxima and minima still occur at the same wavelength. Zero-crossing points were identified in fig. 4 for triphala to estimate guggul. At the zero crossing point of one analyte, other analyte exhibited either maxima or minima. Additionally these maxima or minima helped to determine the concentration of the later analyte in presence of the former. In our study among the various maxima and minima, a wavelength of 371 nm with a correlation coefficient of 0.879 was specifically chosen for the maxima, while a wavelength of 330 nm with an R2 value of 0.895 was selected for the minima intended for Guggul. It was revealed that both of these peaks were appropriate for determining the presence of guggul in a mixture. However, considering its higher mean recovery, we opted to utilize the 371 nm wavelength for the determination of guggul from the polyherbal formulation.

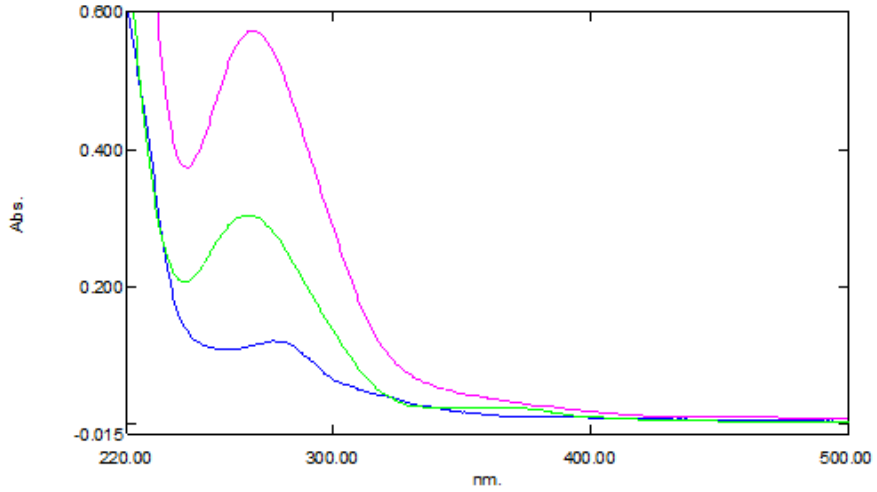

Fig. 1: Zero-order spectra of overlay picture of guggul (blue), Triphala (green) and mixture formulation (Pink)

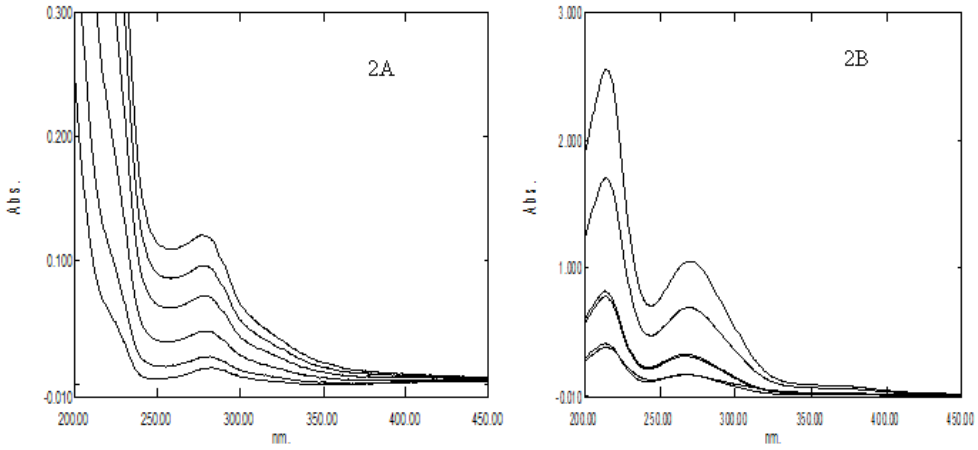

Fig. 2: Zero order spectra of different concentration of (2A): Guggul and (2B): Triphala

