

Comparison Between HPLC and HPTLC Densitometry for the Determination of 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid from *Boswellia serrata* Extract

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ABSTRACT

Objective: Boswellic acids (BAs) are active constituents of plant *Boswellia serrata*. In the present study boswellic acids were extracted from *Boswellia serrata* and dry methanolic extracts were quantified by HPLC and HPTLC. **Method:** A gradient HPLC method was used for the quantification of the boswellic acids; 11-keto- β -boswellic acid (KBA) and 3-acetyl-11-keto- β -boswellic acid (AKBA). A direct HPTLC assay was developed for the determination of KBA and AKBA at 254 nm. The UV detection of analytical assays were used to examine the purity of KBA and AKBA peaks and compared with the standards. **Results:** The quantitative results of both analytical methods did not show any major differences between content of KBA and AKBA, although a trend to slightly lower values were found for content of KBA and AKBA in HPLC. The amount of KBA and AKBA in the *Boswellia serrata* extract was found to be about 3.3 % and 4.6 % by HPTLC and 3.03% and 3.87% by HPLC respectively. **Conclusion:** Both methods are simple and effective for quantification of KBA and AKBA in *Boswellia serrata* extracts and their pharmaceutical formulations.

Key words: Boswellic acids, Quantification, Extraction, *Boswellia serrata* extract.

INTRODUCTION

Boswellia serrata is commonly known for its medicinal values in the Indian traditional systems of medicine. It has been used traditionally in “ayurvedic medicine” as an anti-inflammatory agent. The anti-inflammatory activity is attributed to the presence of four pentacyclic triterpene acids i.e. β -boswellic acid, α -boswellic acid, 11-keto- β -boswellic acid (KBA) and 3-acetyl-11-keto- β -boswellic acid (AKBA)¹ (Figure 1). Out of these KBA and AKBA have more pronounced anti-inflammatory activity. Boswellic acids are selective, non-competitive, non-redox and potent inhibitors of 5-lipoxygenase, which is the key enzyme of leukotriene biosynthesis from arachidonic acid.²⁻⁴ In addition to this,

they also reported to possess diverse biological activities that include immunomodulation⁵, anti-arthritic⁶⁻⁸, anti cancer⁹⁻¹¹ and antiviral¹² properties.

Boswellic acids from *Boswellia serrata* extract can be detected by various analytical methods such as nonaqueous titration method¹³, RP-HPLC method¹⁴, HPTLC method^{15,16} whereas HPTLC¹⁷, HPLC¹⁸⁻²⁰, gas chromatographic–mass spectrometric²¹ and liquid chromatographic–mass spectrometric²² methods are reported for their estimation in human plasma. An HPTLC system has been produced to quantitatively gauge the boswellic acids content in the gum resins of *Boswellia serrata*. HPTLC has turned into a successful

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and intense apparatus for the estimation of synthetic and biochemical markers.²³⁻²⁵ It also offers a better resolution of active constituents with reasonable accuracy in a short time.²⁶ A limited number of HPLC methods are available for the estimation of boswellic acids. The gradient HPLC method have so far been published for estimation of KBA and AKBA in marketed formulation and in crude extract.²⁷ An RP-HPLC method has been reported for the estimation of KBA in plasma. The researchers reported this method which included solid phase extraction and other techniques for the isolation of KBA from plasma followed by gradient elution on a PDA detector with a higher retention time^{28,19-22}.

The purpose of the present work was to provide a simple method to extract the boswellic acids from gum resins of *Boswellia serrata* and development of conditions for separation and quantitative determination of major boswellic acids; KBA and AKBA using the simple, rapid and economical HPTLC method and to make a comparison of results obtained by HPTLC and HPLC.

MATERIALS AND METHODS

Collection of plant material

Gum resins of *Boswellia serrata* for isolation of boswellic acids was procured from local market of Rohtak, Haryana (India) and was authenticated from National institute of science communication and information resources (NISCAIR) by Dr. H.B. Singh, Chief Scientist & Head, Raw Materials Herbarium & Museum (RHMD), NISCAIR vide ref. letter no. NISCAIR/RHMD/Consult/2012-13/2054/62. Standard Markers were procured from Sigma aldirich; Germany via National Facility for Biochemical and Genomic Resources (NFBGR), Institute of Genomics & Integrative Biology, CSIR, New Delhi. All other solvents and reagents were procured from E. Merck Ltd. (Mumbai, India).

