Development and validation of RP HPLC method for simultaneous determination of picroside I, picroside II, phyllanthin and boeravinone B in a polyherbal hepatoprotective tablet formulation

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Abstract

Purpose: To develop and validate RP-HPLC method for simultaneous determination of four active ingredients; picroside I (PSI), picroside II (PSII), phyllanthin (PHY) and boeravinone-B (BVB) in a polyherbal hepatoprotective tablet formulation.

Methods: The study was carried out using Waters X-Bridge, C18 (250 mm x 4.6 mm, 5 μm) column with mobile phase consisting of 5 mM ammonium acetate in 10 % methanol and acetonitrile, with gradient programme at dual wavelengths of 220 nm and 274 nm and flow speed of 1 mL.min⁻¹. The procedure was validated with respect to specificity, linearity, precision, accuracy, system suitability, limit of detection (LOD) and of quantification (LOQ), and robustness in line with ICH specifications.

Results: The method was linear within the concentration range of 25 to 200 %, and the values of correlation coefficients (R²) were > 0.999. Intra-day and inter-day RSDs of PAs and RTs were < 5.0 %, with recovery in the range of 100.0 - 106.0 %.

Conclusion: The four active ingredients have with good resolution with regard to the method used. The method is rapid, simple, highly selective, sensitive and cost effective, which make it an efficient method for quality assurance.

Keywords: Boeravinone B, Phyllanthin, Picroside I, Picroside II, RP-HPLC

INTRODUCTION

The liver is one of the vital organs which helps in maintaining the health of the body when modern lifestyles and habits over stress it, making it to malfunction [1]. Nature has bestowed on man certain herbs with the property to prevent, treat and cure hepatic disturbances. This has led to a huge quest for plant-based medications, making it important to ascertain that these medicines are safe, effective and of high standard [2].

Kutki (Picrorhiza kurroa) is a crucial herbal medicine source utilized in folk and orthodox treatments for hepatic ailments because of its...
hepatoprotective properties [3,4]. The important chemical constituents of Kutki are iridoid glycosides such as kutkosides, picroside I (PSI) and picroside II (PSII). Chromatographic methods have been developed for PSI [5] and PSII [5-7]. Bhumi Amla (Phyllanthus niruri) is an effective hepatoprotective [8] and antigenotoxic herb [9]. Phyllanthin (PHY) and hypophyllanthin (HPY) are two major lignans of the Phyllanthus genus known to have very good hepatoprotective properties [8]. Liquid chromatography and UV spectrophotometric procedures have been designed for estimation of PHY alone in parts of Phyllanthus niruri [10,11]; while HPTLC procedures are available for concurrent analysis of PHY & HPY [12, 13]. An HPLC-UV method is available for the evaluation of lignans [14]; there is a fluorescence detection technique for PHY-related compounds in plasma [15], and a GC-MS method for PHY and HPY is available [16]. Micellar electro-kinetic chromatography [17] and HPLC-SPE-NMR are other published methods, but they are quite costly and not easily available [18].

Punarnava (Boerhaavia diffusa) is an Ayurvedic rasayana herb with hepatoprotective property. The principal bioactive constituent of B. diffusa is boeravinone-B (BVB). It possesses potent hepatoprotective property, and it has been determined using a rapid quantitative UPLC-PDA method [19]. Identification of BVB in methanol extract of B. diffusa root was performed with a validated chromatographic method i.e., TLC and advanced HPLC techniques [20], while HPTLC technique was employed for qualitative and quantitative analysis of BVB in B. diffusa [21,22].

In the present study, we selected polyherbal hevitol tablets having combination of three herbs. Bhumi amla, punarnava, and kutki showed high levels of effectiveness in liver protection during clinical studies. These three herbs contain a variety of bioactive ingredients with liver-protective properties. From these, four bioactive components were selected for quantification: one from bhumi amla (PHY; Figure 1 C), one from punarnava (BVB; Figure 1 D), and two from kutki, (PSI; Figure 1A), and PSII (Figure 1 B). These active ingredients are chemically known as 6’-cinnamoylcatalpol (PSI), vanilloyl catalpol (PSII), (2S, 3S)-(+)1, 4 –dimethoxy-2, 3-diveratrylbutane (PHY), and 6, 9, 11-Trihydroxy-10-methyl-[1] benzopyran- [3, 4-b] [1] benzopyran-12(6H)-one (BVB).

