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Development and Validation of HPLC Method for Charantin from Momordica Charantia Linn

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KEYWORDS

HPLC, Momordica charantia Linn, Charantin, Herbal formulations.

ABSTRACT:

A simple, sensitive, precise, rapid, and reliable HPLC method for the estimation of charantinin *Momordica charantia Linn* as well as in herbal formulation was developed. Charantin were successfully isolated from respective medicinal plants namely charantin (*stigmasterolglucoside* and *β-sitosterolglucoside*). The purity of isolated Charantin was checked by HPLC. HPLC methods were developed and optimized for various parameters such as column (stationary phase), column parameters, mobile phase composition, flow rate of mobile phase, column oven temperature, detection wavelength and injection volume. These developed and validated HPLC method justify their application for quality control of herbal extracts and formulations. These methods can be employed to determine batch to batch variations and routine analysis of herbal formulations by herbal manufacturers.

1. Introduction

Brief About Herbal Medicine:

Different diseases are cured by medicinal plants and their extracts since old ages by humans.[1] Among all healthcare systems Ayurveda is oldest healthcare system which is practicing in entire world in Ayurveda we use different medicinal plants to cure various diseases.[2] As we know the medicinal plants and their parts such as seeds, fruits, roots, leaves barks etc.[3] were used in several preparations to cure different types of diseases such as preparations are known as herbal medicines. According to WHO, Herbal medicines are described as active substances that are component parts of plants or material from plants in their raw or processed condition that have therapeutic action.[4,5] WHO reports a yearly demand of nearly US$14 billion for herbal medicines. Anticipating future needs, by 2050, the global demand for herbal medicines is estimated to exceed US$5 trillion, highlighting the vital role of herbal remedies in global healthcare. India has rapidly emerged as a leading global producer of herbal medicines, building on its rich historical tradition of herbal remedies. The country is now recognized as the

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fastest-growing market leader in the worldwide herbal medicine industry.[6]

As per current health care trend, herbal medicines have reputed place across all over the world in developed and under developing countries. Current researches from various countries indicates a significant trend: approximately 80% of people now prefer herbal medicines for their basic healthcare needs due to its lesser side effects as compared to allopathic medicines. Herbal medicines are very affordable also in allopathic there are non-availability of medicines for the diseases like memory loss, Age related diseases such as diabetic wounds, immune and liver disorder etc. India encompasses 2.4% of the world's geographical area and 8% of world's biological diversity. Over 1.5 million healthcare practitioners in India use herbal (traditional) medical methods to treat a wide range of illnesses. Approximately 25,000 herbal formulations are used as traditional medicine. According to published data, there are currently about 7800 herbal production units in India. In addition, India needs about 2000 tons of raw medical plant material annually, while the United States and Europe import a significant amount of standardized herbal extracts from India. The majority of these herbal extracts are exported from India to the USA and Europe for use as dietary supplements. [7-9]

Numerous medicinal plants and major pharmaceutical organizations engaged in research fields such as pharmacognosy, chemistry, pharmacology, and clinical therapeutics have enhanced their strategies to promote the discovery of drugs derived from natural products.[10]

2. Materials and Methods

Reagents And Instruments

The solvents and chemicals utilized in this experiment were all of HPLC and AR standards. Chemical such as: methanol, acetonitrile, water, isopropyl alcohol, trifluoro acetic acid, glacial acetic acid, phosphoric acid, formic acid, triethyl amine, Triss buffer, ammonium acetate buffer, are of HPLC grade. Chemical such as: methanol, strong ammonia solution (25 %), diethyl ether, chloroform, ethyl acetate, toluene, petroleum ether (600-800 C), isopropyl alcohol, glacial acetic acid, formic acid, concentrated hydrochloric acid, concentrated sulfuric acid, activated charcoal, sodium sulphate and potassium hydroxide are of AR grade. All the above mentioned chemicals and standard compounds of charantin were procured from Nectar Herbs and Drugs, Sidcul (Sigaddi Groth Center), Kotdwar, Uttarakhand.

For detection at 210nm, an HPLC system consisting of LC-10 ATVP Pumps, SIL-10 ADVP auto injector, SCL-10 AVP system controller and SPD-M 10 AVP photodiode array detector was employed. The column used Phenomenex Luna C18, 5 m (2504.6 mm) at ambient temperature. The HPLC apparatus was calibrated and integrated for all relevant parameters using Class-VP software.