Extraction and isolation of boswellic acids

Crushed lumps of gum exudates of *Boswellia serrata* were soaked in methanol for twelve hours. This process was repeated twice followed by filtration. Residue was discarded and filtrate was concentrated till reddish brown syrupy mass was obtained. This syrupy mass was basified with dropwise addition of aqueous solution of potassium hydroxide (3%) until a pH of ^{9,10} was obtained. The solution was stirred continuously until a uniform emulsion was formed. This emulsion was further extracted with three portions of dichloromethane in a separator. Upper aqueous fraction was separated and acidified with addition of dilute hydrochloric acid until a pH of ^{3,4} was achieved. The precipitated acids

were centrifuged and washed with water until neutral to litmus. The product was then dried in oven at 40-45°C to obtain yellow powder.²⁹

HPLC analysis

Standard and sample solution preparations

The stock solution of both boswellic acids i.e. 11-KBA & 3-AKBA were prepared by accurately weighing 1mg of standards quantitatively transferred into a volumetric flask and made up to volume with methanol to achieve conc. of 1 mg/ml. *Boswellia serrata* extract sample solution was prepared by weighing 5 mg of extract, dissolved in 5 ml methanol to achieve conc. of 1 mg/ml. Further final conc. was made up to 50 ppm by dissolving 1 ml of stock solution in to 20 ml of mobile phase A before inject.

Instrumentation

The HPLC system was Thermo Scientific, Ultimate 3000 series consisting of Dionex ultimate 3000 pump with an auto-sampler and diode array detector. Data acquisition and integration was controlled by chromeleon software; version 6.80 SR. An eclipse C₁₈ column (250×4.6 mm; 5µm) was used.

Chromatographic conditions

The mobile phase was a binary gradient system prepared from 0.1% phosphoric acid in water (eluent A) and 0.1% phosphoric acid in acetonitrile (eluent B), properly filtered and degassed for 15 minutes in ultra sonic bath before to use. The gradient program was: 84-94% B from 0–12.5 minutes, 94-100% B from 12.5-13.5 minutes, 100% B from 13.5-28 minutes. The flow rate was 1ml/min throughout the analysis. Column temperature was maintained at 27±20C. The detection wavelength of DAD was 250 nm. Injection volume was kept at 20 µl with a run time of 28 minutes.

Percentage content of KBA and AKBA in boswellic acids was calculated using the following expression:

$$\frac{A1 \times m1 \times 5 \times p1}{A2 \times m}$$

Where;

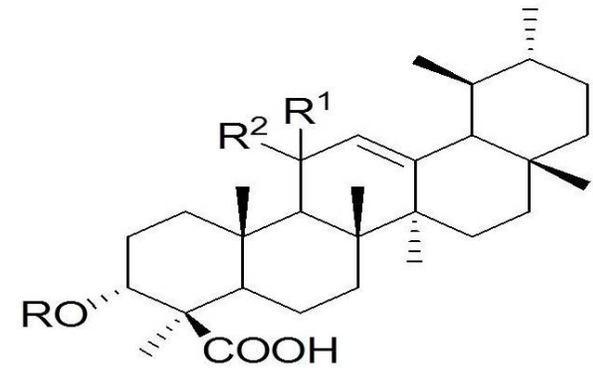
A1 = area of the peak due to KBA/AKBA in the chromatogram obtained with the test solution,

A2 = area of the peak due to KBA/AKBA in the chromatogram obtained with the reference/standard solution,

m = mass of the substance to be examined in grams,

m1 = mass of KBA/AKBA in the reference/standard solution, in grams,

p1 = percentage content of KBA/AKBA in standard.



Chemical Name

- β-boswellic acid
- 11-keto-β-boswellic acid
- 3-acetyl-β-boswellic acid
- 3-acetyl-11-keto-β-boswellic acid

Substituents

- R=R¹=R²=H
- R=H, R¹+R²=O
- R=COCH₃, R¹+R²=H
- R=COCH₃, R¹+R²=O

Figure 1: Chemical structure of boswellic acids.