After relevant literature search, there was no reported chromatographic or spectrophotometric method(s) for simultaneous determination of PHY, BVB, PSI, and PSII in tablet dosage form. Hence, the aim of the present research was to design and validate a RP-HPLC procedure for concurrent estimation of these four active ingredients from tablet formulations.

**EXPERIMENTAL**

**Chemicals and reagents**

Standards for PSI (CAS No. 27409-30-9, purity > 95 %); PSII (CAS No. 39012-20-9, purity > 92 %); PHY (CAS No. 10351-88-9, purity > 98 %) and BVB (CAS No. 114567-34-9, purity > 97%) were purchased from NRPL, India. HPLC-grade methanol (Merck, India), HPLC-grade acetonitrile (Merck, India) and Analytical Reagent grade ammonium acetate (Merck, India) were used. Moreover, HPLC-grade water was prepared using Milli-Q® Direct Water Purification System using 46 mm × 0.45 μm nylon membrane filters (Millipore, India) and 13 mm × 0.45 μm PTFE membrane syringe filters (Millipore, India). Hepitol tablet formulation containing four bioactive ingredients (PSI, PSII, PHY and BVB) was developed. It completed satisfactory stability studies, and it will be commercialized once manufacturing permission is granted by Licensing Authority.

**Instrumentation and analytical conditions**

Chromatography was carried out using HPLC, LC2010CHT (Shimadzu, Japan) system equipped with binary quaternary gradient pump, an ultra-fast auto sampler, a column oven, a degasser, and PDA detector, while Labsolution CS software was used for data acquisition and analysis. Active ingredient separation was done using Waters X Bridge C18 column. After optimization, the column was maintained at 25°C. Samples were analyzed using 5 mM ammonium acetate in 10 % methanol (mobile phase A) and acetonitrile (mobile phase B). Both mobile phases were subjected to filtration with a 0.45 μm nylon membrane filter, and to degassing with an ultrasonic bath before analysis. Gradient
elution was used (Table 1) with a flow rate of 1.0 mL/min and UV detection at wavelengths of 220 and 274 nm.

**Mobile phase preparation:**

**Mobile phase (A):** Ammonium acetate (385mg) was dissolved in 1 L of HPLC-grade H₂O and filtered through 0.45 µm nylon membrane. Methanol (100 mL) was added to 900 mL of the above prepared buffer solution, followed by mixing and sonication.

**Mobile phase (B):** HPLC grade Acetonitrile

**Diluent:** HPLC grade Methanol

**Preparation of standard solutions**

**Solution 1:** 5 mg each of PHY, PSI and PSII standards was solubilized in 10-mL volumetric flask with 7 mL methanol, sonicated and made up to mark with methanol.

**Solution 2:** This was prepared by solubilizing 5 mg BVB standard in a 100-mL volumetric flask with 70 mL methanol. Following sonication, it was made up to mark with methanol.

**Standard solution:** This was prepared by mixing 5 mL of standard solution 1 and 5 mL of standard solution 2 in a 25-mL volumetric flask, and the volume was made up with methanol. The concentrations of active ingredients used were 0.10 mg/mL, 0.10 mg/mL, 0.10 mg/mL and 0.01 mg/mL, for PSI, PSII, PHY and BVB, respectively.

**Preparation of sample solution**

This was done by weighing 1 g of crushed tablets (5 no.) powder into a 100 mL volumetric flask containing 80 mL methanol as diluent; and sonicating the solution at 40 °C for 60 min. The solution was further made up to 100 mL with additional methanol, and filtered first using Whatman paper, followed by 0.45 µm nylon syringe filter. Sample solutions were prepared in duplicate. For system suitability, blank and standard solutions were injected five times, followed by sample solutions.

**Method validation**

This was done in accordance with the ICH protocol [23] for the following parameters: linearity, LOD, LOQ, precision, accuracy, system suitability, specificity, and robustness.

**Linearity**

Linearity was evaluated using serial dilutions of standards for all bioactive ingredients in six different concentrations (from 25 to 200% of the specification level). Calibration graphs for all four active ingredients were plotted. The linearity correlation coefficient was expected to be more than 0.99. Slope, intercept and % bias were also calculated.

**Limit of detection (LOD) and limit of quantification (LOQ)**

In line with ICH guidelines, the LOD and LOQ of the developed procedure were determined.