Selection of Medicinal Plants

A suitable medicinal plant was chosen based on a survey of the literature, as well as the plant's pharmacological and therapeutic properties. The chosen plant materials were collected from four different parts of India: Gujarat, Uttarakhand, Tamil Nadu and Uttar Pradesh. The plant samples obtained were certified by the Forest Research Institute (FRI) in Dehradun. AAll of the plant material used in this experiment has been dried in the oven at 60 degrees Celsius, crushed, and kept until needed.

Isolation of Compound/Bioactives

Solvent extraction, column chromatography, preparative HPLC, and recrystallization were used to isolate the samples.

HPLC Method Development

An HPLC (High-Performance Liquid Chromatography) method was developed for isolated or purchase compound. The technique involves parameters like wavelength detection, analytical column and its dimensions, mobile phase composition, mobile phase flow rate, elution mode, oven temperature, runtime, and so on.

HPLC Method Validation

Every established technique underwent validation in accordance with ICH guidelines, encompassing assessments for accuracy, precision, linearity, system suitability, specificity, robustness, limit of detection (LOD), and limit of quantification (LOQ).

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Specificity: It refers to the method's capacity to unambiguously measure the analyte in the presence of additional elements. The purity of the analyte compound of interest was tested using a photodiode array to rule out any interference from a blank or sample.[11]

System suitability: The assessment included the evaluation of system suitability parameters, which encompassed Tailing factor, resolution, number of theoretical plates, and injection precisio[12]

LOD: The Limit of Detection (LOD) was assessed utilizing the Signal-to-Noise (S/N) ratio method. Analysis was introduced at progressively increasing concentrations until a Signal-to-Noise ratio of 3:1 was attained.[13]

LOQ: The Limit of Quantification (LOQ) was determined through the Signal-to-Noise (S/N) ratio method. The analysis involved injecting the substance at ascending concentrations until a Signal-to-Noise ratio of 10:1 was reached.[14]

Linearity and range: Linearity was assessed across the designated range of test concentrations, and the correlation coefficient (r2) was determined.[15]

Accuracy: The precision of the established approach was determined by introducing the analyte standard into the sample at three varying concentrations, followed by the calculation of the percentage recovery of the analyte.[16]

Precision: The method's precision was assessed through both intraday and interday precision studies. Precision examinations were conducted at three different concentration levels by replicating injections within a single day and on multiple days. For each concentration level, triplicate sample injections were performed, and the percent relative standard deviation (% RSD) was determined.[17]

Robustness: The method's robustness was evaluated by modifying key parameters, observing their effects on retention time, area, and % w/w of the analyte. Under each adjusted condition, six injections of the standard solution and two injections of the sample were conducted. The percent relative standard deviation (% RSD) of the area from the six standard solution injections was calculated for each variable.[18]

Standardization of Extracts and Ayurvedic Formulations

The methods developed and validated were employed to quantify each marker compound or bioactive in diverse extracts and various Ayurvedic formulations, including Taila, Kadis/pills, herbal tea, churna, etc. Additionally, the methods were applied to modern dosage forms such as capsules, tablets, gel, granules, syrup, etc.

Isolation of Charantin from Fruits of Momordica charantia

Procurement of Momordica charantia fruit

Fruits of Momordica charantia were acquired from three geographically different locations:

Sample 1: Momordica charantia were gathered from Bijnor, Uttar Pradesh in April 2020.

Sample 2: Momordica charantia were gathered from Dehradoon Uttrakhand in April 2020.

Sample 3: Momordica charantia were bought from a nearby local marketplace of Bijnor, Uttar Pradesh in April 2020.

