Analytical Quality by design (QbD) approach for Simultaneous phytochemical analysis of Hesperetin and Harpagoside in methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb. by RP-HPLC technique

[*]Rajashree Deka [1], Jogen Ch Kalita [1]

[1]Animal Physiology and Biochemistry Laboratory, Department of Zoology, Gauhati University, Gopinath Bordoloi Nagar, Jalukbari, Guwahati-781014, Assam, India.

***Corresponding author**

*Email: rajashreedeka41@gmail.com

Article History: Received: 17.01.2022 **Revised:** 31.03.2022 **Accepted:** 21.04.2022

DOI: 10.31838/ecb/2022.11.4.021

ABSTRACT

Background: Hesperetin is a cholesterol lowering flavonoid, derivative of hesperidin. It is used in treatment of various ailments pertaining to its medicinal properties like neuro protective, anti-oxidant and immune boosting properties. Harpagoside is a terpene glycoside reported to produce anti diabetic activity by binding to the pyridoxal phosphate site of glycogen phosphorylase-a and have potent anti- rheumatic, anti-inflammatory and analgesic effects as well. Hesperetin and Harpagoside are present inmethanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.

Objective: To develop a sensitive HPLC method for simultaneous estimation of Hesperetin and Harpagoside in methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb. by HPLC technique. Chromatographic conditions to be optimised by Analytical Quality by design (QbD) approach.

Methods: The Shimadzu Prominence-i LC-2030C was employed for the study which has an integrated vacuum de gasser, automatic sample manager and quaternary pump. A Shimadzu Shim Pack C-18

Column (5μm, 4.6×150mm) was used. The detector employed for the study was UV-Visible detector. The mobile phase is made up of methanol (A) and 0.3% ortho-phosphoric acid (B) in water in the ratio of 80:20. The mobile phase was delivered at a flow rate of 0.8ml/min in isocratic mode. The sample injection volume was 10µL. The column temperature was maintained at 30˚C throughout the chromatographic run. The wavelength for the UV-Visible detector was set at 275nm. Design Expert Version 11 Software (Stat-Ease Inc., USA) was used to optimize chromatographic conditions. 3 level Factorial design was employed for optimizing chromatographic conditions. Lesser retention times and tailing factors are chosen as analytical target profile (ATP), concentration of mobile phase additive (Ortho phosphoric acid), mobile phase ratio were chosen as critical analytical procedure parameters (CAPPs).

Results & Discussion: The HPLC analysis of Hesperetin and Harpagoside were achieved by optimizing chromatographic conditions to obtain a high-resolution chromatogram peak in the HPLC. Mobile phase ratio and combination as 80:20 (Methanol: 0.3% orthophosphoric acid in water) at which LC separation occurs best at 0.8 ml/min as the ideal flow rate. Hesperetin and Harpagoside were eluted at a retention time of 2.148 and 5.438 minutes respectively and the eluents were measured at a wavelength of 275 nm.

Conclusion: Analytical Quality by design (QbD) approach was employed to optimise chromatographic conditions for Hesperetin and Harpagoside simultaneous estimation by HPLC. Newly developed analytical method was validated as per ICH Q2 (R1) guidelines.

Keywords: Analytical Quality by design (QbD), Factorial design, *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb., Methanolic flower extract, RP-HPLC, Phytochemical analysis.

1. Introduction

Phlogacanthus thyrsiformis (Roxb. ex Hardw.) Mabb. a promising medicinal herb from Acanthaceae family majorly found in India, Yunnan-Chinese provinces, and Vietnam (1). It is widely used as folk medicine and prescribed by many traditional healers in north eastern part of India. Several secondary metabolites have been isolated from this plant are phlogantholide-A, phloganthoside- A (2, 3), butenoliode, lactone glucoside , β- Sitosterol, lupeol, botulin, Hesperetin, kaempferol, 3-rhamnoside, lupenone and Harpagoside (4) Hesperetin is a cholesterol lowering flavonoid (5), derivative of

hesperidin (6) abundantly found in citrus fruits (7). This phytoconstituent possess lipid-lowering efficacy (8), vitamin-like activity has potential to be used as supplement for various ailments pertaining to its medicinal properties like neuro protective (9), anti-oxidant and immune boosting properties (9). Harpagoside is a terpene glycoside (10) reported to produce antidiabetic activity by binding to the pyridoxal phosphate site of glycogen phosphorylase-a (11) have potent anti-rheumatic, anti-inflammatory and analgesic effects as well. **Figure 1** depicts the structures of Hesperetin and Harpagoside.