The LOD for the proposed method was calculated in line with Eq 1.

\[
LOD = 3.3 \frac{S}{K} \quad \text{(1)}
\]

while LOQ was calculated using Eq 2.

\[
LOQ = 10 \frac{S}{K} \quad \text{(2)}
\]

where \(S\) is the SD of replicate determinations under similar conditions used for sample when there was no analyte, and \(K\) is sensitivity.

**Precision**

This was assessed in terms of intra-day and inter-day fluctuations in triple analytical data of 5 solutions of known concentrations, on the same day (intra-day) or on two continuous days (inter-day). The RSD of RT and PA were used as indexes of accuracy and reproducibility.

### Table 1: Gradient programming

| Time (min) | Mobile phase A | Mobile phase B | Time (min) | Flow rate (mL/min) | Mobile phase A | Mobile phase B |
|------------|----------------|----------------|------------|--------------------|----------------|----------------|
| Initial    | 95             | 5              | 35         | 1                  | 30             | 70             |
| 3          | 95             | 5              | 38         | 1                  | 10             | 90             |
| 13         | 80             | 20             | 46         | 1                  | 10             | 90             |
| 17         | 70             | 30             | 48         | 1                  | 95             | 5              |
| 20         | 50             | 50             | 55         | 1                  | 95             | 5              |

Flow rate: mL/min
**Accuracy**

The accuracy of the developed method was determined using spiking method by addition of accurately measured quantities of standards to known concentrations of the formulated hepitol tablet. The total amount of each compound was calculated from the appropriate calibration curves, and recovery was obtained using Eq 3.

\[
\text{Recovery (\%)} = \left[\frac{(F-I)}{A}\right] \times 100 \quad \ldots \ldots \ldots (3)
\]

where F and I are concentrations of spiked and non-spiked analytes, respectively.

**System suitability**

System suitability parameters i.e., plate count, tailing factor and % RSD were calculated. The system performance was analyzed for five replicates of each standard solution, and % RSDs of the retention time (RT) for all standards were expected to be less than 1.0 %, while peak areas (PAs) for all standards should be < 5 % of calculated contents of all the four bioactive ingredients.

**Specificity**

The method complied with specificity since there was no interference from blank and placebo in the elution zone of the active ingredients present in the samples. Moreover, the peak purity of each bioactive ingredient was checked with Photodiode-Array (PDA) detector.

**Robustness**

Robustness was verified by altering chromatographic conditions such as flow rate by \(\pm 0.1\) mL/min., and column oven temperature by \(\pm 5 \) °C, while keeping all other method parameters same as per methodology.

**RESULTS**

**Method development and optimization results**

Chromatographic parameters for simultaneous determination of PSI, PSII, PHY and BVB in the tablet formulation were optimized with respect to mobile phase, flow rate, solvent type, duration of analysis, wavelength, and other essential parameters so that the developed procedure will be suitable for use in quality assurance of the formulation.

**Mobile phase and flow-rate optimization**

Trials were conducted using uniform/constant mobile phase (isocratic) and gradient solvent systems. However, an isocratic system with mobile phase of water: acetonitrile (40:60, 50:50, 30:70) resulted in high retention times, rendering this approach inappropriate. Consequently, gradient system was tried with acetonitrile (B) and H\(_2\)O (A) as follows: 0 – 600 sec, 30 % B; 600 – 1200 sec, 30 – 80 % B; and 1200 – 1500 s, 80 – 90 % B. Using this approach, the retention times of PSI, PSII, PHY, and BVB were unstable. Therefore, mobile phase containing solvent A (5mM ammonium acetate in 10 % methanol in water) and solvent B (acetonitrile) with the gradient program as shown in Table 1, was finally chosen, with a flow rate of 1 mL/min which was optimized at room temperature (25 ℃).

**Optimized wavelength**

To identify an effective wavelength for the concurrent estimation of these active ingredients, solutions of the compounds in mobile phase were subjected to scanning in UV/VIS in the \(\lambda\) range of 200 - 400 nm. From the overlaid UV spectra, dual wavelengths of 220 nm for PSII and PHY and 274 nm for PSI and BVB were selected.

The chromatograms of the standard active ingredients with good separation are shown in Figure 2 and Figure 3. The peaks were confirmed via comparison of RTs obtained under the same chromatographic conditions. The chromatographic profiles of the active ingredients in the formulation are shown in Figure 4 and Figure 5. All method validation studies were conducted using replicate injections of the sample and standard solutions.

