EXTRACTION, ISOLATION AND METHOD DEVELOPMENT BY UV AND RP-HPLC OF BOSWELLIC ACID FROM BOSWELLIA SERRATA

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ABSTRACT
Boswellia serrata *(*Salai/Salai guggul*)* the oleo gum resin belongs to a large tree of Burseraceae family from Genus Boswellia. The Boswellia species has been used in Ayurvedic system of medicine as anti Arthritic, anti-inflammatory agent, it inhibits the activity of the enzyme 5-lipoxygenase through a non-redox reaction. The Boswellia serrata is extracted in ethanol from raw oleo gum resin of Boswellia serrata and the reddish-brown syrupy mass is processed to get approximately 90% of the pure compound. A UV Spectrophotometric method is developed for the estimation of Boswellia serrata. The solutions are analysed at 254 nm to get Linearity of 0.9988, LOD at 0.88 and LOQ 2.67 µg/ml, Accuracy of 96.30 %, Precision of 0.26 and 0.47 % RSD and Robustness value of 0.58 % RSD. A RP-HPLC method is also developed for the estimation of Boswellia serrata. The HPLC system was equipped with a Luna C18 column and the chromatographic conditions include mobile phase consisting a mixture of methanol and water in 70:30 ratio. The retention time for BA was found to be 2.43 minutes. The Linearity of 0.9989 was obtained along with LOD at 0.041 and LOQ 0.125 µg/ml, Accuracy of 91.49 %, Precision of 0.318 and 0.319 % RSD and Robustness value of 0.166 % RSD.

KEYWORDS: Boswellic acid, Boswellia serrata, Lobaan, UV, RP-HPLC, Indian Frankincense, anti-inflammatory.

INTRODUCTION:

Boswellia serrata *(*Salai/Salai guggul*)* the oleo gum resin belongs to a large tree of Burseraceae family from Genus Boswellia. The pentacyclic triterpenic acids obtained from the oleo gum resin of different Boswellia species are together known as Boswellic acids (BA) (Farah *et al*., 2017). Since ancient times, resins have been important in the preparation of incense, medicines, cosmetics and perfumes. Boswellic acid (BA) have been indicated in apoptosis of cancer cells, in particular brain tumors and cells affected by leukemia or colon cancer. BA also exhibits anti-inflammatory activity by inhibiting leukotriene synthesis (Safayhi *et al*., 1997). It inhibits the activity of the enzyme 5-lipoxygenase through a non-redox reaction. Clinical trials have investigated the effectiveness of BA in treatment of ulcerative colitis, but a study on chemically induced colitis in mouse models showed little effectiveness. A latter study showed that low doses of Boswelliaserrata extract may have hepatoprotective effects. The higher dose was found to have a milder hepatoprotective effect than the lower dose. BAs are also thought to decrease the symptoms of asthma (Siddiqui *et al*., 2011). Boswellia extracts are marketed in tablet, capsule and tincture form. The active Boswellic acid can be extracted from crude oleo gum resin of Boswelliaserrata by the process of any one of the extraction methods such as maceration, infusion, digestion, decoction, percolation, Soxhlet extraction method etc (Lopez-Bascon *et al*., 2020). The analysis of the obtained Boswellic acid can be done by UV-spectroscopy and RP-HPLC methods (Willard *et al*., 1986). Chemical structure of BA is shown in figure 1.

MATERIAL

Boswelliaserrata oleo gum resin was purchased from local market in Aurangabad, Ethanol and methanol LR was purchased from Fisher scientific Ltd Mumbai.

METHODS

Extraction

The raw oleo gum resin of Boswellia serrata about 250 grams was subjected for maceration for 20 hrs in ethanol (500ml) with continuous shaking at 100 RPM on mechanical shaker. This process is repeated thrice to get Boswellia serrata extract which is further processes for isolation of BA (Sharad *et al*., 2012; Zhang *et al*., 2018)

Isolation

The above methanolic extract was concentrated till a reddish brown syrupy mass is obtained and then basifying the syrupy mass with an aqueous solution of an alkali to get a pH of the solution in the range of 9 to 10. The solution is then acidified with mineral acid to get a pH in the range of 3-5. The obtained precipitate is BA, this precipitate is washed with water several times and pH of the water washing is checked to neutral and lastly filtering the precipitate to get BA (Bohlin 1998; Agrawal and Paridhavi, 2012).

