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Method validation for the simultaneous estimation of three-bioactive components in combined extracts of three hepatoprotective plants using RP-HPLC method

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ABSTRACT

Picrorhiza kurroa Royle ex Benth. (Scrophulariaceae), *Andrographis paniculata* (Burm.f.) Nees (Acanthaceae), *Silybum marianum* (L.) Gaernt (Sm). (Asteraceae) are well-known plants to treat various ailments. Simultaneous quantification and determination of picroside-I, andrographolide, and silybin were conducted in the combined extract of their respective plants, by reverse-phase high performance liquid chromatography (RP-HPLC). Analytical determination of the proposed HPLC method was validated as per referenced protocol. Determination and method validation was carried on RP-HPLC column. Picroside-I, andrographolide, and silybin were quantified as 1.232 ± 0.0102, 0.572 ± 0.0734, 2.037 ± 0.0635 µg/mg, respectively. Validated method for simultaneous estimation of analytes was investigated to be linear, specific, reproducible, and accurate. Recovery of identified analytes was within the specified limit of 99.65%–102.59%. The study reveals that the developed HPLC method exhibits potential for determination and quantification of picroside-I, andrographolide, and silybin in in combined extract of *P. kurroa*, *A. paniculata*, and *S. marianum*.

INTRODUCTION

Natural products specifically plants are leading source of biologically active compounds, which are used to treat various human and animal ailments. It is estimated that worldwide, 80% of the people in developing countries are dependent on traditional medicines and medicinal plants for their basic healthcare requirements (Mahady, 2001). Plant-based medicines consist of a single herb or are combination of many herbs containing multiple chemical components. Thus, quality control of such combinations sets a great challenge. Fingerprinting investigation has emerged as an important analytical method for quality control of synthetic as well as herbal drugs. It offers essential characterization of a complex mixture with a quantitative extent of reliability and aims on the detection and stability evaluation of the components (Alamgir, 2017; Xie *et al*., 2007). Modern analytical methods *viz*. High performance liquid chromatography (HPLC), high performance thin layer chromatography, and gas chromatography are exercised worldwide to obtain the fingerprint analysis of single as well as complex herbal drugs (Martin and Guiochon, 2005). HPLC is a widely accepted and robust method to isolate and separate mixture of components of natural products. Additionally, it permits the identification, quantification, and purification of components in a mixture (Fan *et al*., 2006; Govindarajan *et al*., 2019). This approach is currently being recognized among numerous analytical methods as the primary choice for fingerprinting investigation to validate the quality control of herbal drugs (Gamal *et al*., 2019; Li *et al*., 2008; Liang *et al*., 2020; Srivastava *et al*., 2015; Wang *et al*., 2019). *Silybum marianum* (L.) Gaernt (Sm). (Asteraceae), *Andrographis paniculata* (Burm.f.) Nees (Acanthaceae), *Picrorhiza kurroa* Royle ex Benth. (Scrophulariaceae) are the recognized hepatoprotective plants.

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Picrorhiza kurroa contains iridoid glycosides, cucurbitacin glycosides, apocynin, kutkin, and picrosides as its bioactive constituents. Picroliv or Kutkin is the most important iridoid glycoside of the plant, accountable for the hepatoprotective activity and is a combination of kutkoside and picroside-I (Khare, 2007; Mehta *et al*., 2021a). Numerous research works have highlighted the hepatoprotective potential of *P. kurroa* in different animal models (Shetty *et al*., 2010; Sinha *et al*., 2011; Talmale *et al*., 2010). Picroside-I has been reported as a bioactive component, which is responsible for the hepatoprotective activity of *P. kurroa* (Dwivedi *et al*., 1992). The compound has shown its hepatoprotective potential in hepatic fibrosis by regulating the sphingolipid signaling pathway, biosynthesis of primary bile acid, and peroxisome proliferator-triggered receptor signaling pathway (Xiong *et al*., 2020). *Picrorhiza kurroa* has also exhibited hepatoprotective activity against CCl_4 induced hepatotoxicity and acetaminophen-induced hepatic injury (Gupta *et al*., 2016; Kaur *et al*., 2012).

Andrographis paniculata, commonly known as King of Bitters or kalmegh, has scientifically explored for various biological activities including hepatoprotective, anticancer, antioxidant, antibacterial, neuroprotective, immunomodulatory, anti-diabetic, and anti-inflammatory (Mehta *et al*; 2021b, 2021c; Parveen *et al*., 2019). Diterpene lactones are responsible for the multiple biological applications of *A. paniculata*. Among various diterpene lactones, andrographolide has been widely investigated. It has ameliorated the liver injury by decreasing the oxidative stress and inflammatory responses (Chen *et al*., 2014; Ye *et al*., 2011) and showed a protective effect for acute intrahepatic cholestasis by suppressing NFκB expression and suppression of hepatic stellate cell activation (Khamphaya *et al*., 2016).