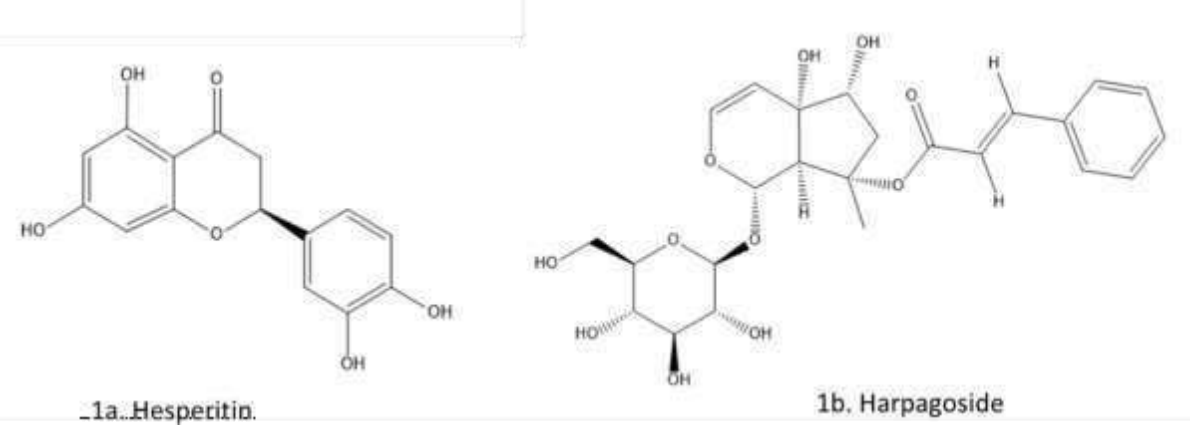

Figure 1: **Structures of Hesperetin and Harpagoside**

Regulatory bodies across the globe have been suggesting to implement Analytical QbD design before proceeding for analytical method development (12, 13) because QbD is a scientific approach enabling deep understanding of the influence of critical factors on the end result (14).

In preliminary phytochemical identification in any herbal sample hyphenated techniques like LCMS/MS, GC-MS/MS are used (15). For specific quantification of constituents HPLC is employed (16). Till date there are no reports delineating the analytical method development and validation pertinent to simultaneous estimation of Hesperetin and Harpagoside in methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb. by HPLC technique. A holistic and scientific approach of employing Analytical Quality by design (QbD) by taking into consideration of critical analytical procedure parameters (CAPPs) to achieve analytical target profile (ATP) was followed in optimising chromatographic conditions.

2. Materials and Methods

Plant material: Collection and identification

The flowers of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb were collected from Hahara gaon, Sonapur, Kamrup Metropolitan District, Assam during February, 2021. The collected flowers were identified by Dr. Souravjyoti Borah, Curator, GUBH, Department of Botany, Gauhati University, Assam.

A herbarium was made readyafter poisoning with mercuric chloride and the voucher specimen bearing accession number 19763 was deposited in the Department of Botany, Gauhati University, Assam for future reference. The fresh flowers collected were utterly cleaned and washed with fresh water, shade dried at room temperature for 14 days, coarsely powdered in mixture grinder (Philips). The powdered sample was stored in an airtight and light- resistant container, properly labeled and sealed and was used in the current investigation.

Preparation of flower extract

Flowers were thoroughly cleaned and washed to eliminate dirt and contamination. They were then shade dried, powdered coarsely. The powdered plant material was macerated with absolute methanol for 72 hours at room temperature while stirring occasionally (17). The extract was filtered using Whatman No.1 filter paper. The filtrate that was obtained was air dried and was stored in 4^{o} C refrigerator for its further use as PTFME (*Phlogacanthus thyrsiformis* flower methanolic extract) (18). The yield obtained from the extract was 8.41 % (w/w).

Chemicals

Hesperetin and Harpagoside were obtained from Sigma-Aldrich, Bengaluru, India. Methanol (HPLC grade) was purchased from M/s Merck life Science, Vikhroli (East) Mumbai, India. HPLC grade water for analysis was obtained from Millipore Milli-Q water purification System (M/s Merck life Science, Mumbai India). Orthophosphoric acid was received from M/s Loba Chemie, Mimbai, India.

Instrumentation and chromatographic condition:

The Shimadzu Prominence-i LC-2030C was employed for the study which has an integrated vacuum de gasser, automatic sample manager and quaternary pump. A Shimadzu Shim Pack C-18 Column (5μm, 4.6×150mm) was used. The detector employed for the study was UV-Visible detector. The mobile phaseis made up of methanol (A) and 0.3% ortho-phosphoric acid (B) in water in the ratio of 80:20. The mobile phase was delivered at a flow rate of 0.8ml/min in isocratic mode. The sample injection volume was 10µL. The column temperature was maintained at 30˚C throughout the chromatographic run. The wavelength for the UV-Visible detector was set at 275nm. Retention times of Hesperetin and Harpagoside were found to be 2.091 minutes and 5.654 minutes respectively.

Application of Analytical quality by design (QbD) for optimizing chromatographic conditions for HPLC technique

QbD is a tool that aids in optimizing chromatographic conditions with lesser time, lesser efforts and reliable results. Chromatographic conditions were optimized by 3 level factorial design by using Design Expert Version 11 Software (Stat-Ease Inc., USA). Defining Analytical target profile (ATP) is the foremost step in QbD process. Desired expected outcome of analytical method should be defined as ATP. Critical analytical procedure parameters (CAPPs) that could influence method performance should be selected and specified. Interaction between these selected parameters can also be studied by QbD design. Statistical parameters like Probability value (P-value), Model fishers value (F-value), Regression coefficient value (R^2), and lack of fit F value the responses were calculated by mathematical model generated by design. Quadratic polynomial equations for response factor and selected best-fit model were developed. ANOVA has been applied to check the significance and suitability of the suggested model. From the desirability of the response variables, the optimized chromatographic conditions were selected and subjected to evaluation.