Extraction of charantin

Fresh Momordica charantia fruits were obtained, dried, and pulverized. A quantity of 20 g of the powder was successively extracted in a Soxhlet apparatus, first with 200 ml of petroleum ether (60°C - 80°C), followed by extraction with 200 ml of ethanol. The extract obtained with petroleum ether was eliminated, and the obtained ethanol extract was utilized for the isolation of charantin.[19]

Isolation of charantin

The ethanol extract underwent concentration to achieve a reduced volume, followed by saponification using KOH. The ethanolic solution was diluted with water and extracted numerous times using diethyl ether after 48 hours to isolate the non-saponifiable portion. The ether extract underwent successive washing with water, HCl, and water. Discard the aqueous and acidic washing. Subsequently, the ether extract was dried using anhydrous sodium sulfate. Allow the ether extract to stand for overnight to give a colorless compound (25

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mg).[20]

HPLC Studies of Isolated Charantin

The isolated charantin's HPLC profile was generated using the chromatographic conditions listed below:

Column: Symmetry C1 S
Column Dimensions: 75 mm X 4.6 mm, 3.5pm
Mobile Phase: Methanol: water (98:02, v/v)
Flow Rate: 0.4 ml/min
Detection Wavelength: 204 nm
Temperature: 25 °C
Injection Volume: 5.0 pl

The reversed-phase HPLC technique was employed to conduct studies on isolated charantin, and the results were compared with a standard. The comparison is presented in Figures 6.4 and 6.5.

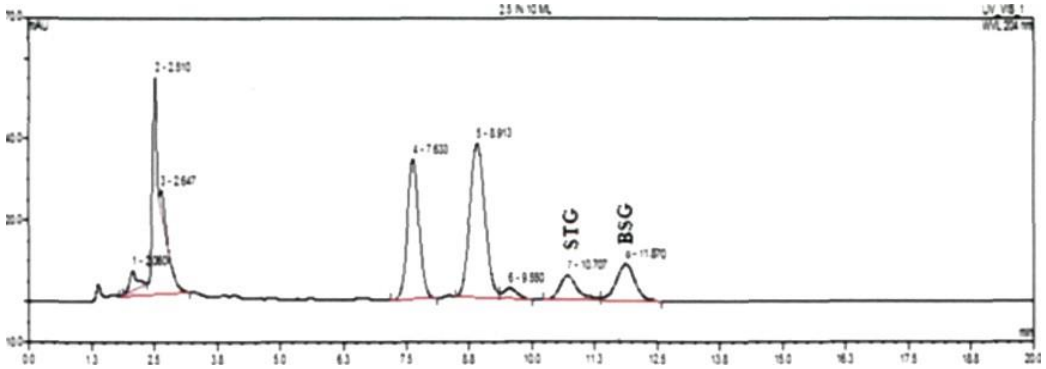

Fig. 1: HPLC chromatogram of standard charantin

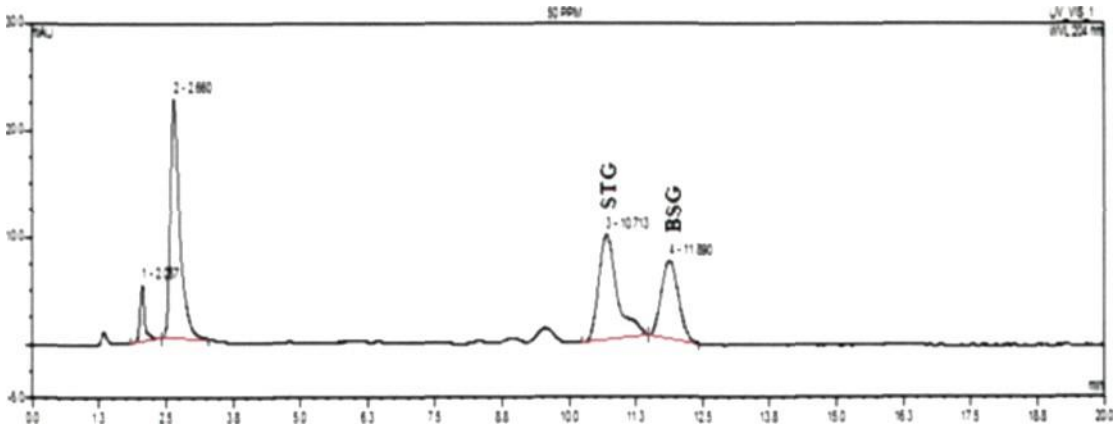

Fig. 2: HPLC chromatogram of Test

Purification of Charantin

The crude charantin produced through the solvent extraction procedure was purified further using preparative HPLC under the chromatographic conditions listed below:

Column: Unisphere Aqua C 18
Column Dimensions: 250 mm X 20 Um, 10 pm (Porc size 100 A")
Mobile Phase: Methanol: water (98:02, v/v)
Flow Rate: 15 ml/min
Detection Wavelength: 2 10 nm
Injection Volume: 5.0 ml

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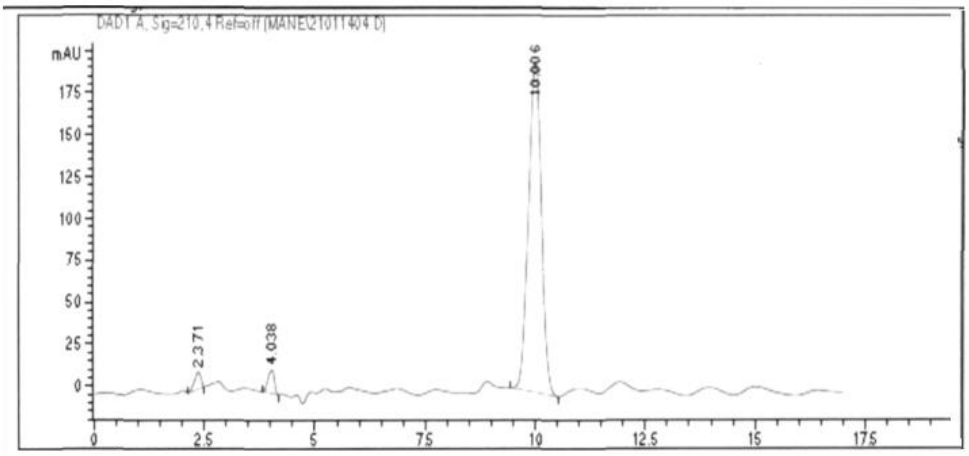

