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RP-HPLC method development and validation of simultaneous estimation of gallic acid and Oleanolic acid in antihyperlipidemic Polyherbal tablets

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Abstract
A new simple, precise, accurate and validated isocratic RP-HPLC method was developed for simultaneous determination of Gallic acid and Oleanolic acid in developed Polyherbal tablet formulation. The chromatographic condition for separation of these two phytoconstituents was C_{18} column diameter (4.6 x 250mm, 5µ), rheodyne manual injector with capacity of 20µL. The mobile phase was composed of mixture of 0.1% Ortho Phosphoric acid and Methanol (5:95), flow rate is 1 ml/min and detection was carried out at 222 nm. The run time was 15 min. Gallic acid was separated at 2.8 min and Oleanolic acid at 9.9 min, Resolution between these peaks was good. Validation of this developed method has been performed to demonstrate Precision, Linearity, Accuracy, Robustness, LOD and LOQ. Applicability of this validated method was carried out in lab scaled formulated dosage form and determine % Assay of both these phytoconstituents. Such type of chromatographic fingerprint analysis may useful tool for quantification of herbal and polyherbal formulation in future.

Keywords: gallic acid, oleanolic acid, chromatographic fingerprinting analysis, quantification, polyherbal analysis

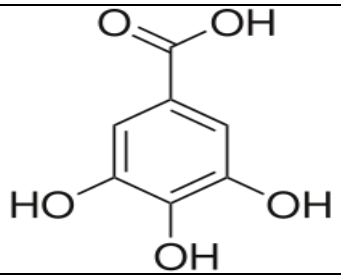
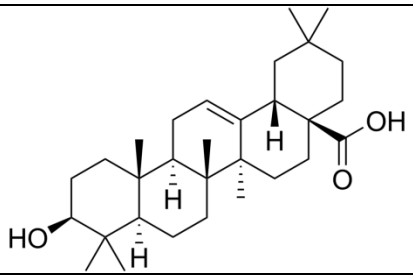
Introduction
As per WHO definition, there are three kinds of herbal medicines: raw plant material, processed plant material and medicinal herbal products. Herbal drugs are finished labeled products that contain active ingredients such as aerial or underground parts of plant or other plant material or combination thereof, whether in the crude state or as plant preparations. The use of herbal medicines has increased remarkably in line with the global trend of people returning to natural therapies. Herbal medicinal products may vary in composition and properties, unlike conventional pharmaceutical products, which are usually prepared from synthetic, chemically pure materials by means of reproducible manufacturing techniques and procedures. Correct identification and quality assurance of the starting material is, therefore, an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy.
The below mentioned factors are influencing the identification and quality of herbal drugs:

1. Herbal drugs are mixtures of many constituents.
2. Quality and sources are different for raw materials.
3. Active constituents are not known.
4. Analytical methods are not available commercially for herbal preparations.

Standardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principles [1].
Standardization of herbal drugs for Global competitiveness such as raw materials needs to be authentic, physico-chemical standards, storage conditions, size and shape. Processing of raw material include material, energy inputs, operational uniformity, safety and occupational health, intermediate quality whereas finished product include physicochemical properties, biological assay, storage stability, user safety *etc*. [2]
Markers are chemically defined constituents of a herbal drug which are of interest for quality control purposes independent of whether they have any therapeutic activity or not. Markers may serve to calculate the amount of active component of herbal drug or preparation in the finished product [3].
Here analytical method development of Gallic acid and Oleanolic acid are developed using Reversed Phase High Performance Liquid Chromatography (RP- HPLC)

and validation parameters such as Accuracy, Precision, Linearity, LOQ, LOD and Robustness are performed.

Table 1: General information of Gallic acid and Oleanolic acid.

Gallic Acid [4]	**Oleanolic Acid [5]**
	
Solubility: alcohol, ether, glycerol, acetone; negligible in benzene, chloroform, petroleum ether	In Methanol
Molecular weight: 170.12gm/mol	456.7 gm/ mol
Formula: $C_7H_6O_5$	$C_{30}H_{48}O_3$
Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants.	Oleanolic acid or oleanic acid is a naturally occurring pentacyclic triterpenoid related to betulinic acid. It is widely distributed in food and plants where it exists as a free acid or as an aglycone of triterpenoid saponins.

2. Chemicals and Reagents

Gallic acid was procured from Sulab (Suvidhinath) Laboratory, Vadodara and Oleanolic acid was procured from Sigma- Aldrich Company, USA. HPLC Grade Methanol and all other reagents are obtained from Rankem Company.

HPLC Grade Water is produced from Double distillation assembly at Laboratory through out the whole study.