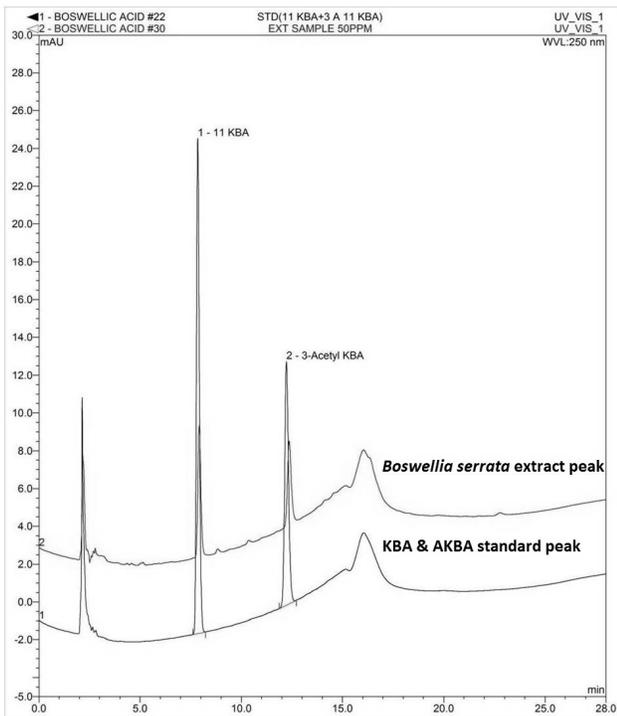


Figure 2: Overlaid HPLC chromatogram showing peaks of KBA and AKBA in extract and reference standard.

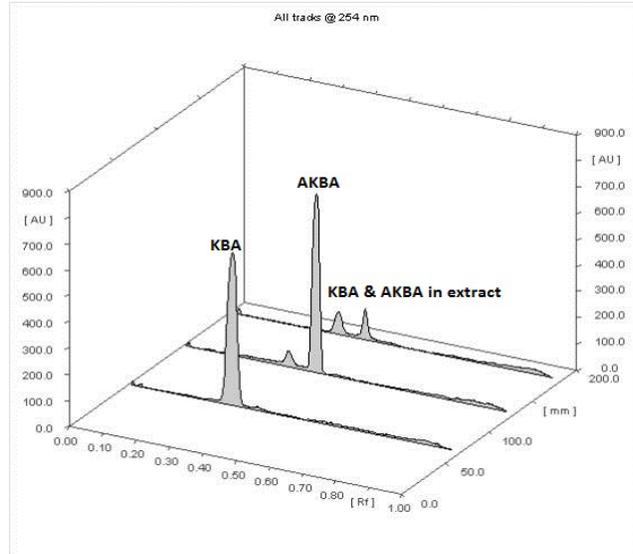


Figure 3: HPTLC chromatogram showing resolved peaks of (a) KBA standard and (b) AKBA standard.

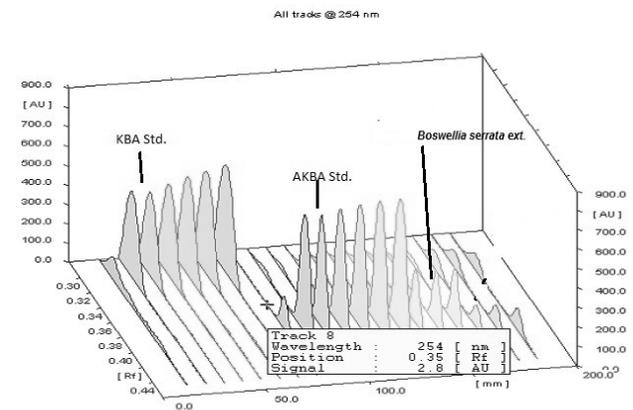


Figure 4: 3D chromatogram of KBA, AKBA standard and extract at 254 nm.

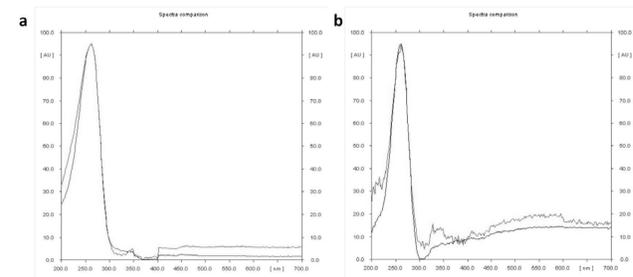
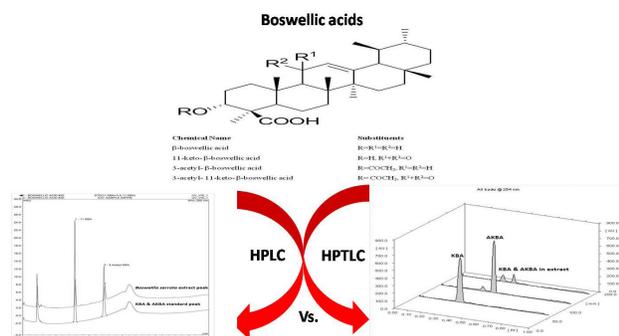