Procedure for Analysis of Extract

For the estimation of BA present in the extract 10 mg of Isolated BA weighed and transferred into 10 ml volumetric flask then dissolved with methanol and Water mixture (70:30). It was kept for ultra-sonication for 30 minutes and filtered through Whatmann filter paper No 41 to get the solution of 1000µg / ml. From this solution 1 ml was taken and diluted to 10ml with selected solvent to give a solution of 100 µg / ml and used for the estimation of BA.

Development and validation of UV-Spectrophotometric method

Linearity

For quantitative analysis of BA, the calibration curves were plotted for each concentration range. The linearity range was estimated between 2-10 µg/ml respectively (Skoog *et al.,* 1993). The data obtained was utilized for the linearity calibration plot.

Limit of detection and Limit of Quantitation

The Limit of detection (LOD) and Limit of Quantitation (LOQ) of BA by the proposed methods were determined using calibration standards. LOD and LOQ values were calculated as 3.3 SD/D and 10 SD/D respectively, where D is the slope of the calibration curve and SD is the standard deviation (Chatwal and Anand, 2007).

Accuracy

The accuracy was determined by standard addition method. Three different levels (80%, 100% and 120%) of standards were spiked to extracted BA in triplicate. After mixing the content was transferred to 100ml volumetric flasks and dissolved in 50ml of methanol: Water mixture (70:30) and volume made up to the mark. The content was kept in Ultrasonicator for 30minutes and filtered through whatmann filter paper No. 41. The solution was analysed at 254 nm and the concentration of BA was calculated.

Precision

The reproducibility of proposed method was determined by performing assay at different time intervals (3 hour interval) on same day (Intra-day precision) and on three different days (Inter-day precision) of BA.

Robustness and Ruggedness

Robustness was studied by determining the effects of small variation in Wavelength (±2) i.e. 252 and 256 nm on absorbance values; The Ruggedness study was done by analyzing the sample by two different analysts (ICH Guidelines Q2 R1, 2005).[12]

Development and validation of RP- HPLC method

Selection of Mobile Phase and column

A different combination of Methanol and Water was on the basis of its polarity. The solvent system was optimized in order to provide a good performance of the assay. Finally, Mobile Phase which containing Methanol and Water (70:30) was selected for further analysis. Luna C18 column was used.

Detection of wavelength

The detection wavelength was measured by running the 20 µg/ml solution of BA in Mobile Phase, and the wavelength of maximum absorption was selected as 254 nm (Shailesh *et al*., 2008).

Preparation of standard solution

10 mg of BA was weighed and transfer into 10 ml volumetric flask. Then drug is dissolved 5ml of methanol by vigorous shaking and then volume was made up to mark with Methanol to obtain final concentration of 1000 mcg/ml named stock solution. Then prepared different concentration of BA by taking the stock solution in respective manner as 0.2, 0.4, 0.6, 0.8, 1.0 ml &dilute further up to 10 ml with Methanol, It gives the concentration of 20, 40, 60, 80 & 100 µg/ml respectively.

Assay of Extracted BA

100 mg of the extract was transferred to a 100 mL volumetric flask and dissolved first in about Methanol and volume is making up to the mark. The solution is ultrasonicated for 30 minutes and then filtered through Whatmann filter paper (No. 41).After suitable dilution, the sample was injected corresponding to 60 µg /ml of BA and peak area was recorded.

Linearity

For quantitative analysis of BA, the calibration curves were plotted for each concentration ranges. The linearity ranges for BA found to be 20-100 µg/ml respectively.

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

The LOD and LOQ of BA by the proposed methods were determined using calibration standards. LOD and LOQ values were calculated as 3.3 SD/D and 10 SD/D respectively, where D is the slope of the calibration curve and SD is the standard deviation.

