Simultaneous Quantification by HPLC of Three Therapeutically Active Components Quercitin, Kaempferol & Naringin in the Ethanolic Extract of Merremia Emarginata (Burm.F)Hallier F. & Its Application as a Quality Control Tool

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***Abstract*— Merremia emarginata(Burm.f)Hallier f.commonly known as Akhuparni is a medicinal Plant belonging to the family Convolvulacea. The plants possess many proven biological activities and phytoconstituents is also used in marketed ayurvedic formulations. The present study reports the HPLC method which has been developed and validated for simultaneous quantification of Quercitin, Kaempferol & Naringin from extract of whole plant of M. emarginata and its formulation. Jasco 2000 series HPLC system was used with PDA detector for the experiment. The method involves use of a C18 column (Finpak, C18 25cm X 4.6 mm, 5µm) as a stationary phase. Mobile phase was gradient mixture of methanol and 0.1% Orthophosphoric acid with a flow rate of 1 ml/min wavelength used for simultaneous analysis is 270nm. The developed method was then validated in accordance with the ICH guidelines in terms of specificity, linearity, LOD, LOQ,precision and recovery.The validated method was successfully applied for quantification of three components in a reliving worm infestation "Krimighna Kashya" containing Ipomea reniformis extract. This method can also be used as a quality control tool for other formulations or dietary supplements containing the extract of M. emarginata.**

***Index Terms*— HPLC, Kaempferol, Naringin, Quercitin..**

I. Introduction

Today, alternative therapies have achieved astonishing popularity even though they are not encouraged by the U.S. Food and Drug Administration, which classifies most plant drugs as dietary supplements or food additives and places severe limitations on labeling. Also, unlike conventional drugs, herbal medicines are not regulated for their purity or potency. So, with the persistent trend of this latest green revolution, health care authorities around the world have found it a daunting task to promote the standardization of herbal medicines.

Merremia emarginata (Burm.f) Hallier f. also known as Ipomea reniformis(Roxb.) Chois which belongs to Convolvulaceae family is procumbent perennial herb spreading up to 1m & possess yellow coloured flowers. Mainly grows in rainy season & winter season. Its properties appear to be fanciful. The leaves are small, kidney shaped to somewhat heart shaped, 6–15mm long, often wider than long & irregular toothed. It is adulterated with Centella asiatica. It is reported to have many important medicinalproperties. In the Indigenous system of Medicine, Ipomoea reniformis has been claimed to be useful for cough, headache, neuralgia, rheumatism, diuretic, inflammation, troubles of nose, fever due to enlargement of liver and in kidney diseases. Powder of leaves is used as a snuff during epileptic seizures, Juice acts as purgative and the root is having diuretic, laxative, and applied in the disease of the eyes and gums. The whole plant decoction is mainlyresponsible for its medicinal uses. HPLC analytical techniques have been included in many of the latest monographs on the identification and determination of the plant constituents as well as the adulterants in herbal medicines. Although there are limitations for these techniques, using HPLC avoids the main problems associated with the use of gas chromatography such as thermal stability of components, their high molecular mass, compound destruction, and polarity of the compound.

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II. LITERATURE SURVEY

Validation of analytical methods is mandatory in implementing a quality control system in any analytical laboratory. It provides an assurance of reliability during normal use and can be referred as a process of providing documented evidence of quality for several herbal and traditional drugs. Separation techniques such as chromatography and electrophoresis have been extensively used for quality control of herbal medicine because of their high efficiency and speed.

Merremia emarginata (Burm.f) Hallier.F, commonly known as Akhurparni, is a is a procument herb spreading upto 1m and possess yellow coloured flowers. Mainly grows in rainy seasons and winter season. Its properties appear to be fanciful. Akhuparni is commonly seen throughout country as a weed, including plain as well as hill grows especially in rainy season.[1]

In recent years, there has been a growing interest in the chemical composition and biological activities of *M.*

emarginata. The therapeutic value of the plant depends on the active constituents present in the small or large quantity. The secondary metabolites are the important substance responsible for the main medicinal properties in the crude drugs. Little work is done in the field of phytochemical investigation of the plant. Shah et al., have showed the presence of resin, glycosides, reducing sugars and starch while alkaloids are absent. Petroleum ether extract was reported to contain fats and fixed oil while aqueous extract was reported to contain amino acids, tannins (condensed tannins, pseudo tannins), and starch. Chemical investigation of *Ipomoea reniformis* shows the presence of caffeic, pcoumaric, ferulic and sinapic acid esters identified in seeds. But very less work has been done in phytochemical analysis of whole plant M. emarginata majorly biological activity of plant has been studied for anticancerous, anti-inflammatory, antioxidant properties, etc [3]. Hence as the isolation and characterisation of compounds from M. emarginata has been performed. In view of these wide therapeutic effects a need was felt for simultaneous quantitation of the compounds identified are being for checked for the presence in marketed formulation of the plant. In this research work, a simple, precise, and accurate HPLC method has been established for simultaneous quantitation of Quercitin, Kaempferol & Naringin in the dried plant powder of *M. emarginata*. Further, the proposed method has been validated as per ICH guidelines and applied as a quality control tool for standardization of a commercially available *relieving worm infestation named "Krimighna Kashya"*. Such a study would not only facilitate standardization of the raw material and commercial products but also facilitate future pharmacological studies and quality control.