3. Experimental procedure

3.1 HPLC instrument

Table 2: HPLC instrumentation

HPLC equipment	**SHIMADZU LC-20AD Prominence**
Column	Hyperchrom 5µ C18 (250 mm x 4.6 mm, 5 µm)
Detector	SHIMADZU SPD-20A Prominence UV/VIS Detector
Injector	Rheodyne 7725 injector valve with fixed loop at 20µl
Software	LC solution
System controller	SBM 20Alite

3.2 Preparation of standard solution

Gallic acid stock preparation

Gallic acid 10 mg dissolved in 10 ml Methanol to prepare 1000 µg/ml solution.

Form this solution 1 ml was taken and diluted up to 10 ml with methanol to prepare 100 µg/ml.

Oleanolic acid stock preparation

Oleanolic acid 10 mg dissolved in 10 ml Methanol to prepare 1000 µg/ml solution.

Mixture

From Gallic acid stock solution, 0.4 ml taken and 4 µg/ml and from Oleanolic acid stock solution 0.8 ml taken and diluted to 10 ml with methanol to make 4, 80 µg/ml for injection in HPLC.

3.3 Preparation of Sample Solution

Polyherbal Tablet A and B were formulated in Laboratory using herbal extracts in which these phytoconstituents GA and OA were present. Approximately five tablets were crushed and 500 mg tablet powders dissolved in 50 ml of methanol. From this solution, 1 ml was to be diluted up to 10 ml with methanol and injected in HPLC after filtered through 0.22 micron syringe filter.

3.4 Selection of wavelength (Iso-absorptive point)

Selection of wavelength of both makers was done by using UV spectrophotometer. Standard solutions of Gallic acid (100µg/ml) and Oleanolic acid (1000µg/ml) were scanned between 200-400nm under UV-Vis spectrophotometer and intercept at 222nm as shown in figure, which was selected as detecting wavelength.

3.5 Optimization of Mobile Phase

Based on sample solubility and suitability various chromatographic condition such as mobile phase, pH, wavelength etc. were tried to get good resolution and sharp peaks.

Table 3: Trials for optimization of mobile phase for HPLC method (ACN = Acetonitrile, OPA= Ortho phosphoric acid)

Mobile phase	**Ratio**	**Gallic acid**		**Oleanolic acid**	
		RT(min)	**Tailing factor**	**RT(min)**	**Tailing factor**
Water : Acetonitrile	50:50	3.6	2.5	-	-
Water(0.3 %OPA) : Acetonitrile	15:85	5.3	1.2	-	-
Water : ACN : Methanol	45:10:45	2.9	1.3	-	-
Water: Methanol	95:5	2.9	1.2	11.7	1.05
OPA (0.1%) : Methanol	10:90 (0.8ml/min)	2.8	1.0	17.2	1.0
OPA (0.1%) : Methanol	2:98 (0.8ml/min)	3.5	1.9	9.5	1.0
OPA (0.1%) : Methanol	2:98 (1 ml/min)	2.8	1.5	7.5	1.1
OPA (0.1%) : Methanol	3:97 (1 ml/min)	2.8 (Shape was not good)	1.5	7.9	1.1
OPA (0.1%) : Methanol	5:95 (1 ml/min)	2.8	1.2	9.5	1.0

3.6 Chromatographic condition

After all these trial performed mobile phase 0.1% Ortho Phosphoric acid: Methanol (5:95) was selected for HPLC method which had given sharp and symmetric peaks for both the markers with good resolution.

Table 4: Optimized method parameters for HPLC

Column	Hyperchrom ODS BP C18 (Size: 250*4.6 mm, 5µ)
Flow rate	1.0 ml/min
Detection wavelength	222 nm
Mobile Phase	Ortho Phosphoric acid 0.1 % in Water: Methanol (5:95) It was filtered through 0.45 µm Nylon filter and sonicated for 5 min.
Injection Volume	20 µl through rheodyne manual injector.
Temperature	Ambient
Retention Time	2.8 min for Gallic acid and 9.9 min for Oleanolic acid

3.7 HPLC Method Validation (6)

The method was validated according to ICH guidelines for Linearity, Precision, Accuracy, Limit of Detection and Limit of Quantification.

3.7.1 Linearity

Linearity of the method was performed by analyzing both the markers in combination as following concentration range.

Table 5: Concentration of Gallic acid (GA) and oleanolic acid (OA)

Linearity Solution	**Concentration of GA (µg/ml)**	**Concentration of OA (µg/ml)**
1	1	50
2	2	60
3	3	70
4	4	80
5	5	90
6	6	100

Now calibration curve was plotted against Area of peak verses Concentration of injected linearity standards. From the graph, correlation co-efficient and regression line equation were to be determined.