Fig. 3: HPLC chromatogram of fraction I V

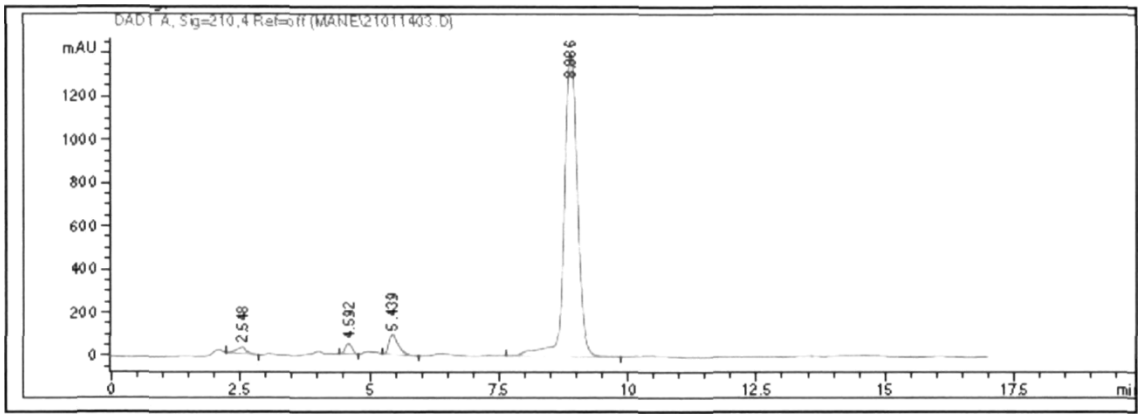

Fig. 4: HPLC chromatogram of fraction V

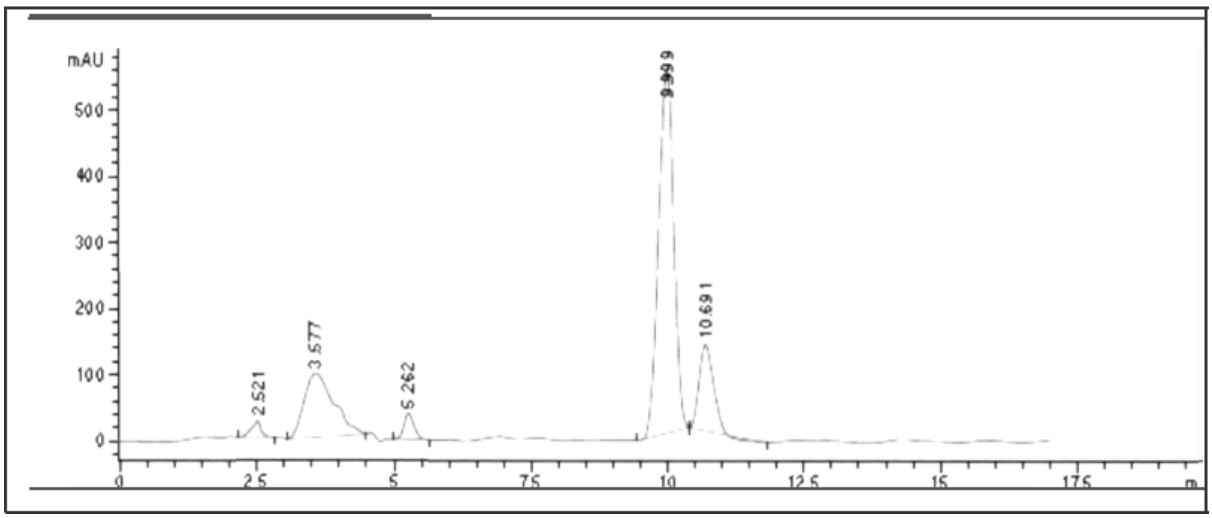

Fig. 5: HPLC chromatogram of fraction VI

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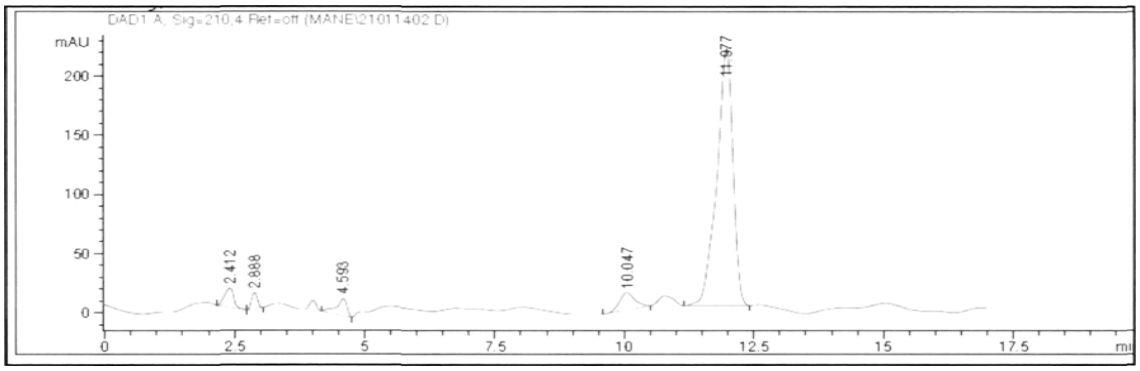

Fig. 6: HPLC chromatogram of fraction VII

Development and Validation of HPLC Method for Charantin

As a result, the optimum chromatographic parameters for charantin analysis were as follows:

Column: Symmetry C 18

Column Dimensions: 75 mm X 4.6 mm, 3.5pm

Mobile Phase: Methanol: water (98:02, v/v)