Table 1: Critical analytical procedure parameters (CAPPs) with three levels as per factorial design for optimizing chromatographic conditions

Analytical target profile (ATP)				
No Response	**Response name**	**Units**	**Desired outcome**	
01	Retention time	minutes	Lesser retention time	
02	Tailing factor	-	Higher tailing factor	
Critical analytical procedure parameters (CAPPs)				
No Factor	**Factor name**	**Level 1**	**Level 2**	**Level 3**
1	Concentration of mobile phase additive	0.1%	0.2%	0.3%
2	Mobile phase ratio	20%B (Ortho phosphoric acid in water)	50%B (Ortho phosphoric acid in water)	80%B (Ortho phosphoric acid in water)

Chromatographic conditions optimization by Analytical quality bydesign (QbD)

Preparation of Standard Solution

10 mg of Hesperetin was weighed and taken into 10 mL volumetric flask, made up the volume with

methanol and 10 mL of Harpagoside was dissolved in 10 mL methanol to obtain a 1000 µg/mL concentration solution. Further dilutions were carried out, leading to final concentrations of 5, 10, 20, 40, 80, 160 µg/mL for Hesperetin and 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 µg/mL for Harpagoside.

Preparation of Sample for RP-HPLC Analysis

10 mg of finely powdered extract was dissolved in 10 mL of methanol to obtain 1000µg/mL stock solution. Solution was sonicated for 10 minutes. Filtered with 0.22 µm syringe filter to remove particulate matter and carefully filled into HPLC vials.

Method Validation

The method validation for RP-HPLC was performed according to ICH Q2 R1 guidelines. The validation process has been done for Hesperetin and Harpagoside for the following parameters:

Linearity

It denotes the proportional relationship of peak response versus analyte concentration. The method linearity was determined by generating the standard calibration graphs. The least square regression analysis of calibration graphs yields correlation coefficient, slope and y-intercept.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Visual determination method is used to determine both LOD and LOQ. LOD is the least concentrationof an analyte that can be identified. LOQ refers to the smallest amount of analyte that can be reliably quantified.

Precision

The method precision was verified using intraday and inter-day accuracy studies. Preciseness of thepeakareas obtained is taken into consideration. Relative standard deviation (RSD) is calculated.

Accuracy

The analytical procedure compares the recoveryof analyte value with that of conventional true value.

Robustness

Alteration in flow rate and mobile phase ratio were considered to check robustness for current analytical method employed, the flow rate (±0.8 mL/min, 1.2 mL/min and 1 mL/min), study was

performed in replicates (n = 6).

Stress studies

Sample was subjected to acidic, basic and oxidative conditions as per ICH guidelines.

3. Results and Discussion

Optimization of chromatographic conditions for Digoxin tablets

Pertinent combination of selected factors for analytical method that could yield lesser retention times and tailing factor is the prominent challenge in the current study. Application QbD in current study is intended to study the influence of minor changes in selected parameters on final outcome. 3 level factorial design suggested 13 trail runs for factors (Concentration of mobile phase additive, Mobile phase ratio) at three levels for response factors (retention time and tailing factor). **Table 2** portrays summaryof results obtained for 13 trail runs suggested bythe design.

Table 2: Summary of results obtained for 13 trail runs suggested by the design.

	Factor 1	**Factor 2**	**Response 1 (Hesperetin)**	**Response 2 (Hesperetin)**	**Response 1(Harpagoside)**	**Response 2 (Harpagoside)**
Run	Concentration of mobile phase additive(OPA)	Mobile phase ratio	Retention time	Tailing factor	Retention time	Tailing factor
1	0.1	20	6.2	1921	8.5	3001

2	0.2	50	5.1	3212	7.1	4121
3	0.2	20	6.1	3312	8.2	4234
4	0.2	80	2.1	3121	5.8	4121
5	0.3	80	2.2	5234	5.2	7212
6	0.1	80	2.8	2001	5.7	3121
7	0.1	50	5.2	1952	7.3	3012
8	0.2	50	5.3	3213	7.4	4128
9	0.2	50	5.1	3412	7.5	4278
10	0.2	50	5.2	3129	7.6	4312
11	0.3	20	6.2	5122	8.4	3121
12	0.3	50	5.2	4925	7.9	4276
13	0.2	50	5.3	4812	7.8	4212

Three-dimensional response surface plots portrays cumulative influence of concentration of mobile phase additive and mobile phase ratio on response factors retention time and tailing factor as represented in **Figure 2**. Results clearly shows the influence of the parameter, concentration of mobile phase additive is in directly proportional relation with response factor tailing factor, where as other parameter mobile phase ratio has inversely proportional relation with retention time. This inference can be justified that increase in mobile phase additives can improve peak shape by decreasing in tailing factor of eluted peak. Whereas changes in mobile phase ratios can shift peaks to different retention times. Perturbation plots portrays how closer the responses towards the design space. From figures it is clearlyevident that all the responses are falling closer and are in linear fashion.