Silymarin is the standardized extract of *S. marianum* (Milk Thistle) seeds, consisting of various flavonolignans (about 70%–80% *w/w*) and oxidized and polymeric polyphenolic components including the combination of flavonoids. The key components of silymarin are silybin, isosilybin, silychristin, isosilychristin, silydianin, and silimonin (Poppe and Petersen, 2016). Silybin is the active compound of the extract, which have been validated by different scientific reports and present in a range of 20%–40% in various pharmaceutical products containing silymarin (Bijak, 2017; Bijak *et al*., 2014). Silymarin and silybin have shown significant hepatoprotective, antioxidant, chemo-preventing, and anti-inflammatory activities (Abenavoli *et al*., 2016; Mastron *et al*., 2015; Serviddio *et al*., 2014; Zhu *et al*., 2016).

Several methods are available to estimate bioactive constituents individually and/or in combination with other components in their respective plants (Korany *et al*., 2017; Patel *et al*., 2008; Singh *et al*., 2005). As per the literature survey, although these three plants are explored individually and in combination with other drugs to treat various hepatic diseases, but to the best of authors' knowledge, there is no report of any analytical study published for these three plants. Therefore, we selected these plants and developed the HPLC method for the simultaneous determination of the three bioactive components of these hepatoprotective plants *viz*. andrographolide (for *A. paniculata*), picroside-I (for *P. kurroa*), silybin (for *S. marianum*), when they are used in a mixture. Our aim is to establish and validate HPLC approach for simultaneous determination of the three bioactive markers, in the combined extract attained by mixing kalmegh, kutki, and milk thistle. The developed method was validated as per the referenced protocol.

Experimental

Plant material and authentication

Andrographis paniculata (aerial parts), *P. kurroa* (roots), and *S. marianum* (seeds) were provided as gift samples by AIMIL Pharmaceuticals India Limited, Ranjeet Nagar, New Delhi and authenticated by Dr. H.B. Singh-Chief Scientist and Head, Raw Materials Herbarium and Museum, National Institute of Science Communication and Information Resources, New Delhi vide certificate no. A/RM/2017/1352 (*A. paniculata*), A/RM/2017/1311 (*P. kurroa*), A/RM/2017/1383 (*S. marianum*).

Material and Methods

HPLC system (LC-2010 CHT series chromatographic system, Shimadzu, Japan), equipped with SPD-10 AVP UV–Visible spectrophotometric detector was used for the present study. All the chemicals and solvents used for present study were of analytical grade (AR) and are procured from the Merck (Mumbai, India) and SD Fine Chemicals (New Delhi, India). Water was obtained in the laboratory using (PURELAB Flex 2, ELGA) water purification system.

Physicochemical evaluation

The physicochemical parameters like ash values, extractive values, loss on drying (LOD) *A. paniculata*, *P. kurroa*, and *S. marianum* were determined as per the standard protocol of The Ayurvedic Pharmacopoeia of India (Anonymous, 2007).

Preparation of extracts

Each powdered (100 g) drug (*P. kurroa, A. paniculata* and *S. marianum*) was weighed using an analytical balance. The crude material of each drug was soaked in 700 ml of hydroalcoholic solvent (7:3, *v/v*) for overnight at room temperature followed by extraction using the reflux method for 8 hours at 60ºC. The extracts were then filtered. The filtrates were evaporated on a water bath (60°C) to get dried residues which were kept in airtight containers for further study (Farahmandfar *et al*., 2019; Mukherjee *et al*., 2008).

Sample and stock solutions preparation

The stock solutions of all samples were prepared along with reference marker compounds, picroside-I, andrographolide, and silybin. In brief, 1 mg of each sample and marker compound were dissolved in methanol and filtered through 0.22 µ PEP filter before analysis. Furthermore, all the drug samples were mixed in 1:1:1 *v/v/v* ratio to obtained mix sample.