Figure 5: Overlapped peak of (a) KBA and (b) AKBA standard with KBA and AKBA in plant extract at similar Rf value.



HPTLC analysis

Standard and sample solution preparations

A 1000 µg/ml (1 mg/ml) of *Boswellia serrata* extract, KBA and AKBA reference standards solutions were prepared in methanol as sample and reference standard stock solution.

Instrumentation

A CAMAG (Muttenz, Switzerland) HPTLC framework outfitted with a specimen tool Linomat V with CAMAG test syringe, 100 µl, twin harsh plate advancement chamber (20 x 10 cm), TLC Scanner 3 and joining programming WINCATS 1.4.8 was used. An aluminium supported HPTLC plate (20 x 10 cm) pre-covered with silica gel 60F 254 (E. Merck) was used as an adsorbent. CAMAG TLC plate heater was used to pre-activation of the HPTLC plates and for drying the developed plates.

Chromatographic conditions

Mixture of glacial acetic acid, n-hexane, ethyl acetate and toluene (0.3: 1: 8: 2) (v/v/v/v) were used as a mobile phase. The experimental conditions maintained were temperature 25 ± 2°C, relative humidity 40%.

The different volume of standard stock solution 2, 4, 6, 8, 10, 12 and 14 µl were spotted on HPTLC plate (20 x 10 cm) both for KBA and AKBA in order to deliver concentrations of 2, 4, 6, 8, 10, 12, and 14 µg/spot of KBA and AKBA respectively using vial I & II followed by spotting of 4 µl of sample stock solution in triplicate. Samples were applied as bands 4 mm wide, 12 mm apart, by CAMAG Linomat V applicator using 100 µl test syringe with a steady application rate of 150 nLs⁻¹. After sample application plates were developed in a developing chamber pre-saturated with the mobile phase (20 ml) for 20 min. The plate was developed in CAMAG horizontal twin tough glass developing chamber (20 x 10 cm) at the room temperature up to 8 cm (80% of the total plate size). Ascending mode was used for the development of Thin Layer chromatography.

After development, plates were dried on CAMAG TLC plate heater at 120°C temperature for 5 minutes. The developed plate was then scanned at 254 nm using CAMAG TLC densitometric scanner 3 incorporated with WINCATS 1.4.8 programming. The calibration curve was readied using standard concentration range of 2-14 µg/spot for KBA and 4-14 µg/spot for AKBA. Each concentration peak area was plotted against the concentration of KBA and AKBA spotted in densitometric analysis with in the integrated software. Quantification of KBA and AKBA was done as the peak area of sample spots were recorded and the amount of KBA and AKBA was figured using standard curve. The superimposing so as to distinguish proof of KBA and AKBA was affirmed by the UV spectra of test and standard inside of the same R_f window.

RESULTS AND DISCUSSION

Extractive value of the boswellic acids isolated from gum exudates of *Boswellia serrata* was found to be 38.23%. The procedure for the determination of KBA and AKBA in boswellic acids using HPTLC densitometry in comparison with a HPLC method is reported. All chromatographic systems were chosen experimentally.

HPLC analysis

A HPLC method with gradient elution was developed for quantification of KBA and AKBA in boswellic acids isolated from *Boswellia serrata* extract. The sample solution was applied directly to the HPLC system without any additional step. A typical overlaid chromatogram of the boswellic acids extract and KBA and AKBA standard is shown in (Figure 2). The identity of the KBA and AKBA in extract was confirmed by comparison of retention time and overlaying peak of KBA and AKBA in extract and standard. The amount of KBA and AKBA in the *Boswellia serrata* extract was calculated to be 3.03% and 3.87%.