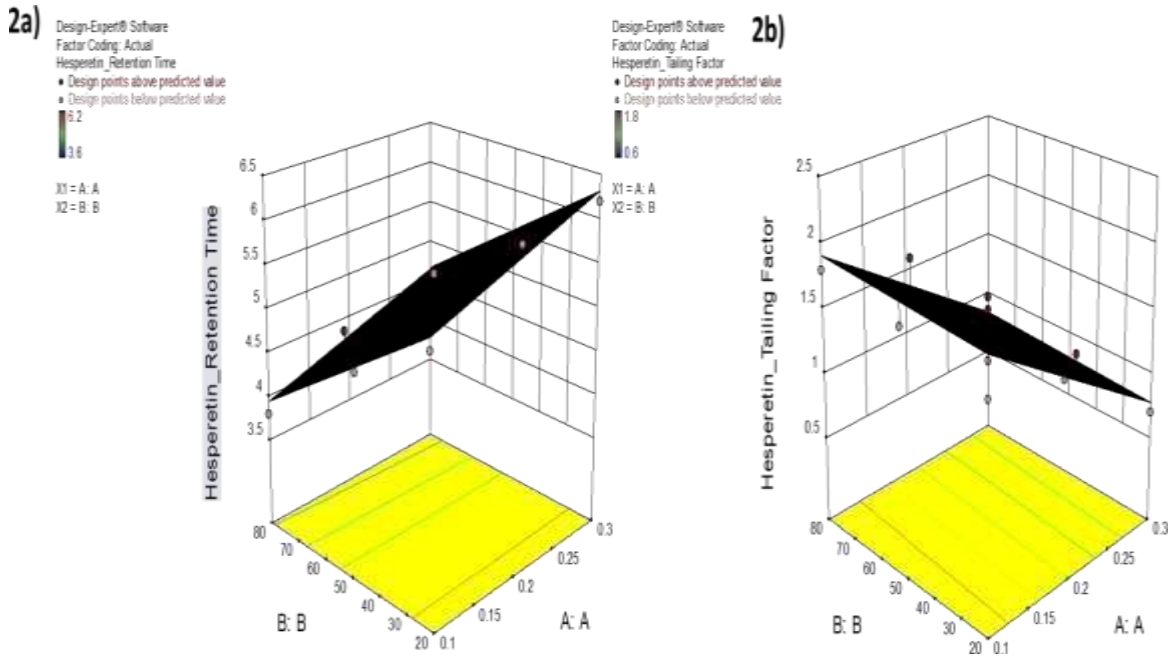

Figure 2: 3D response surface plot portraying minimal standard error of design for hesperetin drug for factors 2a) Retention time (RT) 2b) Tailing factor

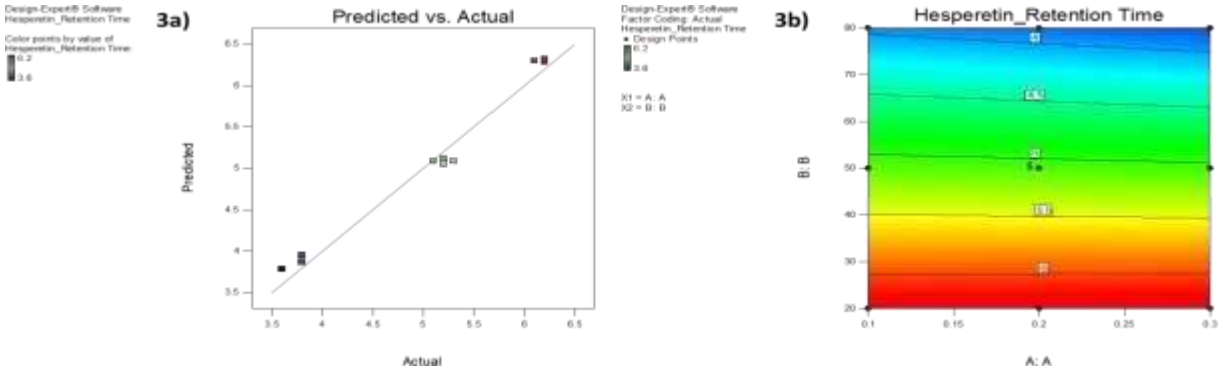

Figure 3: 3a) Perturbation plot and 3b) Contour plot for factor retention time (RT) for hesperetin drug

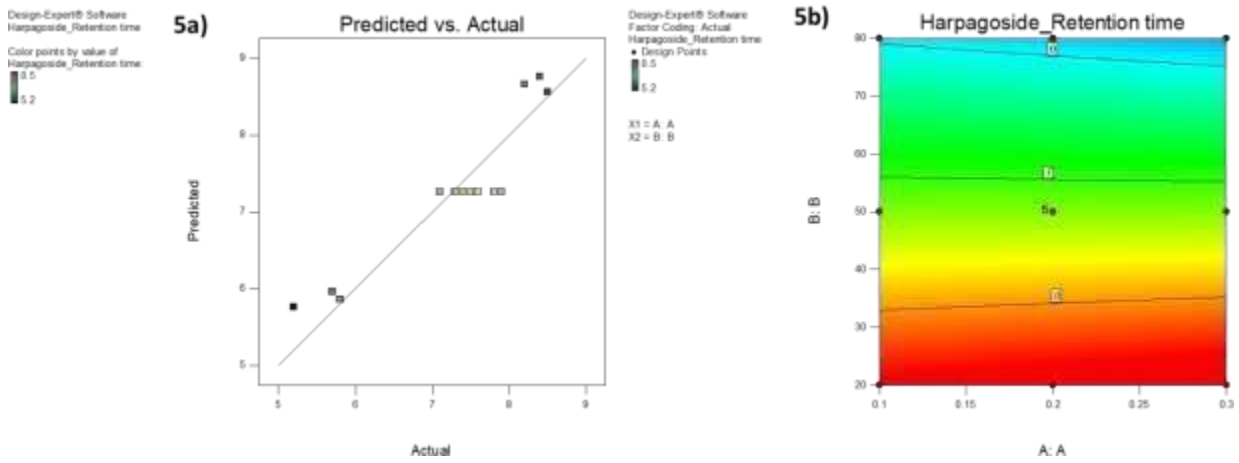

Figure 5: 5a) Perturbation plot and 5b) Contour plot for factor retention time (RT) for harpagoside drug