III. EXPERIMENTAL

A. Chemicals and Reagents

HPLC grade methanol, and water were procured from E.Merck, Mumbai, India.

Reference standards of Naringin (purity >97%) , Quercitin (purity >97%) & Kaempferol (Purity > 90%) were purchased from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany).

B. Plant Material

Whole plants of *Merremia emarginata* (Burm.f.) Hallier F. were collected from Saphale in Palghar district, near railway station, Maharashtra, India in the month of September. The plant was authenticated, and voucher specimen no. HS-1 was deposited in The Botanical Survey of India, Pune, India. Collected whole plants were washed with tap water and air-dried thoroughly under shade at room temperature for 2 weeks to avoid direct loss of phytoconstituents from sunlight. The shade dried materials were powdered using grinder and sieved through an ASTM 80 mesh. It was then homogenized to fine powder and stored in an air-tight container for further analysis.

C. Formulation

Relieving worm infestation "**Krimighna Kashya**" [1],ayurvedic formulation was procured from Nagarjuna pharmaceuticals, which mentions each 10ml contains 10mg of I.reniformis; B.No 149.

D. Sample Preparation

The powdered plant material weighing 5gm of M. emarginata was introduced in JASCO Make Super critical extraction. Flow rate of CO2 was at 5.0mL/min, ethanol was used as modifier at 0.8mL/min. BPR was kept maintaining pressure at 15MPa. And each extraction was carried out for 30mins. The extracts were collected in 8ml glass tubes provided with system, the CO2 gets evaporated easily and ethanolic extract was further diluted 10times with Methanol and subjected to HPLC for quantification.

E. Formulation

For analysis of the krimigana kashya syrup, 5gm of syrup was weighed & taken into a 100ml volumetric flask. 40 mL of ethanol was added to the flask and the mixture was sonicated on ultra sonictor for about 5 mins. Then volume was make upto 100ml ethanol, The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India).

F. Preparation of Stock Solutions

Standard stock solutions of pure compounds were prepared separately by dissolving 10 mg of each compound in 10 mL of methanol to get concentration of 1000 µg/mL. For calibration curve, aliquots of 0.5-600 µg/mL, 1.0-600 µg/mL and 2.0-500 µg/mL were prepared from the above stocks for Naringin, Quercitin & Kaempferol, respectively. Also three quality control levels (LQC, MQC, HQC) each of Naringin (1.0, 100, 400 µg/mL), Querciti (2.0, 100, 400 µg/mL), and Kaempferol (3.5, 100, 400 µg/mL) were prepared for precision, accuracy and ruggedness studies.

G. Chromatography

A sensitive and accurate high-performance liquid chromatographic (HPLC) method has been developed, the method developed is such that the compounds in ethanolic extract are well resolved a simple, reproducible and efficient reverse phase high performance liquid chromatographic method has been developed. Jasco LC-2000 system was used for the experiment. The method involves use of a C18 column (Finpak, C_{18} 25cm X 4.6 mm, 5µm) as a stationary phase. Column oven temperature was 25 ^{0}C & Jasco MD-2010 PDA detector was used as it will help us to heck purity of compound isolated. Mobile phase was gradient mixture of methanol and 0.1% Orthophosphoric acid with a flow rate of 1 ml/min wavelength used for simultaneous analysis is 270nm.

For calibration curve, solutions from 0.5-600 µg/mL, 1.0-600µg/mL and 2.0-500 µg/mL were prepared from the above stocks for Naringin, Quercitin and Kaempferol, respectively. Also three quality control levels (LQC, MQC, HQC) each of Naringin (1.0, 100, 400 µg/mL), Quercitin (2.0, 100, 400 µg/mL) and Kaempferol (3.5, 100, 400 µg/mL) were prepared for precision, accuracy and ruggedness studies.

The Method of analysis is already optimised for isolation of compounds and same method is used for quantification of compounds by HPLC. The method involved in analysis and

validation is as follows. Gradient programming method of HPLC is as follows

Time	% Orthophosphoric	Methanol
0.0	30	70
5.0	30	70
5.1	25	75
15.0	25	75
15.1	30	70

IV. VALIDATION OF THE METHOD

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method [17].