Accuracy

The accuracy was determined by standard addition method. Three different levels (80%, 100% and 120%) of standards were spiked to commercial tablets in triplicate. After mixing the content was transferred to 100ml volumetric flasks and dissolved in 50ml of methanol: Water mixture (70:30) and volume made up to the mark. The content was kept in Ultrasonicator for 30minutes and filtered through whatmann filter paper No. 41. The solution was analysed at 254 nm and the concentration of BA was calculated.

Precision

The reproducibility of proposed method was determined by performing tablet assay at different time intervals (3-hour interval) on same day (Intra-day precision) and on three different days (Inter-day precision) BA.

Robustness and Ruggedness

The Robustness study was carried out by determining effect of small variation in wavelength and in Ruggedness sample was analyzed by two different analysts (Markus and Ikhlas, 2001).

RESULTS AND DISCUSSIONS:

Analysis of Extract

Various dilutions were prepared and Absorbance was recorded at 254nm. The amount of extracted BA from crude oleo gum resin was found to be 90 %.

UV- Spectrophotometric Method Development and Validation.

In the present study, we have described a method that was developed to profile methanol soluble Boswellic acid, which are mainly pentacyclic triterpenic acids of oleo gum resin which show the intense absorption in UV region.

Linearity:

The absorbance is plotted against the corresponding concentrations to obtain the calibration graphs. The regression equations for the calibration curve of BA was obtained as $y = 0.1481x + 0.038$. The absorbance of the samples in the range of 2-10 µg/ml was found to be linear with a correlation coefficient $R^2 = 0.9988$. The UV-spectra of BA is shown in Figure 2.

LOD and LOQ

The LOD and LOQ parameter was evaluated by using the slope of line and standard deviation obtained from accuracy studies. LOD and LOQ were found to be 0.88 and 2.7 respectively.

Accuracy:

Accuracy (recovery) studies were carried out on extracted BA by adding known amount of standard BA i.e. 1.6 mg (80%), 2 mg (100%) and 2.4 mg (120%). The mean of percentage recoveries and the % RSD was calculated. The results are given in Table 1. The Results of the study were found to be between the ranges of 94.44-97.95 % exhibiting that the developed method is an accurate method for BA determination.

Precision:

The Inter-day and Intra-day precision of the developed UV-spectroscopic method was obtained as Relative Standard Deviation (RSD). The study was done by assaying the drug in triplicate per day for 3 consecutive days, and the results obtained are 0.265 and 0.475 RSD for Inter-day and Intra-day precision respectively. The results of the study as shown in Table 2 of the developed method, validated sufficient sample stability and method reliability where all the RSDs were <2%.

Robustness and ruggedness:
The results of the Ruggedness study indicate that there is not significant change in the absorbance values at the same concentration by two different analyst and the RSD was found to be less than 1% demonstrating the method is rugged. It was also found that there was no significant change in SD and RSD value of absorbance obtained at minor wavelength changes indicating the robustness of method. The results are shown in Table 3.

RP-HPLC METHOD DEVELOPMENT AND VALIDATION
The Primary aspect of this study was to develop a rapid and specific isocratic HPLC method for the estimation of Boswellic acid. The proposed method utilizes an isocratic technique at room temperature without tedious sample preparation procedure and PDA detection.

Chromatographic Condition:
RP-HPLC method was developed for BA, which can be conveniently employed for routine quality control in pharmaceutical dosage forms. The chromatographic conditions were optimized in order to provide a good performance of the assay. Different combinations of solvents such as Methanol and water were tried for mobile phase based on their polarity. Finally Mobile phase of Methanol: Water was selected in the ratio of 70:30 for further analysis and the flow rate was set at 0.9 ml/min. These chromatographic conditions gave a retention time of 2.45 minutes for BA. The real advantage of the method is its low retention time: 2.45 and use of cost effective solvent like water and methanol. It reduces total run time for HPLC, leads to low solvent consumption, and makes the method more economical. Herbal extract for this method has good solubility in methanol; therefore, methanol was selected for extraction. The Chromatogram of standard and extracted BA is shown in Figure 3&4.