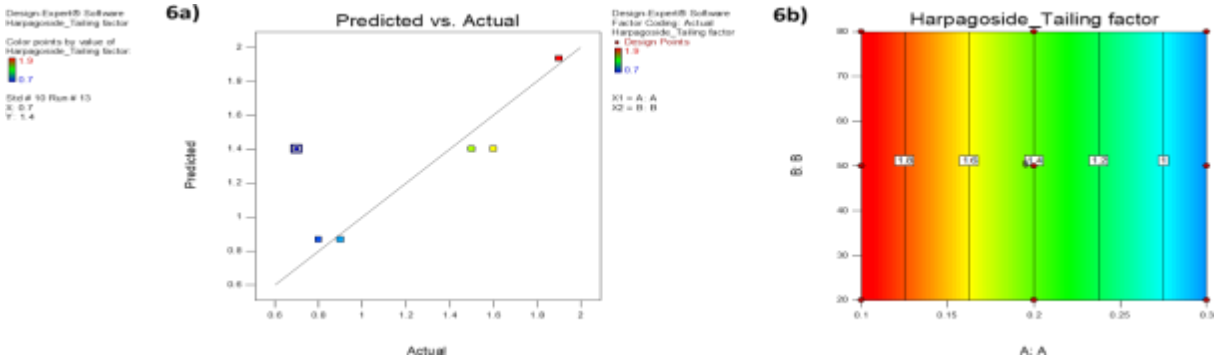

Figure. 6: 6a) Perturbation plot and 6b) Contour plot for factor tailing factor for harpagoside drug

Polynomial Equations predicting response in terms of selected factors is represented as follows:

Hesperetin

Retention Time=7.012393+0.5 * A -0.03722 * B-0.01667 * A *B

Tailing Factor =380.4145+15545 * A -0.52778 * B +2.666667 * A *B

Harpagoside

Retention Time=9.261538+1.666667 * A-0.04 * B-0.03333* A * B

Tailing Factor =4434.218-7420.83 * A-43.4167 * B+330.9167 * A *B

NOTE: A= Concentration of mobile phase additive; B= Mobile phase ratio

Table 3: ANOVA for Quadratic model of Hesperetin drug for the response variables

Hesperetin – Retention time						
Source Model	Sum of Squares	df	Mean Square	F Value	p-value	Significant
	8.898	3	2.966111	103.233	2.73 E-07	
A-A	0.007	1	0.006667	0.232028	0.641521	
B-B	8.882	1	8.881667	309.119	2.82 E-08	
AB	0.010	1	0.01	0.348042	0.569737	
Residual	0.259	9	0.028732			
Lackof Fit	0.219	5	0.043718	4.371795	0.08897	Not significant
Pure Error	0.040	4	0.01			
Core Total	9.157	12				

Hesperetin – Tailing factor						
Source	Sum of Squares	df	Mean Square	F Value	p-value	Significant
Model	1.713333	2	0.856667	13	0.001654	Significant
A-A	1.706667	1	1.706667	25.89883	0.000471	
B-B	0.006667	1	0.006667	0.101167	0.756976	
AB	0.658974	10	0.065897			
Residual	0.198974	6	0.033162	0.288369	0.914822	
Lackof Fit	0.46	4	0.115			Not significant
Pure Error	2.372308	12				
Core Total	17014457	12				

Table 4: ANOVA for Quadratic model for Harpagoside drug for the response variables

Harpagoside – Retention time						
Source	Sum of Squares	df	Mean Square	F Value	p-value	Significant
Model	11.8	3	3.93	21.44455	0.000195	Significant

A-A	0	1	0	0	1	
B-B	11.76	1	11.76	64.11556	2.2E-05	
AB	0.04	1	0.04	0.21808	0.651606	
Residual	1.650769	9	0.183419			
LackofFit	1.382769	5	0.276554	4.127669	0.097174	Not significant
Pure Error	0.268	4	0.067			
Core Total	13.45077	12				
Harpagoside – Tailing factor						
Source	Sum of Squares	df	Mean Square	F Value	p-value	Significant
Model	1.706667	2	0.853333	13.06122	0.001626	Significant
A-A	1.706667	1	1.706667	26.12245	0.000457	
B-B	2.22E-16	1	2.22E-16	3.4E-15	1	
AB	0.653333	10	0.065333			

Residual	0.065333	6	0.010889	0.074074	0.9963	
Lackof Fit	0.588	4	0.147			Not significant
Pure Error	2.36	12				
Core Total	1.706667	2	0.853333	13.06122	0.001626	