Flow rate: 0.4 ml/min

Detection Wavelength: 204 nm

Temperature: 25 °C

Injection Volume: 5.0 pl

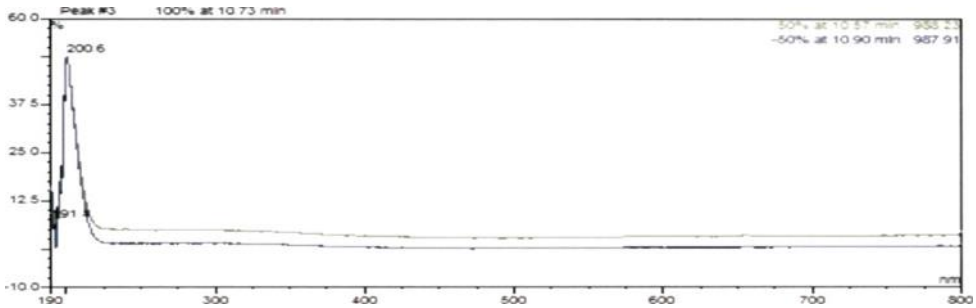

Fig. 7: HPLC Spectrum of stigmasterolglucoside

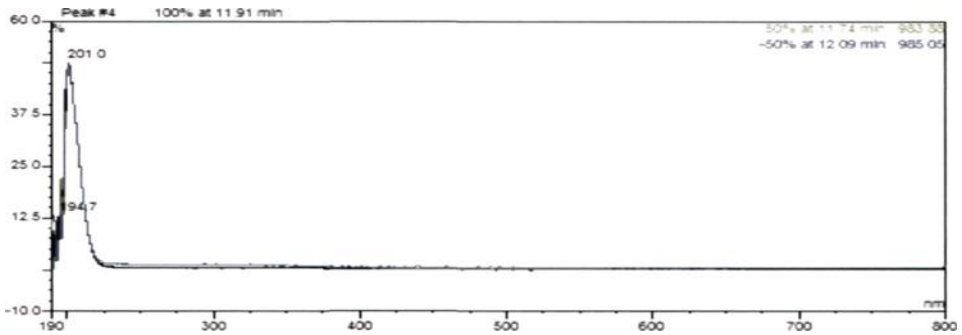

Fig. 7: HPLC Spectrum of β-sitosterolglucoside

Fig. 8: Overlay HPLC spectra of stigmasterolglucoside and β-sitosterolglucoside

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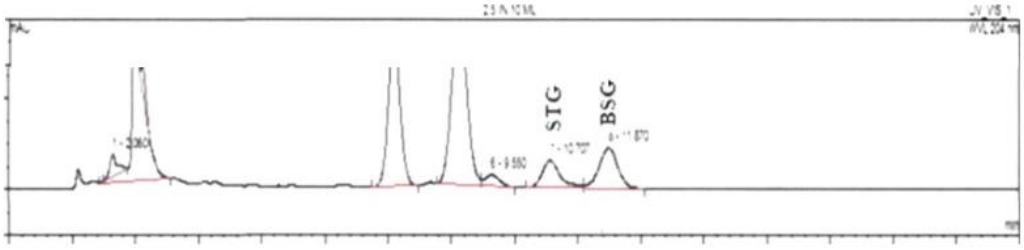

Fig. 9: HPLC chromatogram of standard charantin

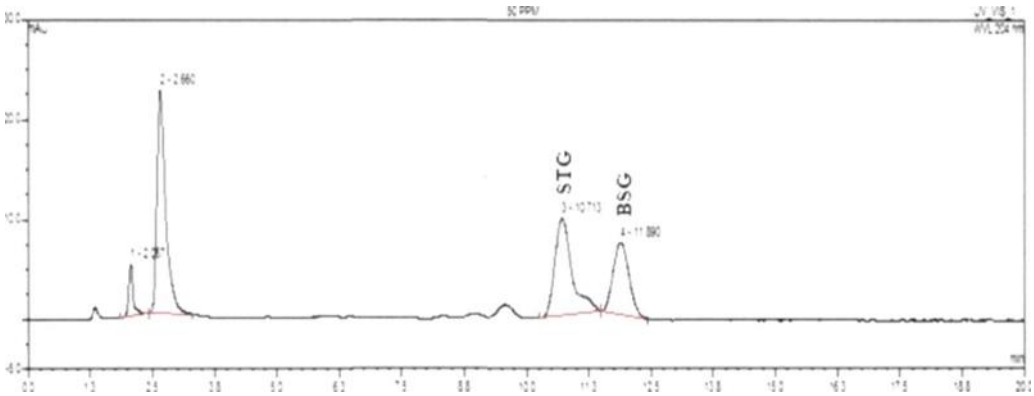

Fig. 10: HPLC chromatogram of semi-purified extract of *Momordica charantia*

3. Results And Discussion

Charantin were successfully isolated from respective medicinal plants namely *Momordica charantia* (*stigmasterolglucoside* and *β-sitosterolglucoside*). The purity of isolated Charantin was checked by HPLC. HPLC procedures were created and optimized for a variety of variables, such as Column (stationary phase), column parameters, composition of the mobile phase, flow rate of the mobile phase, temperature of the column oven, detection wavelength, and injection volume.