**Linearity**

Linearity results are as shown in Table 2 which indicates that PSI, PSII, PHY and BVB exhibited linearity at concentration ranges between 25 to 200%. The linear regression equations for the four active ingredients are as follows:

- PSI: \(Y = 6E + 07X + 30062, R^2 = 0.999\);
- PSII: \(Y = 4E + 07X + 25947, R^2 = 0.999\);
- PHY: \(Y = 4E + 07X + 41530, R^2 = 0.999\),
- BVB: \(Y = 3E + 08X - 1351, R^2 = 0.999\),

and the correlation coefficients were 0.9999, 0.9998, 0.9999 and 0.9998 for PSI, PSII, PHY and BVB respectively, indicating a linear correlation between the peak areas and drug concentrations.
Accuracy

Table 3 shows that the new procedure produced good recovery in the quantitation of PSI, PSII, PHY and BVB in the tablet formulation.

Precision

Precision results are shown in Table 4. The results were well within laboratory variation on two different days. In both intra- and inter-day precision studies, the values obtained were within acceptable limits (RSD < 5.0 % and SD < 1.0 %).

Robustness

Results for robustness (Table 5) revealed that the developed procedure was appropriate for concurrent determination of PSI, PSII, PHY and BVB. The robustness values of these four active markers in tablet formulation were 0.32, 0.50, 0.26 and 0.005%, respectively.

Selectivity

Sharp peaks were obtained for PSII and PHY at 220 nm, and for PSI and BVB at 274 nm, with retention times of 17.4 and 31.4 min and 20.5 and 28.7 min respectively for the compounds, relative to placebo solution.

LOD and LOQ

The results for LOD and LOQ are as shown in Table 6. The LOD values were 1.72, 0.84, 0.62 and 0.010 μg/mL (signal-to-noise ratio of 3:1), and the LOQ values were 5.31, 2.56, 1.89 and 0.04 μg/mL for PSI, PSII, PHY and BVB, respectively. These results indicate that the developed method is highly sensitive.

Table 2: Linearity data

| Linearity (%) | Picroside II | Picroside I | Phyllanthin | Boeravinone B |
|---------------|-------------|-------------|-------------|--------------|
|               | Conc. (mg/mL) | Area        | Conc. (mg/mL) | Area        | Conc. (mg/mL) | Area        | Conc. (mg/mL) | Area        |
| 25            | 0.0118       | 488703      | 0.0064       | 397433      | 0.0046       | 244568      | 0.0001       | 26257       |
| 50            | 0.0237       | 978421      | 0.0127       | 791719      | 0.0092       | 454831      | 0.0002       | 53111       |
| 100           | 0.0473       | 1885276     | 0.0254       | 1512328     | 0.0185       | 845676      | 0.0004       | 103679      |
| 125           | 0.0592       | 2417617     | 0.0318       | 1928942     | 0.0231       | 1073629     | 0.0005       | 133849      |
| 150           | 0.0710       | 2870456     | 0.0381       | 2285824     | 0.0277       | 1270147     | 0.0006       | 160019      |
| 200           | 0.0947       | 3786014     | 0.0508       | 3019677     | 0.0369       | 1675098     | 0.0008       | 214670      |
| 0.9998        | ---          | 0.9999      | ---          | 0.9999      | ---          | 0.9999      | ---          | 0.9998      |
| 25947         | ---          | 30062       | ---          | 41530       | ---          | 1352        |
| 39879122      | ---          | 59052957    | ---          | 44289718    | ---          | 269113700   |
| 23637         | ---          | 16892       | ---          | 9365        | ---          | 1474        |
| 1.38          | ---          | 1.99        | ---          | 4.91        | ---          | -1.30       |

Table 3: Results of accuracy (recovery) study

| Run | Level | PSII Mean Assay | % SD | % RSD | PSI Mean Assay | % SD | % RSD | PHY Mean Assay | % SD | % RSD | BVB Mean Assay | % SD | % RSD |
|-----|-------|-----------------|------|-------|----------------|------|-------|----------------|------|-------|----------------|------|-------|
| 1   | 50%   | 0.53            | 0.01 | 2.2   | 0.32           | 0.01 | 1.8   | 0.28           | 0.01 | 2.1   | 0.006          | 0.002 | 3.6   |
| 2   | 100%  | 0.50            | 0.00 | 0.0   | 0.32           | 0.01 | 1.8   | 0.26           | 0.01 | 2.2   | 0.005          | 0.0002 | 3.0   |
| 3   | 150%  | 0.51            | 0.01 | 1.1   | 0.32           | 0.01 | 1.8   | 0.26           | 0.00 | 0.0   | 0.005          | 0.0001 | 1.1   |

| Overall Statistical Data | 0.51 | 0.01 | 1.1 | 0.32 | 0.01 | 1.8 | 0.27 | 0.004 | 1.4 | 0.005 | 0.0001 | 2.5 |
| Average Recovery % | 102.0 | --- | --- | 106.0 | --- | --- | 103.8 | --- | 100.0 | --- | --- | --- |