Table 5: Fit Statistics

Drug name	**Hesperetin**		**Harpagoside**	
Factor	**Retention time**	**Tailing factor**	**Retention time**	**Tailing factor**
Std. Dev.	0.16	0.256705	0.428274	0.255604
Mean	5.08	1.346154	7.261538	1.4
C.V. %	1.33	19.06951	5.897844	18.25742
R-Squared	0.97176	0.722222	0.877273	0.723164
Adj R-Squared	0.962347	0.666667	0.836364	0.667797
Pred R-Squared	0.898385	0.618272	0.540678	0.655978
Adeq Precision	26.94323	9.190401	12.62819	8.687051

Effects of the selected factors on response factors

In current design A, B and AB are significant model terms. ANOVA results for selected model are presented in **Table 3 and Table 4**. The results obtained for F-value and P-value intends the significance of the model. Fit statistics data in **Table 5** presents statistical terms like R-Square value, Coefficient of variance (C.V. %), Adeq Precision. Data depicts clear correlation of R-Square value for adjusted and predicted value from the design. Coefficient of variance (C.V. %) is more than 1% for all the factors. Adeq Precision is also more the 4, intending that the model chosen can navigate the design space.

Optimized chromatographic conditions by A-QbD

Design Expert Version 11 Software finally concluded an optimised chromatographic conditions based on desired optimal response (Lesser retention time and higher tailing factor). Mobile B with ratio of 20% (Ortho phosphoric acid in water) and Concentration of mobile phase additive (OPA) of 0.3%.

Optimisation of chromatographic method

The high-performance liquid chromatography analysis of Hesperetin and Harpagoside were achieved by optimizing chromatographic conditions to obtain a high-resolution chromatogram peak in the HPLC method. Several mobile phase ratios and mobile phase combinations were tested to get the final mobile phase ratio and combination as 80:20 (Methanol: 0.3% orthophosphoric acid in water) at which LC separation occurs best. The effect of variation in flow rate on the peak shape and elution speed is studied and obtained 0.8 ml/min as the ideal flow rate. Berberine and Eugenol were eluted at a retention time of 2.148 and 5.438 minutes and the eluents were measured at a wavelength of 275 nm. The standard and sample chromatograms with Hesperetin and Harpagoside peaks is shown in **Figures 7 and Figures 8.**

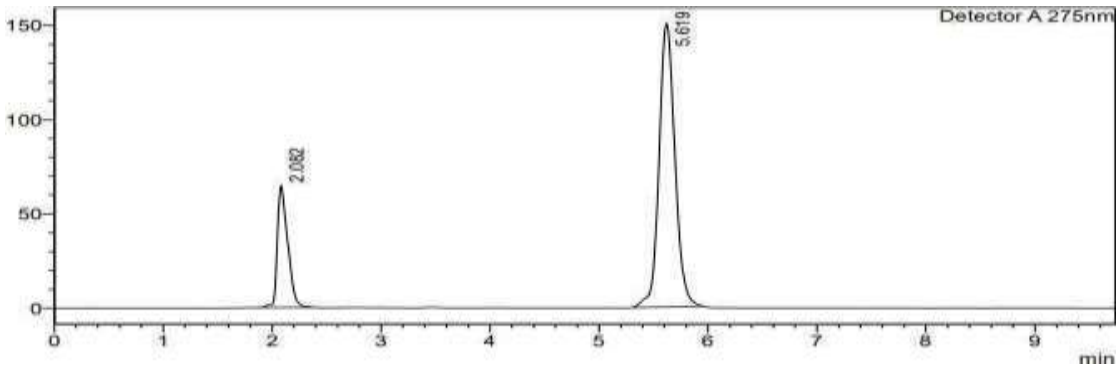

Figure 7: RP-HPLC Chromatogram of standard

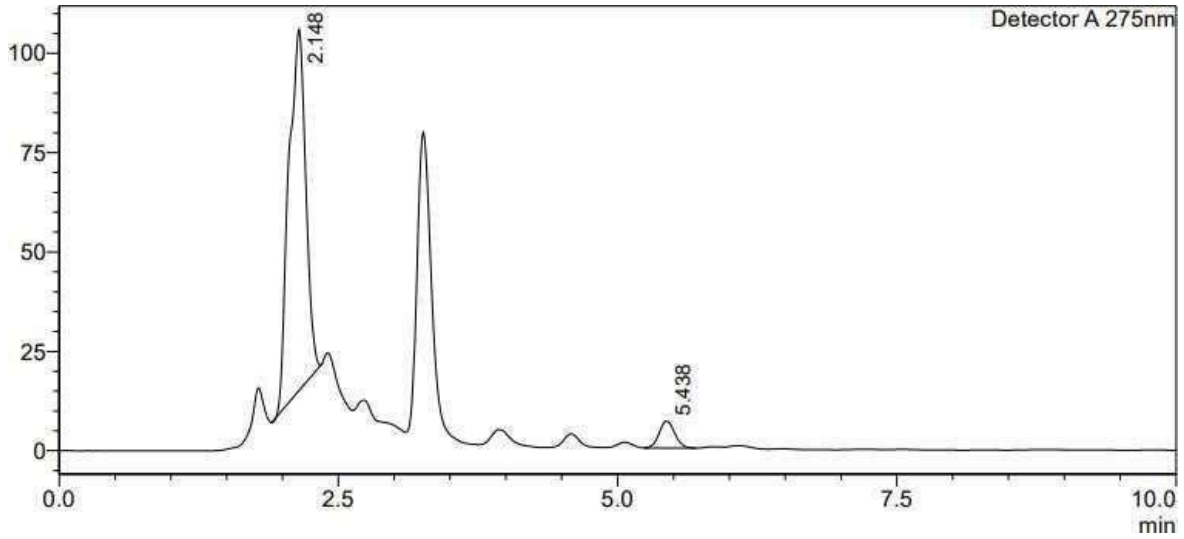

Figure 8: RP-HPLC Chromatogram of sample (methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) Mabb.)