RP-HPLC Method Validation.
Linearity:
Linearity was determined for BA in the range 10-50 µg/ml. The absorbance is plotted against the corresponding concentrations to obtain the calibration graphs with R^2= 0.99889.

LOD and LOQ:
LOD and LOQ were found to be 0.041 and 0.125 respectively, which specifies that it is acceptable and governs according to ICH guidelines.

Accuracy:
Accuracy (recovery) studies were carried out on extracted BA and the prepared samples was analysed at 254 nm and the concentration of BA was calculated. The accuracy studies performed in this method development leads to positive effect with reference to percentage recovery rate and it shows gradual increase in recovery rate. The results are given in Table 4.

Precision:
The Intra-day precision of the developed RP-HPLC method was obtained as RSD of 0.31. The Inter-day precision was also determined by assaying the drug in triplicate per day for 3 consecutive days, and the values for which is 0.32. The results of the Intra-day and Inter-day are shown in Table 5.

Robustness and ruggedness of the method:
The results of the Robustness study indicates that there is not significant change in the absorbance values at the same concentration by two different analyst and the RSD was found to be less than 1% demonstrating the method is rugged. Robustness was studied by determining the effects of small variation in Wavelength (±2) i.e. 252 and 256 nm on R_f values; it was found that there was no significant change in Standard Deviation (SD) and RSD value of peak area indicating the robustness of method. The result of the study is shown in Table 6.

Conclusion:

The extraction and isolation of Boswellic acid from raw oleo gum resin of Boswellia serrata was performed and the analytical method were developed and validated for Boswellic acid in bulk and isolated Boswellic acid. The analytical method development and validation includes UV-Spectrophotometric method and RP-HPLC method. The developed method is cost effective and can be incorporated for routine drug analysis of Boswellic Acid. The method is validated by performing accuracy, precision, robustness and ruggedness studies. This technique provides us chromatogram of herbal compounds present in methanolic extract of plants Therefore, this method in future studies must be coupled to HPLC-MS and/or NMR analyses for complete identification of separated compounds.

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Tables:

S.No.		Level of Recovery		
		80%	100%	120%
1.	Extracted BA present (mg)	2.0	2.0	2.0
2.	Standard BA (mg)	1.6	2.0	2.4
3.	Recovery (mg)	3.4	3.86	4.31
4.	Recovery (%)	94.44	96.5	97.95
5.	Mean	96.296		
6.	SD	1.763		
7.	RSD (%)	1.831		

Table 1: UV-Spectrophotometric method Accuracy study

S. No.	Intra-day Precision			Inter-day Precision		
	Absorbance measured					
	0 Hr.	3 Hr.	6 Hr.	Day 1	Day 2	Day 3
1.	0.956	0.939	0.946	0.956	0.959	0.962
2.	0.951	0.941	0.951	0.951	0.96	0.959
3.	0.951	0.943	0.948	0.951	0.958	0.968
Mean	0.953	0.941	0.948	0.953	0.959	0.963
SD	0.003	0.002	0.003	0.003	0.001	0.005
RSD (%)	0.303	0.212	0.265	0.303	0.104	0.475

Table 2: Intra-day and inter-day Precision of UV-spectroscopic method.

S. No.	Absorbance		
	252 nm	256 nm	257 nm
1.	0.941	0.949	0.945
2.	0.946	0.942	0.948
3.	0.955	0.953	0.957
Mean	0.947	0.948	0.950
SD	0.005	0.045	0.005
%RSD	0.543	0.587	0.575

Table 3: UV-Spectrophotometric method Robustness Study

S.No.		Levels of Recovery		
		80%	100%	120%
1.	Extracted BA present (mg)	10	10	10
2.	Standard BA (mg)	8	10	12
3.	Recovery (mg)	16.52	18.13	20.25
4.	Recovery (%)	91.77	90.65	92.04
5.	Mean	91.49		
6.	SD	0.74		
7.	RSD (%)	0.80		