Mean Assay %. Average Recovery %, Serial no. indicates triplicate samples injected for each level.
Table 4: Results of Precision and Intermediate Precision

| Variable   | Sample | PSII  | PSI   | PHY   | BVB   |
|------------|--------|-------|-------|-------|-------|
| Method     | 1      | 0.50  | 0.38  | 0.23  | 0.0045|
|            | 2      | 0.51  | 0.38  | 0.23  | 0.0045|
| Precision  | 3      | 0.50  | 0.37  | 0.23  | 0.0043|
|            | 4      | 0.51  | 0.38  | 0.22  | 0.0045|
|            | 5      | 0.51  | 0.40  | 0.23  | 0.0045|
|            | 6      | 0.53  | 0.39  | 0.23  | 0.0042|
|            | 1      | 0.51  | 0.35  | 0.25  | 0.0050|
|            | 2      | 0.52  | 0.36  | 0.25  | 0.0047|
| Intermediate | 3     | 0.51  | 0.36  | 0.25  | 0.0048|
| Precision  | 4      | 0.51  | 0.35  | 0.24  | 0.0049|
|            | 5      | 0.51  | 0.35  | 0.24  | 0.0047|
|            | 6      | 0.50  | 0.35  | 0.24  | 0.0046|
| Overall statistical data | 0.51 | 0.37 | 0.24 | 0.0046 |
| SD        | 0.009  | 0.017 | 0.010 | 0.0002 |
| Cumulative % RSD | 1.8 | 4.6 | 4.2 | 4.3 |

Table 5: Robustness results

| Parameter | Condition | PSII | PSI | PHY | BVB |
|-----------|-----------|------|-----|-----|-----|
|           |           | Rt   | Area | Assay (%) | Rt | Area | Assay (%) | Rt | Area | Assay (%) | Rt | Area | Assay (%) |
| Flow rate | 0.9 L/min | 0.1  | 0.6  | 0.50 | 0.2 | 1.6  | 0.32 | 0.4 | 1.7  | 0.25 | 0.4 | 1.8  | 0.0062 |
|           | 1.1 L/min | 0.1  | 0.8  | 0.54 | 0.1 | 1.1  | 0.31 | 0.1 | 1.8  | 0.25 | 0.1 | 2.0  | 0.0062 |
| Column temp. | 20°C | 0.2  | 0.4  | 0.49 | 0.1 | 0.3  | 0.32 | 0.2 | 0.5  | 0.25 | 0.2 | 0.6  | 0.0054 |
|           | 30°C      | 0.1  | 1.1  | 0.56 | 0.1 | 1.3  | 0.31 | 0.1 | 1.0  | 0.24 | 0.1 | 1.0  | 0.0050 |

Rt: retention time

Table 6: Results of LOD, LOQ

| Active ingredient | Content of active ingredient | LOD (%) | LOQ | Concentration (mg/mL) | LOD | LOQ |
|-------------------|-----------------------------|---------|-----|-----------------------|-----|-----|
|                   | Level (%) |                     |       |                       | 0.00172 | 0.00531 |
| PSII              | 3.70      | 11.21               | 0.00084 | 0.00256 |
| PSI               | 3.32      | 10.07               | 0.00062 | 0.00189 |
| PHY               | 3.37      | 10.22               | 0.00001 | 0.00004 |
| BVB               | 2.50      | 10.00               | 0.00001 | 0.00004 |

Specificity and system suitability

The retention time of the standard active ingredients (Figures 2 and 3) and the four active ingredients in sample solution (Figures 4 and 5) were identical, thereby confirming the specificity of the method. In addition, peak purity data of all active markers in sample indicated that peaks had no co-eluting peaks. Therefore, the developed procedure is specific for the intended use (Table 7). System suitability results (Table 8) revealed that % RSD values of retention time of all the active markers in replicate injection were less than 1.0%, and % RSD of area of all active markers were less than 2.0%, highlighting the suitability of the analytical method.