Validation of the method

Specificity:

Interfering peaks were not found for berberine and eugenol the retention time of the sample was found at 2.148 min for Hesperetin and 5.438 min for Harpagoside.

Linearity:

A set of linear concentrations of 5-160µg/ml of standard Hesperetin and 0.05-1.6µg/mL of standard Harpagoside was prepared from the stock solutions. **Table 6 and Table 7** shows the concentration range and peak area for Hesperetin and Harpagoside respectively. The linearity was established by plotting the concentration of Hesperetin and Harpagoside against their respective peak area as shown in **Figure 9 and Figure 10**. Regression Coefficient was 0.999 for Hesperetin and 0.996 for Harpagoside, hence, signifying the excellent linearity.

Table 6: Linearity of Hesperetin

Concentration	Peak area
5	58934
10	108852
20	206638
40	405503
80	807414
160	1641689

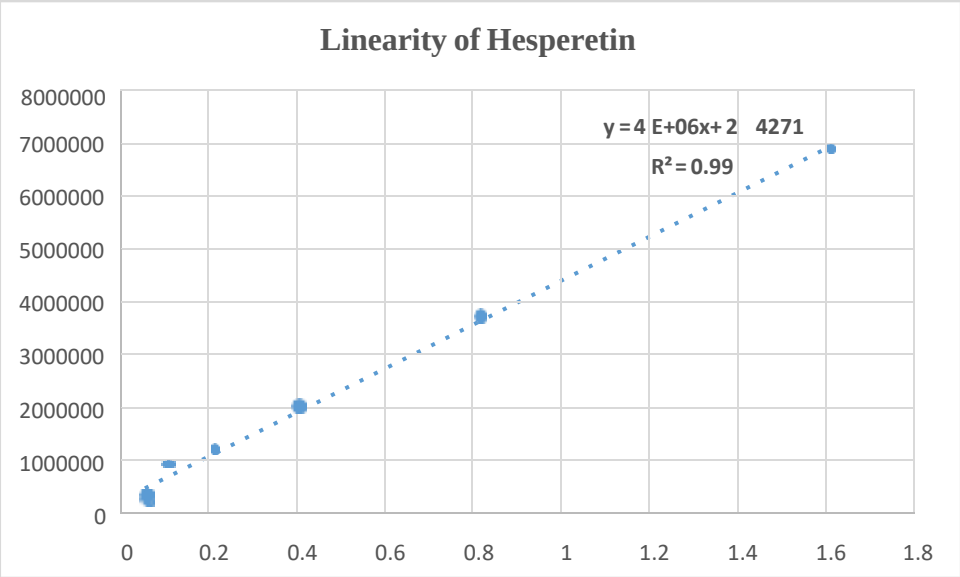

Figure 9: Linearity graph of Hesperetin

Table 7: Linearity of Harpagoside

Concentration	Peakarea
0.05	175586
0.1	790876
0.2	1107697
0.4	2008746
0.8	3606325
1.6	6800759

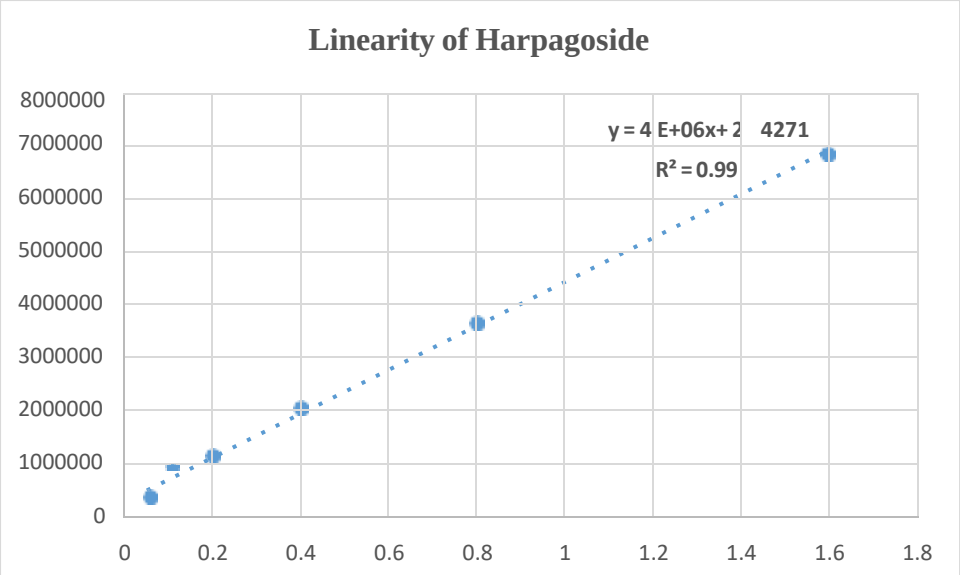

Figure 10: Linearity graph of Harpagoside

Limit of Detection and Limit of Quantification

The LOD and LOQ were calculated and found to be 0.19 µg/mL and 0.58 µg/mL for Hesperetin and 0.49µg/mL and 1.5µg/mL for Harpagoside respectively.