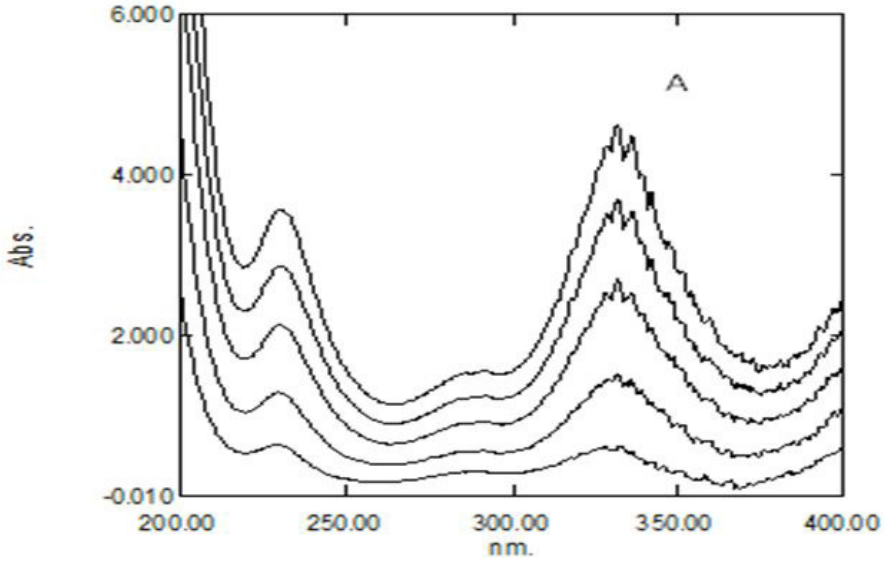

Fig. 3: Ratio spectra of guggul by divisor concentration of triphala

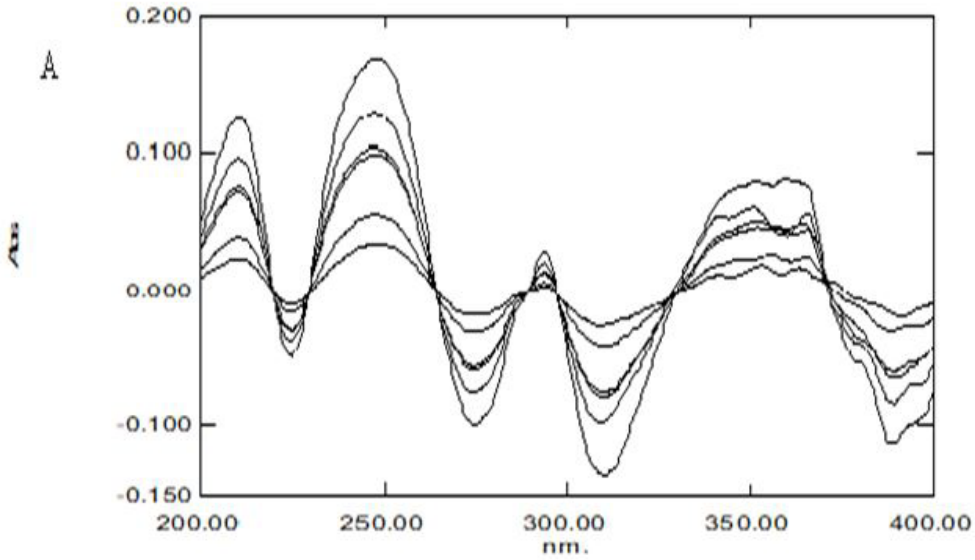

Fig. 4: Zero crossing point of Triphala by divisor concentration of guggul (A, 1st order derivative)

The wavelength that demonstrated the optimal linear response of the analyte concentration is obtained from calibration curve. Guggul exhibited band with a maximum amplitude at 280 nm where is regression equation. In RDS analytical method, the linearity range of the guggul was 1-5 mg/ml where the value of R2 was 0.998.

Validation parameters were tabulated in Table 1. Based on these findings, there were no significant difference in the assays conducted on intra-day and inter-day. This study demonstrated accuracy, precision and applicability of the proposed RDS method. The statistical evaluation was conducted to verify the effectiveness of the RDS method through replicate estimation of polyherbal formulation. Recovery studies were also conducted to determine the accuracy and precision. Due to high percentage of recovery data and low RSD value, the excipients used in the formulations showed no interference in the proposed method.