A. *Selectivity and Specificity*

During the experiments, an UV scan ranging from 200 to 400 nm in the time window of the analytes using PDA detector was performed with the aim of revealing eventual interfering compounds and evaluating the selectivity of the method. Specificity of the intended method was established by comparing the HPLC retention time and absorption spectra of target peaks from the analysed samples with those of the reference compounds.

B. *System suitability*

System suitability experiment was performed by injecting six consecutive injections (50 µg/mL) of each bioactive marker, namely Naringin, Quercitin and Kaempferol during the start of the method validation. Values with % CV of ≤2% were accepted.

C. *Calibration curves*

Linearity of the components was determined in triplicate at eight different concentrations for Naringin and seven different concentrations for Quercitin and Kaempferol, respectively. Calibration curve was plotted as mean peak area versus concentration. The linear regression equation was obtained using a least-square method and used to estimate the concentration of the three components in the analysed samples. RSD of standard peak areas for solutions of the same concentration were less than 2%, indicating there was no statistically significant variation.

D. *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

Sensitivity of the method was evaluated by determining the values of LOD and LOQ. Stock solution of each standard was serially diluted with methanol to prepare the series of samples with least concentration and injected into the HPLC system. The limit of detection (LOD) and quantification (LOQ) for Naringin, Quercitin and Kaempferol were determined by measuring the signal to noise ratio (S/N). LOD and LOQ was considered at S/N of 3:1 and 10:1 respectively.

E. *Precision*

Variability of the method was studied by analyzing quality control samples of Naringin (1.0, 100, 400 µg/mL), Quercitin (2.0, 100, 400 µg/mL) and Kaempferol (3.5, 100, 400 µg/mL) on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy values within the range of 85 – 115% and % CV of ≤2% were accepted.

F. *Recovery*

Recovery tests were carried out to further investigate the accuracy of the method by adding three different concentration levels of the mixed standard solutions to known amounts of M.emarginata samples and formulation prior to extraction. The resultant samples were then extracted and analyzed with the described method. The mean percentage recoveries were calculated using the formula: Recovery (%) = [(amount found – original amount) / amount added] x 100. Values within the range of 85 – 115% were accepted.

G. *Ruggedness*

Ruggedness of the method was studied by determining the effects of small variations of mobile phase composition (±2%), and flow rate (1.00 ± 0.05 mL/min). Effect of these deliberate changes on the response (area) and retention time of QC samples of Naringin, Quercitin and Kaempferol was observed during the analysis. The results were expressed in terms of % mean difference. Values within a difference range of ±5% were accepted. Stability The stability of the stock solutions of all the three standards was evaluated by storing the solutions in refrigerator at 2-8oC for 72 hours and then comparing the results against freshly prepared stocks for each standard. Samples in triplicate were also subjected to bench top stability at 0.0 h and 6.0 h respectively. Values within a difference range of ±5% were accepted.

V. ESTIMATION OF NARINGIN, QUERCITIN & KAEMPFEROL IN M. EMARGINATA PLANT POWDER AND AYURVEDIC FORMULATION CONTAINING M. EMARGINATA PLANT POWDER.

The extract of M.emarginata and Krimigana kashya syrup were injected seven times separately and analysed using the optimized chromatographic conditions. Peak areas were recorded for each analyte of interest and the amount of all the three analytes (Naringin, Quercitin & Kaempferol) was calculated by use of the calibration plot.

VI. RESULTS AND DISCUSSION

Initial trial experiments were conducted to select a suitable mobile phase for accurate analysis of the standards. Of the various mobile phases tried in both isocratic and gradient mode, Methanol and 0.1% orthophosphoric acid in gradient mode gave the best resolution between the three .These standards were also resolved from other components present in the sample extract enabling simultaneous quantification. HPLC chromatograms corresponding to the three standards, M.emarginata leaf extract are represented in Figure 1 & 2. All the analytes exhibited he UV maximum absorption at 270 nm and hence this wavelength was chosen for the detection of components eluting out from the column.

A. *Method Validation*

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method

Selectivity and Specificity: During the UV scan no appreciable difference was found in the spectra of reference standards and the analysed samples. Hence, the method demonstrated a high degree of selectivity.

System suitability: System suitability tests are used to verify whether the resolution and reproducibility of the chromatographic system are adequate for the analysis. For Naringin, Quercitin and Kaempferol the %CV values for area and retention time was found to be <2% indicating that the system was suitable to carry out further analysis.

Linearity: The method was found to be linear from 0.5-600 µg/mL for Naringin, 1.0- 600 µg/mL for Quercitin and 2.0-500 µg/mL for Kaempferol respectively. The correlation coefficient was found to be ≥0.995 for all the three components. Results of regression analysis are summarized in Table 1.