Most of the gradients employed in the study utilized binary gradients, which consisted of varying percentages of acetonitrile in acidic water. Each established method underwent validation in accordance with ICH guidelines, assessing various parameters including, accuracy, system suitability, LOQ, linearity specificity, range, LOD, precision and robustness and results of the same were given in the tables below. All the methods demonstrated linearity,robustness, precision, accuracy and sensitivity.

Table 1: Results of system suitability parameters for STG and BSG

Conc. In pg/ml	Retention time (Rt) in min		Area		Peak asymmetry		Number of Theoretical plates(N)		Resolution
	STG	BSG	STG	BSG	STG	BSG	STG	BSG	
50	10.673	11.843	3.9495	2.6484	1.56	1.11	5772	6386	2.03
50	10.690	11.860	3.9716	2.6667	1.55	1.11	5744	6319	2.02
50	10.713	11.890	3.9646	2.6786	1.56	1.12	5757	6327	2.03
50	10.730	11.907	3.9893	2.6806	1.55	1.12	5775	6363	2.03
50	10.733	11.910	3.9971	2.6694	1.57	1.12	5767	6379	2.03
50	10.730	11.907	3.9671	2.6864	1.58	1.13	5787	6315	2.03

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Extract	10.707	11.870	2.4921	3.8471	1.58	1.13	5300	5016	1.85
Extract	10.703	11.867	2.4635	3.8046	1.58	1.13	5332	5008	1.86
RSD			0.4354	0.5053					

Linearity and range

Table 2: Results of linearity studies for STG and BSG

Concentration (pg/ml)	Retention Time (min)		Area		Average area	
	STG	BSG	STG	BSG	STG	BSG
12.0	10.710	11.874	0.6262	0.4510	0.5714	0.4612
12.0	10.709	11.875	0.5167	0.4714		
12.5	10.703	11.877	0.7010	0.6328	0.7085	0.6279
12.5	10.687	11.853	0.7160	0.6230		
25.0	10.710	11.880	1.8690	1.2086	1.8790	1.2162
25.0	10.700	11.880	1.8891	1.2238		
37.5	10.703	l 1.880	3.0350	2.0405	3.0228	2.0328
37.5	10.727	11.907	3.0106	2.0251		
50.0	10.737	11.913	3.8857	2.5632	3.9200	2.5693
50.0	10.730	11.903	3.9543	2.5754		
62.5	10.767	l 1.947	5.0276	3.2950	5.0219	3.2870
62.5	10.737	11.913	5.0163	3.2790		
75.0	10.767	11.947	5.7413	3.7423	5.7473	3.7357
75.0	10.740	11.917	5.7534	3.7292		
100	10.777	11.960	8.2106	5.2217	8.1234	5.1924
100	10.797	11.980	8.0363	5.1632		

Accuracy:

Table 3: Results of accuracy studies for STG and BSG

Amount present (pg./ml) ± SD		Amount Found (pg./ml) ± SD		% Recovery ± SD	
STG	BSG	STG	BSG	STG	BSG
40.46 ± 0.02	37.8l ± 0.09	37.94 ± 0.31	37.42 ± 0.44	93.78 ± 0.76	98.95 ± 1.18
48.32 ± 0.04	48.72 ± 0.08	49.83 ± 0.20	48.76 ± 0.22	103.12 ± 0.50	100.08 ± 0.60
57.34 ± 0.11	59.20 ± 0.26	57.56 ± 0.25	57.85 ± 0.27	100.38 ± 0.51	97.7l ± 0.37

n=3, triplicate injections

Precision:

Table 4: Results of intraday and interday precision studies for STG and BSG

Component	Amount level (mg/10ml)	Intraday (% RSD)				Interday (% RSD)	
		Day 1	Day 1	Day 1	Day 1	Day 2	Day 2
		STG	BSG	STG	BSG	STG	BSG
BSG/STG	2.0	0.9840	0.0068	0.9164	0.9669	0.8186	0.7136
	2.5	0.6095	0.7051	1.2084	0.9580	1.7122	0.8925
	3.0	0.8396	0.9843	0.6238	1.0686	0.3226	0.2391

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n=3, triplicate injections

Robustness:
sample (2.5 mg in 10 ml of methanol) were applied.