Table 7: Specificity results

| Parameter              | PSI       | PSII      | PHY       | BVB       | Acceptance Criteria |
|                       |          |           |           |           | Peak purities of each active ingredient comply |
| Peak purity in standard | 0.078   | 0.25     | 0.216     | 0.221     |
| Purity Angle          | 0.015    | 0.206    | 0.122     | 0.221     |
| Threshold             | 0.230    | 0.225    | 0.216     | 0.221     |
| Peak purity in sample | 0.209   | 0.132    | 0.408     | 0.665     |
| Purity Angle          | 0.053    | 0.226    | 0.226     | 0.665     |
| Threshold             | 0.211    | 0.226    | 0.226     | 0.665     |
| *There were no peaks at RTs of PSII, PSI, PHY and BVB in the chromatogram of diluent and placebo solutions. No interference in the elution zone of the active ingredients present in the samples from blank and Placebo. |

Note: Peak purity of all the active ingredients passed.
Table 8: System suitability data

| Parameter | PSI | PSII | PHY | BVB |
|-----------|-----|------|-----|-----|
| RT (min)  |     |      |     |     |
| Size      |     |      |     |     |
| RT (min)  |     |      |     |     |
| Size      |     |      |     |     |
| Mean      | 18.28 | 3887400 | 21.36 | 3661230 |
| SD        | 0.02 | 3219.97 | 0.1 | 0.7 |
| % RSD     | 0.1 | 0.8 | 0.1 | 1.2 |

SS: system suitability injection no., RT: retention time

DISCUSSION

The new developed method has advantages such as efficient separation, repeatability, effectiveness, and sharp resolution of active components. There was no interference peak due to interaction between the active ingredients and non-drug components in the tablet. The developed method was validated in line with ICH guidelines regarding linearity, accuracy, precision, selectivity, LOD, LOQ, specificity and robustness.

Considering all these advantages, we conclude that the developed procedure is suitable for use in the concurrent determination of the four active ingredients, thereby facilitating quality assurance of polyherbal formulas. The method can also be applied in laboratory investigations and industrial decision making.

CONCLUSION

This research work has produced and validated RP HPLC procedure which was shown to be simple, reproducible, and economical for concurrent analysis of four bioactive components i.e., PSI, PSII, PHY, and BVB from polyherbal hepatoprotective tablet formulation.

The supporting data indicate that the new method satisfies the ICH requirements with respect to reliability, linearity, repeatability, specificity, LOD, LOQ and robustness, thereby proving the reliability of the method.

The total run time of the four components was < 45 min, which is suitable for routine quality assurance. The new procedure is of advantage in the separation of the four active markers simultaneously at two different wavelengths. In addition, the optimized procedure is simple, and it is suitable for regular use for quantification of herbal formulations, and for decision making regarding quality compliance.
DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all the liabilities pertaining to claims relating to the content of this article will be borne by the authors. Sunil V. Shanbhag planned the research work, designed and carried out all the experiments, data compilations, statistical analysis, and preparation of the manuscript. Madhusudan T. Bachute made critical observations in the manuscript and developed the final document. Both the authors read and approved the final manuscript.