Qualitative and quantitative analysis using HPLC method

Qualitative and quantitative analysis were performed for estimation of picroside-I, andrographolide, and silybin in *P. kurroa, A. paniculate*, and *S. marianum,* respectively, using HPLC system (LC-2010 CHT series chromatographic system, Shimadzu, Japan) which consisted of a Model LC-10 ATVP binary pump

Table 1. Physicochemical parameters of the *A. paniculata, P. kurroa* and *S. marianum.*

Plant name	Foreign matter (% *w/w*)	LOD (% *w/w*)	Total ash (% *w/w*)	Acid insoluble ash (% *w/w*)	Alcohol soluble extractive (% *w/w*)	Water soluble extractive (% *w/w*)
A. paniculata	0.02	6.99	7.88	2.13	12.3	25.56
P. kurroa	0.12	10.79	5.66	0.95	15.4	32.4
S. marianum	0.12	7.2	6.42	0.65	10.4	16.8

and equipped with SPD-10 AVP UV–Visible spectrophotometric detector. Class-VP 5.032 software was stated for routine drug analysis. The separation of compounds was conducted using C_{18} column (25 × 4.6 mm, 5 µ) at ambient temperature (30°C) by injection of 10 µl volume of respective solutions. Methanol and water in ratio of 50:50 *v/v* with 0.1% of formic acid in isocratic elution mode was used for conducting the analysis, and it was delivered at a flow rate of 0.7 ml minute^{-1}. The chromatographic separation time for the system was set up to 30 minutes. All the measurements were taken in triplicate to detect the accuracy of the detector response at every concentration level. Detection was carried out at 270 nm. Moreover, the calibration curve was plotted between obtained area and respective concentration (20–1,250 ng/inj) of each reference compounds. The validation parameters such as sensitivity, linearity, LOD, and limit of quantification (LOQ), precision, accuracy, and recovery of analytes were determined to validate the developed method as per referenced protocol (Dar *et al*., 2019; Zahiruddin *et al*., 2016).

RESULTS

Physicochemical evaluation

The results of the physicochemical parameters have shown in the Table 1.

Qualitative and quantitative analysis using HPLC method

Qualitative and quantitative validation of picroside-I, andrographolide, and silybin in *P. kurroa, A. paniculate*, and *S. marianum,* respectively, was performed successively using methanol and water (50:50 *v/v*) with 0.1% of formic acid at isocratic mode showed good separation and resolution. Any further changes in the ratio of methanol: water revealed either poor resolution or tailing. Besides that, for the combined extracts of all the drugs, the same established HPLC method was used. The experimental outcomes reveals that picroside-I, andrographolide and silybin were found to be 1.232 ± 0.0102, 0.572 ± 0.0734, and 2.037 ± 0.0635 µg/mg (Table 2) at the retention time 11.496, 19.452, and 27.027 minutes, respectively. The validation analysis of developed method revealed good linearity at the range 20–1,250 ng/inj with regression equation 757.91*x* + 11,153, 570.57*x* + 14,903, 871.18*x* + 23,419, regression coefficient 0.9966 ± 0.0005, 0.9983 ± 0.0007, 0.9984 ± 0.0003, respectively. The LOD and LOQ for the developed method for estimation of picroside-I, andrographolide, and silybin were found to be 3.639, 4.225, 3.895 ng/inj and 11.029, 15.329, 14.137 ng/inj, respectively. The LOD and LOQ observation reveals excellent sensitivity of the system with respect to the analytes. The inter-day and intra-day precision were determine with respect to percentage relative standard deviation (%RSD) and the results were expressed in the range which was found to be 0.860–1.268, 1.232–1.653, and 0.779–2.086 for inter-day precision and 0.753–1.975, 0.952–1.667, and 0.753–1.975 for intra-day precision. The results of inter and intra-day analysis demonstrated that the assessments was within the limit which represents good precision of developed method. Furthermore, the resulted outcomes form accuracy data suggest accurate and robust validation analysis which was confirmed based on percentage drug recovery found as 99.65%–101.43%, 98.75%–101.12%, and 99.79%–102.59%, respectively. The simultaneous estimation and determination of picroside-I, andrographolide, and silybin by HPLC method was validated, and found with all the specified parameters within the range. The validated method revealed the adequate separation of analytes in reference mixture with no merging of unknown peaks. It indicated the peak purity was good and thus verifies the method selectivity. Thus, the validation results revealed that the method developed is reproducible with adequate selectivity, sensitivity, and accuracy and precision. The experimental outcomes of validation method have been summarized in Tables 2 and 3 and the chromatograms are displayed in Figures 1 and 2.

Table 2. Reverse-phase high performance liquid chromatography quantification of picroside-I, andrographolide, silybin in the combined extract of all the plants.

Standard	Retention time (minute)	Concentration (µg/mg)
Picroside-I	11.496	1.232 ± 0.0102
Andrographolide	19.452	0.572 ± 0.0734
Silybin	27.027	2.037 ± 0.0635

Table 3. Method validation parameters for the quantification of picroside-I, andrographolide, and silybin by proposed HPLC method.