The focus of our study was to determine guggul as the specific analyte in polyherbal mixture. Therefore, we exclusively employed ratio derivative analysis to determine the presence of guggul in the

polyherbal mixture. This approach has the potential in the simultaneous detection of the components in binary mixtures without the need for separating the components beforehand.

To evaluate the performance of ratio derivative spectrophotometric method for estimation of guggul in polyherbal formulation, HPLC method was performed.

Guggul, a substance used in traditional medicine can be determined using reverse phase HPLC from binary mixtures containing guggul and triphala. To determine the most suitable HPLC conditions for separating guggul, various mobile phase systems were tested. The chosen mobile phase consisted of methanol and 1 % acetic acid. The retention time for guggul in combined pharmaceutical dosage was found to be 3.08±0.025 min, with a flow rate of 1.0 ml per min (fig. 5). The detection of guggul was optimized at a wavelength of 280 nm, which yielded best results. For quantitative analysis, a calibration graph was constructed with a correlation coefficient of 0.9773 for guggul in the binary mixture. This indicated a good level of precision in the HPLC procedure. To further assess the accuracy and precision of the method, a recovery study was conducted, which yielded satisfactory results. The LOD and LOQ for guggul were estimated to be 1.05 mg/ml and 3.18 mg/ml, respectively. These values provided important information about the sensitivity and reliability of the HPLC method for detecting and quantifying guggul in binary mixtures.

The comparative data of the two methods are summarized in Table 1-Table-3. At a 95 % confidence level, no significant disparities were observed between the results obtained using the HPLC method and ratio derivative spectroscopy for the same batch.

TABLE 1: VALIDATED PARAMETERS TO ESTIMATE GUGGUL FROM POLYHERBAL FORMULATION USING BY RATIO DERIVATIVE SPECTROSCOPY AND HPLC

Parameters	HPLC	Ratio derivative spectroscopy
System Suitability		
Retention Time	3.08±0.025	-
Sensitivity		
LOD (mg/ml)	1.05	0.016
LOQ (mg/ml)	3.18	0.048
Precision		
Intra-day		
Repeatability (mg/g of formulation) (n=6)	550.49	549.66
% RSD	0.095	0.181
Inter-day		
Mean drug content (mg/g of formulation) (d 1/d 2/d 3) (n=3)	550.86/550.45/549.95	550.95/549.45/549.78
Intermediate Precision	550.75	550.25
% RSD (d 1/d 2/d 3) (n=6)	0.065/0.09/0.086	0.085/0.170/0.162

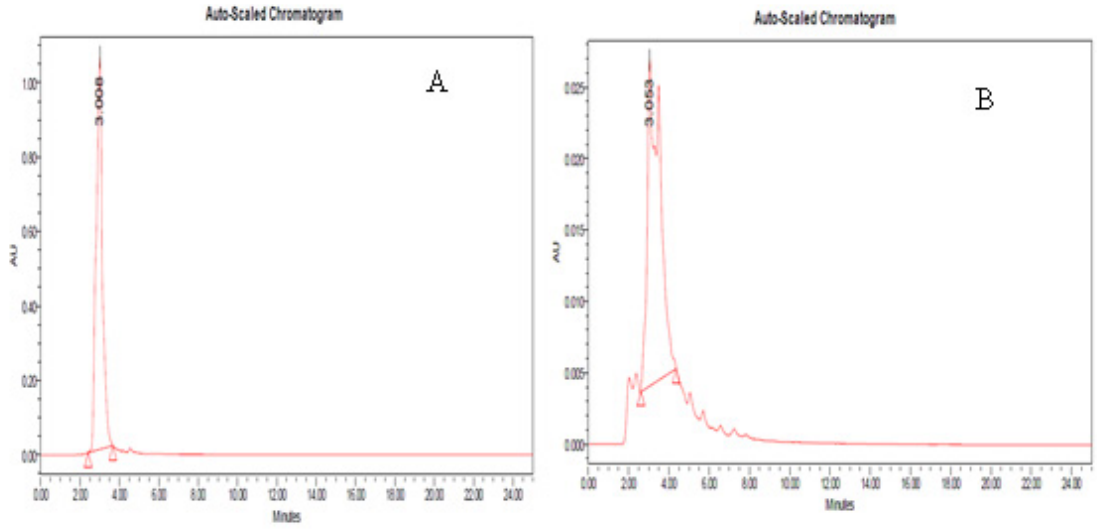

Fig. 5: (A): Chromatogram of Raw guggul and (B): Chromatogram of Raw guggul in polyherbal formulation