Sensitivity: Sensitivity of the method was affirmed in terms of LOD and LOQ for Naringin, Quercitin and Kaempferol, respectively. The values for both LOD and LOQ were low enough as per ICH guidelines, which indicated that the method is capable of detecting and quantifying trace amount of the three components in plant samples.

Precision: In the repeatability study intra-day and inter-day precision of the HPLC method were investigated using replicate injection (n=3) of quality control samples of all the three standards. The developed method was found precise with % CV<2%.

Stability: Stock solution stability study of all the three standards stored for the period of 72 hours at 2-8°C showed % CV <2% with the % mean difference within ±5%. During bench top stability study, similar results were obtained. Stability studies showed that Naringin, Quercitin and Kaempferol were found stable for at least 6.0 h at room temperature and 72 hours at 2-8°C of storage condition.

Recovery: The recovery values for all the three components were within acceptable limits (85.0 to 115.0%). This indicated that the method was reliable and accurate.

Ruggedness: Proposed method was not influenced by the factors considered for ruggedness study. Change in flow rate and mobile phase composition affected the retention time of the three analytes but the results were satisfactory since % CV was < 2%.

B. Assay:

The assay value for samples of M. emarginata plant powder was found to be 0.32%, 0.06 % and 0.78% for Narignin, Quercitin and Kaempferol respectively, while for the formulation it was found to be 0.92 %, 0.29% and 0.33% for Narignin, Quercitin and Kaempferol respectively. The method is specific for all the three components because it resolved all standards well in the presence of other phytochemicals in M.emarginata. The proposed HPLC method was found to be suitable for qualitative and simultaneous quantitative analysis of Narignin, Quercitin and Kaempferol in the methanolic extract of M.emarginata.

VII. CONCLUSION

A precise, accurate and reproducible HPLC method is validated for simultaneous quantification of three bioactive markers Naringin, Quercitin and Kaempferol. Proposed HPLC method can be used as an analytical tool for quality evaluation of plants and formulations containing Naringin, Quercitin and Kaempferol as chemical markers. It is an efficient method to screen M.emarginara samples in order to assess its quality and authenticity. Hence, it can be demonstrated that HPLC is a powerful practical tool for comprehensive quality control of plant raw materials and its formulations.

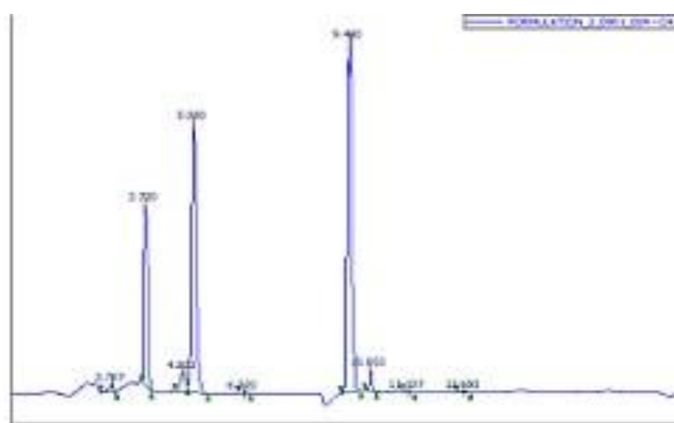

Fig 1 – A typical chromatogram of standard Naringin, Quercitin and Kaempferol.

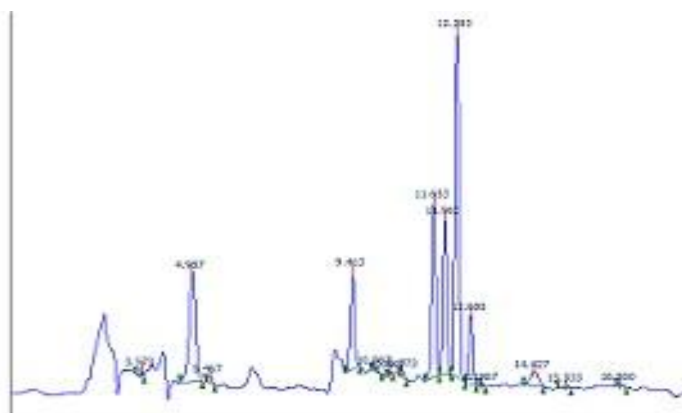

Fig 2 – A typical chromatogram of Plant extract M.emarginata

Table 1: The regression data, linearity, LOD and LOQ and retention times for three bioactive compounds by HPLC

Compounds	Retention time	Regression equation	r2	Linear range(µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Naringin	4.891	y = 4540.x + 1048	0.9997	0.5-600	0.3	0.5
Quercitin	9.387	y = 2588.x + 8834	0.9998	1.0-600	0.2	0.5
Kaempferol	10.040	y = 1618.x + 6114	0.9995	2.0-500	0.3	1.0

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