Method precision

Precision was investigated using homogeneous samples for both Hesperetin and Harpagoside. Six determinations were done covering the reportable range for the procedure and the parameters like SD and the RSD were determined. The summarized result of the precision study indicates that the % RSD is within the limit (%RSD<2) denoting the developed method was precise **Table 8**.

Table 8: Method Precision results of Hesperetin and Harpagoside

Sl. No	Hesperetin (Peak Area)	% Drug	Harpagoside (Peak Area)	% Drug
1.	1308760	100.2502	1001063	102.3423
2.	1299047	99.49027	987390	100.7687
3.	1287868	98.61562	976539	99.51985
4.	1301545	99.68571	975670	99.41984
5.	1301252	99.66279	980579	99.98481
	Average	99.54092	**Average**	100.4071
	SD	0.528706	**SD**	1.078503
	RSD	0.531144	**RSD**	1.07413

Intermediate precision

Six determinations were done covering the reportable range for the procedure and the parameters like SD and the RSD were determined. **Table 9** summarizes result of the precision study indicating that the % RSD is within the limit (%RSD<2) denoting the developed method was precise.

Table 9: Intermediate Precision results of Hesperetin and Harpagoside

Sl. No	Hesperetin (Peak Area)	% Drug	Harpagoside (Peak Area)	% Drug
1.	406848	99.07489	940651	99.70962
2.	405008	98.62413	937225	99.31389
3.	408345	99.44162	947732	100.5275
4.	407702	99.2841	935242	99.08484
5.	408344	99.44138	950628	100.862
	Average	99.17322	**Average**	99.89958
	SD	0.305748	**SD**	0.687537
	RSD	0.308297	**RSD**	0.688228

Accuracy

The percentage recovery of six replicates is used for accuracy and the response of each solution is measured in HPLC. **Table 10** shows the results were found to be accurate.

Table 10: Accuracy results of Hesperetin and Harpagoside

Sl. No	Hesperetin (PeakArea)	% Drug	Harpagoside (Peak Area)	% Drug
1.	1308760	100.25	1001063	102.34
2.	1299047	99.49	987390	100.76
3.	1287868	98.615	976539	99.51
4.	1301545	99.685	975670	99.41
5.	1301252	99.662	980579	99.98

	Average	99.54	**Average**	100.4
	SD	0.528	**SD**	1.07
	RSD	0.531	**RSD**	1.074

Robustness

Robustness was studied by changing the operational parameters such as flow rate and oven temperature. The results obtained are summarized in **Table 11**.

Table 11: Robustness results for Hesperetin

Hesperetin	**Peak area at different flow rate**		**Peak area at different temperature**	
Conc (µg/mL)	**0.7 mL/min**	**0.9 mL/min**	**28^0C**	**32^0C**
20	408503	409234	404976	405641
20	407779	408976	405789	406860
20	410007	410791	406899	404991
20	406678	408918	406985	407865
20	405923	410312	407124	408432
Average	99.30	99.76	98.95	99.05

SD	0.348	0.183	0.205	0.31
%RSD	0.351	0.187	0.207	0.32

Table 12: Robustness results for Harpagoside

Harpagoside Conc (µg/mL)	**Peak area at different flow rate**		**Peak area at different temperature**	
	0.7 mL/min	**0.9 mL/min**	**28^0C**	**32^0C**
0.2	39777639	39997182	40122352	40205426
0.2	40125899	39812548	39896817	39542201
0.2	40331241	40354210	39897965	39098998
0.2	40114876	40252107	39997652	39722109
0.2	39891148	39903458	40211450	40143298
Average	99.50	99.54	99.45	98.74
SD	0.485	0.516	0.311	1.01
%RSD	0.487	0.518	0.313	1.03

Stress Studies

Harpagoside was subjected to more degradation.

Table 13: Results for stress studies of Hesperetin and Harpagoside

Stress Conditions	**Peak Area Hesperetin**	**%Mean Difference**
HCl	81695	4.75
NaOH	68389	14.24
H_2O	71964	13.52
H_2O_2	82212	4.97
	Peak Area Harpagoside	**%Mean Difference**
HCl	28459	40.12
NaOH	17488	51.91
H_2O	8349	70.66
H_2O_2	13523	51.91

4. Conclusion

Precise, accurate and sensitive RP-HPLC-UV method for the estimation of hesperetin and harpagoside in methanolic flower extract of *Phlogacanthus thyrsiformis* (Roxb. ex Hardw.) developed and validated according to ICHQ2(R1) guidelines. Methanol and 0.3 % Orthophosphoric acid in Milli-Q water (80:20 v/v) provided the better separation (retention time 2.148 and 5.438 for Hesperetin and Harpagoside) with the tailing factor 1.29. Linearity, LOQ, LOD and precision studies confirmed the sensitivity of the developed method. Stability of the drug also established through stress degradation studies.

Author Contributions

Rajashree Deka, Jogen Ch Kalita conceptualized, wrote, proofread and edited the manuscript.Both the authors reviewed the final version of the manuscript.

Acknowledgement: We acknowledge Department of Zoology, Gauhati University for providing us the facilities to carryout our research.

Funding: NIL

Conflict of Interests

The authors declare no conflict of interest.

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