TABLE 2: ACCURACY STUDY OF GUGGUL ESTIMATION WAS USED BY RATIO DERIVATIVE SPECTROSCOPY

Sample	Average theoretical concentration of sample solution (mg/ml)	Solution (%)	Theoretical concentration from spiked solution (mg/ml)	Theoretical excess amount added (mg/ml)	Average actual assay from sample solution (mg/ml)	Assay from spiked solution (mg/ml)	Observed excess amount (mg/ml)	Recovery difference (mg/ml)	Accuracy (%)	% RSD
		80	0.44	0.11		0.4	0.13	0.02	90.9	
Guggul	0.55	100	0.55	0	0.53	0.51	0.02	0.02	92.72	1.65
		120	0.66	0.11		0.62	0.09	0.02	93.93	

TABLE 3: ACCURACY STUDY OF GUGGUL ESTIMATION WAS USED BY HPLC

Sample	Average theoretical concentration of sample solution (mg/ml)	Solution (%)	Theoretical concentration from spiked solution (mg/ml)	Theoretical excess amount added (mg/ml)	Average actual assay from sample solution (mg/ml)	Assay from spiked solution (mg/ml)	Observed excess amount (mg/ml)	Recovery difference (mg/ml)	Accuracy (%)	% RSD
		80	0.44	0.11		0.41	0.13	0.02	93.18	
Guggul	0.55	100	0.55	0.00	0.54	0.52	0.02	0.02	94.54	1.06
		120	0.66	0.11		0.63	0.09	0.02	95.15	

As per the guideline provided by the ICH, LOD was determined as 3.3 divided by the standard deviation of the intercept of the regression line multiplied by the sensitivity (S), which represented the slope of the calibration curve. However, the limit of quantification, also known as LOQ, was set at 10×σ/s. The determination and measurement thresholds for the crude drug; guggul were calculated and are displayed in Table 1. The value of LOD was authenticated and experimentally verified[5].

The effectiveness of the implemented techniques was assessed by determining guggul from the mixture. The obtained results indicated a commendable level of accuracy, as reflected by the low percentage relative error (Er %) values, and a remarkable level of precision, as indicated by the low percentage relative standard deviation (RSD %) values. The findings, which are presented in Table 1-Table 3, demonstrated that both the Er % and RSD % values remain below 2 %, thus affirming the reliability and consistency of the implemented techniques[5].

The study established that the proposed ratio derivative method for Guggul quantification in polyherbal formulation was simple, economic and time saving and comparable to HPLC method. Moreover the results of the propositioned method were in good agreement with the HPLC outcome.

In conclusion, the derivative and ratio-derivative spectrophotometry was simple, sensitive, accurate, precise, reproducible, time saving method. This technique was independent of purification and interference of excipients. HPLC is considered as a better and more precise method but it is expensive and time consuming. Statistical data conveyed no significant difference found between the results of RDS and HPLC. However, RDS can be a good alternative to HPLC for regular quality control assessment of the investigated drug from polyherbal formulations where HPLC is not affordable. Hence this method can be used for the simultaneous quantification of guggul from a mixture with triphala in a polyherbal formulation.

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Author contribution:

This research idea was formulated by Anumita Dey, Helen Chattapadhyay and Sriparna Datta. Anumita Dey carried out the execution of the work and took responsibility for writing the manuscript. Anumita Dey and Helen Chattapadhyay were responsible for data analysing and formatting the manuscript, while Sriparna Datta and Mradu Gupta undertook the editing of the manuscript.

Conflicts of interests:

The authors affirm that they do not have any known conflicting financial interests or personal relationships that could have impacted the findings reported in this manuscript.

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