Table 4: Recovery study of RP-HPLC method

S. No.	Intra-day Precision			Inter-day Precision		
	Mean Peak Area					
	0 Hr.	3 Hr.	6 Hr.	Day 1	Day 2	Day 3
1.	534.12	559.45	553.65	534.12	542.45	552.65
2.	532.33	560.85	550.32	532.33	540.85	549.32
3.	531.02	562.17	551.01	531.02	542.17	550.01
Mean	532.49	560.82	551.66	532.49	541.82	550.66
SD	1.57	1.36	1.76	1.56	0.85	1.76
RSD (%)	0.3	0.24	0.31	0.29	0.15	0.32

Table 5: Intra-day and inter-day Precision of RP-HPLC method.

	Peak Area		
S.No.	252 nm	256 nm	257 nm
1.	564.35	569.12	571.32
2.	563.14	569.57	565.61
3.	562.96	567.75	569.11
Mean	563.48	568.81	569.46
SD	0.755	0.947	0.954
%RSD	0.134	0.167	0.161

Table 6: Robustness Studies RP-HPLC method

Figures

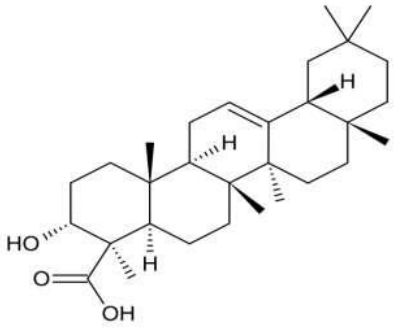

Figure 1: Chemical Structure of Boswellic acid

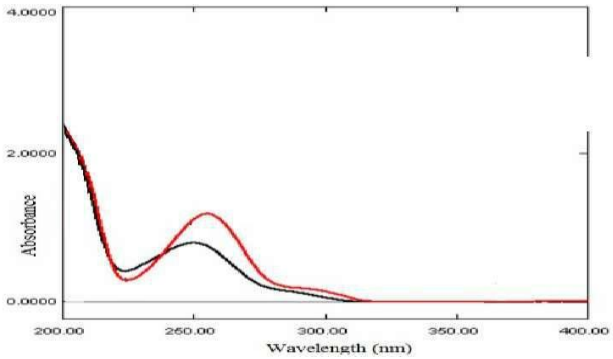

Figure 2: UV-spectra of BA

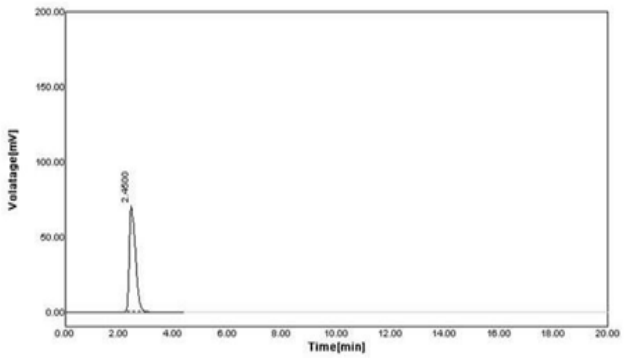

Figure 3: HPLC Chromatogram of Standard BA

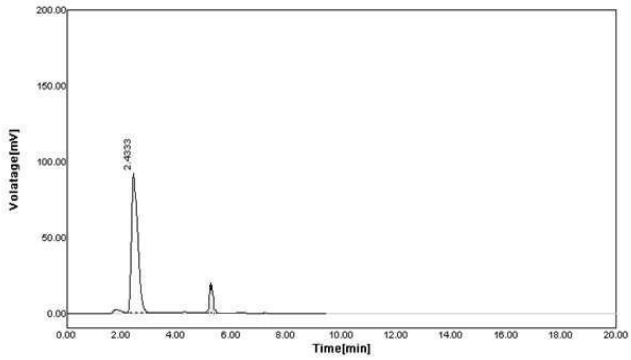

Figure 4: HPLC Chromatogram of Sample