HPTLC analysis

The developed mobile phase consisting of glacial acetic acid, n-hexane, ethyl acetate and toluene (0.3: 1: 8: 2) (v/v/v/v) gave better, sharp and well defined peak resolution for both standards (KBA and AKBA) as well as sample (Figure 3). The spot at R_f 0.39 was identified as KBA and spot at R_f 0.42 was identified as AKBA with the help of chromatogram of the standard compound and HPTLC plate at 254 nm. The well defined spots were obtained upon complete saturation of the solvent chamber for 30 minutes.

The TLC plate was scanned at 254 nm and the identity of KBA and AKBA bands in the sample chromatogram were confirmed by the 3D (Figure 4) chromatogram obtained after densitometric scanning and by comparing peak of retention factor of KBA and AKBA from sample and standard solution. This was further authenticated by overlaying spectra of KBA and AKBA standards with extract (Figure 5). The peak corresponding to KBA and AKBA from the sample solution had same retention factor as that from standard KBA and AKBA (Rf 0.39 and 0.42 respectively).

The calibration curves of KBA and AKBA was constructed by plotting peak area against concentration of standard KBA and AKBA respectively. They confirm the linearity of the standard curves over the range studied. The linear regression of KBA standard curve was determined with $R^2 \pm SD = 0.984 \pm 1.68\%$. with regression line; $y = 380.75x + 12716.380$. The regression curve of AKBA was determined with $R^2 \pm SD = 0.997 \pm 0.75\%$. with regression line; $y = 446.083x + 8885.082$.

The contents of KBA and AKBA quantified using calibration curve was found to be 3.3% and 4.6 % respectively in the extract of *Boswellia serrata*.

HPTLC versus HPLC

There was no significant difference between the content of KBA and AKBA obtained by HPTLC and HPLC, although the HPLC method showed slightly lower values compared with HPTLC. Similar studies comparing HPLC and HPTLC methods for determination of ethanobotanicals have also been reported.^{30,31} The major advantage of HPTLC over HPLC is reducing analysis time and cost per analysis.³² Another advantage of HPTLC discussed in the previously reported study is the capability to detect more compounds than HPLC. In this regard, the compounds which cannot be eluted still can be detected. Moreover, the compounds having no UV absorption, e.g. sugar, still can be detected by reagent spraying.³³ As HPLC method is widely accepted over HPTLC as per pharmacopeial standards but looking at the advantages of HPTLC over HPLC here in this study; HPTLC-densitometry could be used for the quantitative determination of KBA and AKBA in *Boswellia serrata* extracts and their pharmaceutical definitions and as an alternative to HPLC in rapid screening or in routine analysis.

CONCLUSION

Present study was conducted in order to compare HPLC and HPTLC methods for qualitative and quantitative determination of bioactive boswellic acids. Both

methods are simple and effective for quantification of KBA and AKBA in *Boswellia serrata* extracts and their pharmaceutical formulations.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

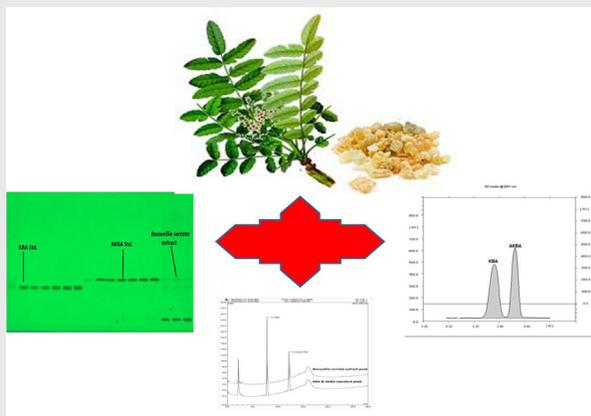
The authors have no conflicts of interest.

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PICTORIAL ABSTRACT



SUMMARY

- An HPTLC method was developed in comparison to HPLC protocol for quantitative analysis of KBA and AKBA in *Boswellia serrata* extract .
- The gradient HPLC method was and direct HPTLC assay was used for the determination of KBA and AKBA at 254 nm.
- Analytical assay showed lower values for content of KBA and AKBA in HPLC as compared to HPTLC. The amount of KBA and AKBA in the *Boswellia serrata* extract was found to be about 3.3 % and 4.6 % by HPTLC and 3.03% and 3.87% by HPLC respectively.
- The developed methods are simple, effective and reliable for quantitative analysis of *Boswellia serrata* extracts and their pharmaceutical formulations.