3.7.2 Accuracy

The accuracy was determined by calculating % recoveries of GA and OA (Spiking method). It was carried out by adding known amounts of each analyte corresponding to three concentration levels (80, 100, and 120%) of the labeled claim to the excipients. At each level, two determinations were performed, and the accuracy results were expressed as percent analyte recovered by the proposed method.

3.7.3 Precision

Precision of an analytical method is usually expressed as the standard deviation. The repeatability studies were conducted by estimating the response of GA and OA in six times.

Reproducibility of methods was checked by performing intra-day precision (three times a day) and inter-day precision (repeated triplicates for three consecutive days). Results are expressed in terms of standard deviation and % Relative standard Deviation (RSD).

Intraday precision was determined by estimation of mixture of standard markers solution in lower, middle and higher concentration in triplicates on the same day.

Interday precision was determined by estimation of mixture of standard markers solution in lower, middle and higher concentration on three different days.

3.7.4. Robustness

Robustness of the method was investigated under a variety of conditions including changes of composition of buffer in the mobile phase, flow rate, and temperature. This deliberate change in the method has no effect on the peak tailing, peak area, and theoretical plates and finally, the method was found to be robust.

3.7.5 Limit of Detection (LOD)

The LOD can be defined as the lowest amount of analyte that can be detected but not quantitated.

LOD can be calculated as per following equation:

$$LOD = 3.3\ \sigma/S$$

Where σ is standard deviation of regression line and S is slope of calibration curve

3.7.6 Limit of Quantification

Quantification limit of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precesion and accuracy.

$$LOQ = 10\ \sigma/S$$

Where σ is standard deviation of regression line and S is slope of calibration curve

3.8. Quantification of GA and OA in polyherbal tablet

Applicability of proposed method for the laboratory based formulation tablet was quantified for the marker components – Gallic acid and Oleanolic acid. The content of all two markers were determined by injecting the prepared laboratory sample as per proposed chromatographic condition. The concentrations of markers were determined by following equation.

$$\% \text{ Assay} = \frac{Area\ of\ sample\ x\ Std\ wt\ taken\ x\ Sample\ dilution}{Area\ of\ std\ x\ Std\ dilution\ x\ Sample\ wt\ taken} \text{ x } 100$$

4. Results and Discussion

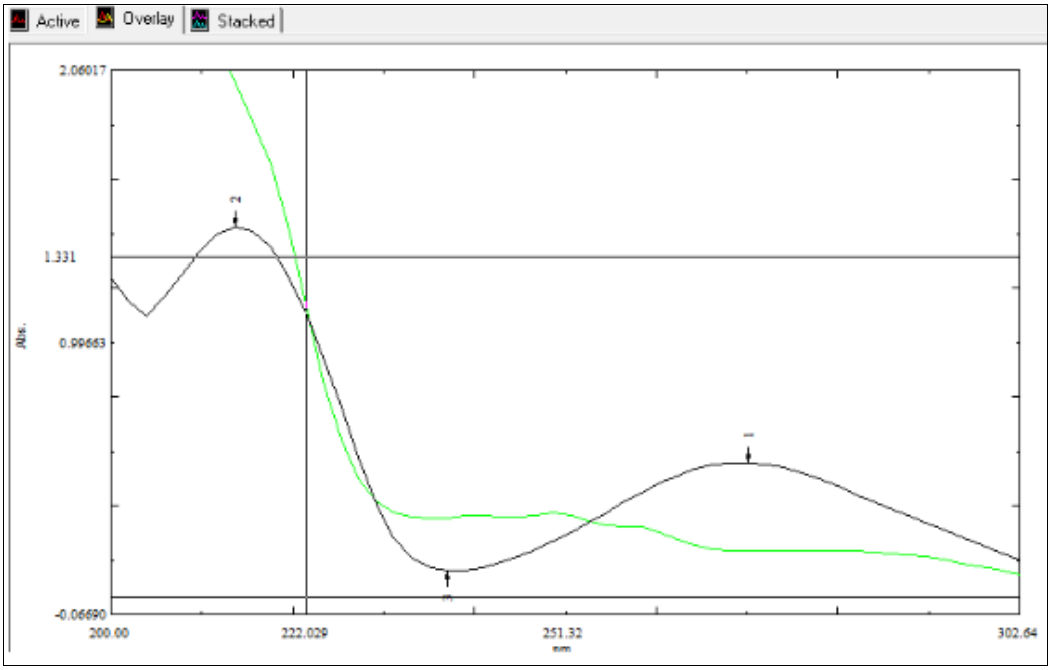


Fig 1: Overlay spectra for both markers GA and OA.