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REFERENCES

1. Abraham G. A Review on Hepato-protective herbs used in Ayurveda. Global J Res Med Plants & Indigen Med 2014; 3(7): 303-311.
2. Akhade MS, Agraval PA, Laddha KS. Development and Validation of RP-HPLC Method for Simultaneous Estimation of Picroside I, Plumbagin, and Z-guggulsterone in Tablet Formulation. Indian J Pharma Sci 2013; 75(4): 476-482.
3. Handa SS. Perspectives of Indian medicinal plants in management of liver disorder. New Delhi: Indian Council Med Res 2008; p. 245 270.
4. Vaidya AB, Antarkar DS, Doshi JC, Bhatt AD, Ramesh VV, Vora PV, Perissond DD, Baxi AJ, Kale PM. Picrorhiza kurroa (Kutaki) Royle ex Benth as a hepatoprotective agent—experimental & clinical studies. J Postgraduate Med 1996; 42(4): 105-108.
5. Sultan P, Jan A, Pervaiz Q. Phytochemical studies for quantitative Estimation of indoid glycosides in Picrorhiza kurroa Royle. Bot Stud 2016; 57(7): 2-6.
6. Weinges K, Kloos P, Henkels WD. Natural products from medicinal plants XVII. Picroside II, a new 6 vanilloyl catapol from Picrorhiza kurroa Royle and Benth. Justus Liebigs Ann Chem 1972; 759: 173 182.
7. Bhusari S, Borre G, Randhe M, Wakte P. Validated RP-HPLC for Simultaneous Estimation of Etoposide and Picroside-II in Patented Pharmaceutical Formulation and the Bulk. Int J Pharma Phyto Pharma Res 2019; 9(2): 83-90.
8. Ezzat MI, Okba MM, Ahmed SH, El-Banna HA, Prince A, Mohamed SO, Ezzat SM. In-depth hepatoprotective mechanistic study of Phyllanthus niruri: In vitro and in vivo studies and its chemical characterization. PLOS ONE 2020; 15(1): e0226185.
9. Singh T, Ruchi, Singh A, Kumar R, Singh JK. Acute toxicity study of Phyllanthus niruri and its effect on the cyto-architectural structure of nephrocytes in Swiss albino mice Mus-musculus. Pharmaco J 2016; 8(1): 77-80.
10. Venkatesh S, Reddy BM, KasiViswanath C, Ramesh M. Spectrophotometric Determination of Hepatoprotective Principle of Phyllanthus niruri Linn. Ind J of Pharma Sci 2004; 336-337.
11. Annamalai A, Lakshmi PT. HPTLC and HPLC Analysis of Bioactive Phyllanthin from Different Organs of Phyllanthus amarus. Asian J Biotechnol 2009; 1: 154-162.
12. Tripathi AK, Verma AK, Gupta MM, Gupta SP, Khanuja. Quantitative determination of phyllanthin and hypophyllanthin in phyllanthus species by high performance thin layer chromatography. Phytochemical Analysis 2006; 17(6): 394-397.
13. Dhalwal K, Biradar YS, Rajani M. High performance thin layer chromatography densitometric method for simultaneous quantitation of phyllanthin, hypophyllanthin, gallic acid and ellagic acid in Phyllanthus amarus. JAOAC Int 2006; 89(3): 619-623.
14. Sharma A, Singh RT, Handa SS. Estimation of Phyllanthin and Hypophyllanthin by High performance liquid chromatography in Phyllanthus amarus. Phytochemical Analysis 1993; 4: 226-229.
15. Murugaiyah V, Chan KL. Analysis of lignans from Phyllanthus niruri L. in plasma using a simple HPLC method with fluorescence detection and its application in a pharmacokinetic study. J Chromatography B 2007; 852(1-2): 138-144.
16. Novellino KVA, Bernardo SR, Tappin MRR, Alves RN, Da Silva DB, Da Silva MJ. Phyllanthin and hypophyllanthin determination by gas chromatography mass spectrometry of six Stonebreaker species from different regions of Brazil. Med Plant Res 2020; 14(4): 175-184.
17. Kuo CH, Lee SS, Chang HY, Sun SW. Analysis of lignans using micellar electro kinetic chromatography. Electrophoresis 2003; 24(6): 1047-1053.

18. Bairwa K, Srivastava A, Jachak SM. Quantitative analysis of Boeravinones in the roots of Boerhaavia diffusa by UPLC/PDA. Phyto chem Anal 2014; 25: 415-420.

19. Wang CY, Lee SS. Analysis and identification of lignans in Phyllanthus urinaria by HPLC-SPE-NMR. Phytochem Anal Mar-Apr 2005; 16(2): 120-126.

20. Krishnamoorthy PK, Muthukumaran S, Maheswaran A, Sukumaran P. Isolation, purification and characterization of Boeravinone B from Boerhaavia diffusa Linn. Int Res J Pharm 2017; 8: 140-144.

21. Pooja, Lal VK, Verma A. HPTLC densitogram profile of aqueous distillate (Arka) from roots of Boerhaavia diffusa. Br J Pharm Res 2016; 9: 1-6.

22. Singh A, Sharma H, Singh R, Pant P, Srikant H, Dhiman KS. Identification and Quantification of Boeravinone-B in Whole Plant Extract of Boerhaavia diffusa Linn and in its Polyherbal Formulation. J Natural Remedies 2017; 17(3): 88-95.

23. International Conference on Harmonization. ICH. Topic Q2 (R1) Validation of Analytical Procedures: Text and Methodology. Int. Conf. Harmon 2005.