Table 5: Results of robustness study for STG

Parameters	Mean Retention time (min)	Mean area	%RSD of area	% w/w of STG
Mobile phase composition (v/v)				
Methanol: water (97.5:2.5)	12.472	4.2912	0.4753	11.5958
Methanol: water (98:02)	10.711	3.9732	0.4354	11.8614
Methanol: water (98.5:1.5)	11.814	4.2472	0.5533	11.8109
Flow rate (ml/min)				
0.3	14.240	5.3533	0.4053	11.8721
0.4	10.711	3.9732	0.4354	11.8614
0.5	8.607	3.1595	0.3070	12.3481
Column oven temperature (°C)				
24	11.042	3.9757	0.3018	11.8668
25	10.711	3.9732	0.4354	11.8614
26	10.460	4.0344	0.2230	11.8931
Detection wavelength (nm)				
203	10.711	4.3711	0.4735	11.6145
204	10.711	3.9732	0.4354	11.8614
205	10.711	3.5740	0.4364	12.1740

n=6, six injections

Table 6: Results of robustness study for BSG

Parameters	Mean Retention time (min)	Mean area	% RSD of area	% w/w of BSG
Mobile phase composition (v/v)				
Methanol: water (97.5:2.5)	13.914	2.8499	0.5438	27.2133
Methanol: water (98:02)	11.886	2.6716	0.5053	28.0677
Methanol: water (98.5:1.5)	13.157	2.8681	0.4114	27.6625
Flow rate (ml/min)				
0.3	15.804	3.5933	0.3757	27.9700
0.4	11.886	2.6716	0.5053	28.0677
0.5	9.551	2.1058	0.4748	28.2287
Column oven temperature(°C)				
24	12.258	2.6803	0.2649	28.3970
25	11.886	2.6716	0.5053	28.0677
26	11.606	2.7044	0.1627	28.2853
Detection wavelength (nm)				
203	11.886	2.8772	0.4796	27.6840

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204	11.886	2.6716	0.5053	28.0677
205	11.886	2.4592	0.4432	28.3901

n=6, six injections

Stability studies

Table 7: Stability studies of STG in extract containing *Momordica charantia*

Extract	**Temperature**					
	4°C			**25 °C**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
% of STG in extract of *Momordica charantia*	97.71	96.52	96.60	98.47	97.59	99.02

Table 8: Stability studies of BSG in extract containing *Momordica charantia*

Extract	**Temperature**					
	4 °C			**25 °C**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
% of BSG in extract of *Momordica charantia*	99.18	98.88	99.66	98.44	98.68	98.06

Quantitative analysis of extracts and formulations containing *momordica charantia* for charantin content

Table 9: Percent content of STG and BSG in extracts and formulations containing *Momordica charantia*

Extracts/ Formulations	**% w/w of STG ± SD**	**mg of STG ± SD**	**% w/w of BSG ± SD**	**mg of BSG ± SD**
Extract—I (Gujarat)	4.54 ± 0.28	-	11.01 ± 0.52	-
Extract-II (Tamil Nadu)	6.81 ± 0.05	-	17.23 ± 0.15	-
Extract-III (Maharashtra)	ND	-	14.82 ± 0.71	-
Tablet	0.13 ± 0.02	0.0017 ± 0.00/Tablet	0.54 ± 0.10	0.0070 ± 0.00/Tablet
Capsule	0.l2 ± 0.03	0.0069 ± 0.00/Capsule	4.13 ± 0.35	0.2332 ± 0.02/Capsule
Granules	0.07 ± 0.00	0.0243 ± 0.00/Teaspoon	l.07 ± 0.07	0.3679 ± 0.02/Teaspoon
Churna (Brand I)	0.09 ± 0.00	0.0569 ± 0.00/Teaspoon	0.88 ± 0.01	0.5295 ± 0.00/Teaspoon
Churna (Brand II)	0.08 ± 0.00	0.0418 ± 0.00/Teaspoon	0.44 ± 0.01	0.21 ± 0.00/Teaspoon

n=3, triplicate injections

ND = Not detected

4. CONCLUSION

The HPLC methods developed and validated in this study substantiate their suitability for quality control purposes in herbal extracts and formulations. These methods can be effectively utilized for assessing batch-to-batch variations and conducting routine analyses of herbal formulations by manufacturers in the herbal industry.

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