Parameters	Picroside-I	Andrographolide	Silybin
Scanning wavelength	270 nm	270 nm	270 nm
Linearity range (ng/inj)	20–1,250 ng/inj	20–1,250 ng/inj	20–1,250 ng/inj
Regression equation	757.91*x* + 11,153	570.57*x* + 14,903	871.18*x* + 23,419
Regression coefficient ± SD	0.9966 ± 0.0005	0.9983 ± 0.0007	0.9984 ± 0.0003
Slope ± SD	757.91*x* ± 2.845	570.57*x* ± 4.0183	871.18*x* ± 1.9175
LOD (ng/inj)	3.639	4.225	3.895
LOQ (ng/inj)	11.029	15.329	14.137
Interday Precision (%RSD range)	0.860–1.268	1.232–1.653	0.779–2.086
Intraday Precision (%RSD range)	0.753–1.975	0.952–1.667	0.753–1.975
Accuracy (% data recovered)	99.65%–101.43%	98.75%–101.12%	99.79%–102.59%

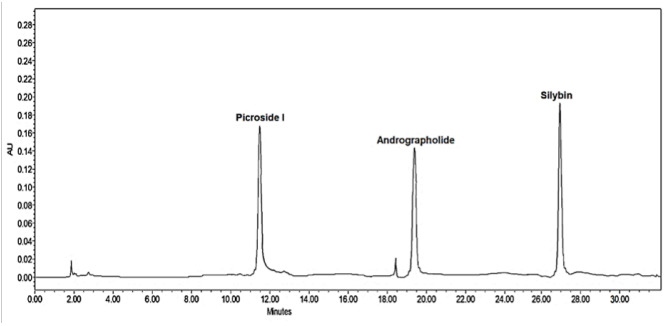

Figure 1. Simultaneous separation of picroside-I, andrographolide, and silybin through HPLC.

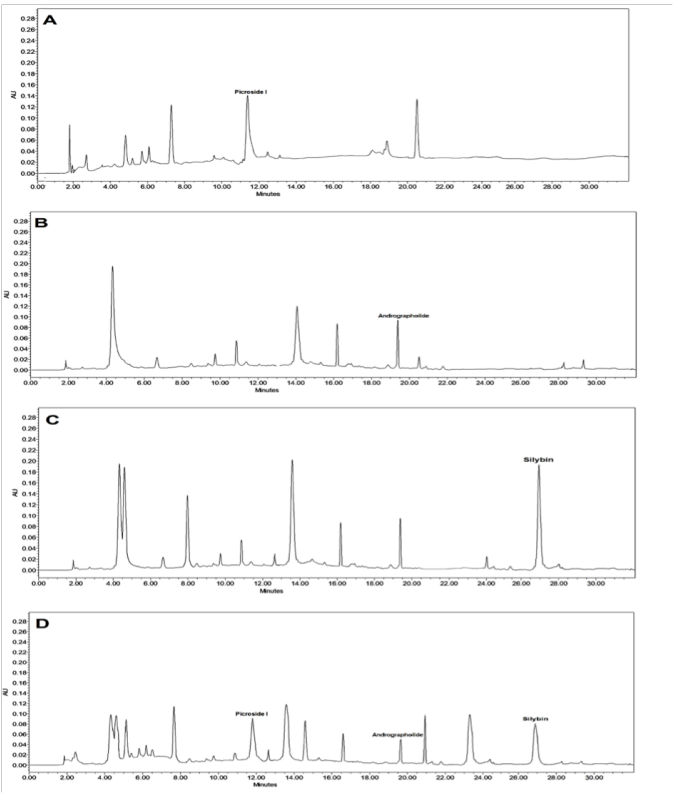

Figure 2. Represents HPLC chromatograms of various plant extracts, where (A) represents the chromatogram of *P. kurroa*, (B) represents the chromatogram of *Andrographis paniculata*, (C) represents the chromatogram of *S. marianum* and (D) represents the chromatogram of combined sample of all the drugs in 1:1:1 *v/v/v* ratio.

CONCLUSION

In the present work, a validated HPLC method has been developed and validated for simultaneous estimation and quantification of three components *viz*. picroside-I, andrographolide, and silybin from the combined extract, comprised kutki, kalmegh, and milk thistle extracts. The proposed method was revealed to be precise, accurate, simple, and robust although the time taken for silybin to completely elute out was long. The optimum value of HPLC system for chromatographic separation of silybin was set for 30 minutes, so that elution can be read. However, new method can be developed in future in reducing the elution time of silybin. This method can be used for the quantification and quality control of polyherbal formulations those contain *P. kurroa*, *A. paniculata*, and *S. marianum* as their ingredients. Furthermore, in future, synergistic studies on these three hepatoprotective plants can be taken into consideration for developing novel antihepatic formulations.

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CONFLICT OF INTEREST

The authors declare that they have no known competing conflict, financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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