4.1 Isoabsorptive point (Wavelength selection)

Scanning of Gallic acid standard and Oleanolic acid standard were run by UV Visible spectroscopy and both the markers were intercept at 222 nm. Therefore 222nm was selected as detection wavelength for further study.

4.2 System suitability parameters

After various trials the mobile phase 0.1 % Orthophosphoric acid and methanol with the ratio of 5:95 would give a good resolution and sharp peak. The below mentioned chromatogram passed the system suitability parameters such as tailing factor, theoretical plates and Resolution.

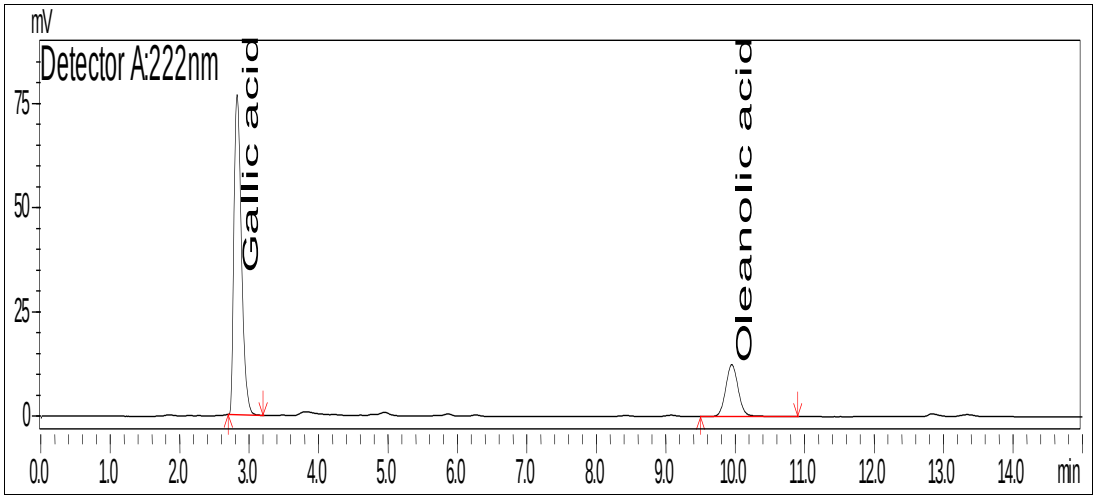


Fig 2: HPLC Chromatogram of Simultaneous estimation of Gallic acid and Oleanolic acid.

Table 6: peak symmetry for Gallic acid and Oleanolic acid

Name	Retention time	Peak start	Peak End	Height	Area	Area %	Tailing factor	Theoritical plate	Resolution
Gallic acid	2.844	2.700	3.200	61108	575350	78.65	1.218	3088.996	-
Oleanolic acid	9.949	9.500	10.90	11714	156109	21.34	1.076	14402.220	26.501

4.3. Method Validation parameters for HPLC fingerprinting

4.3.1 Linearity Parameter

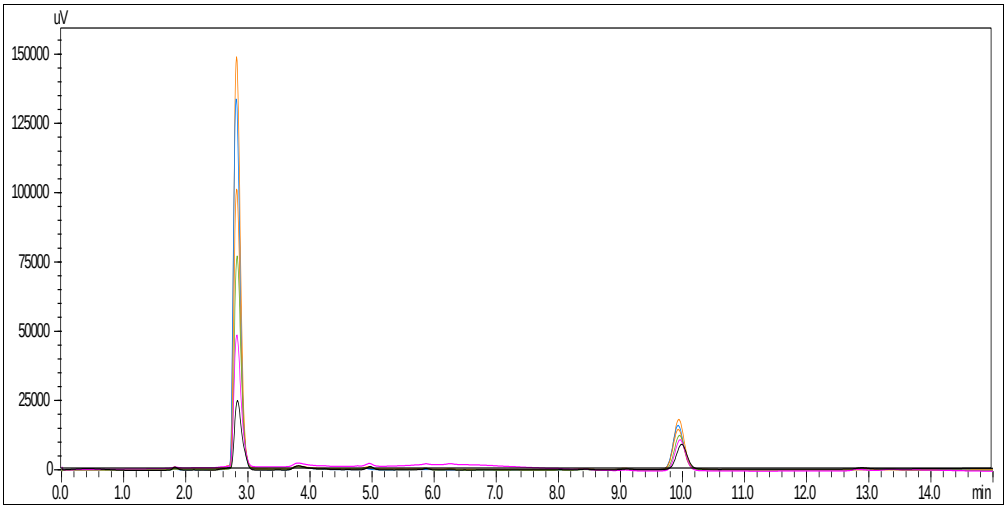


Fig 3: Overlay HPLC Chromatogram for different linearity concentration for both markers.

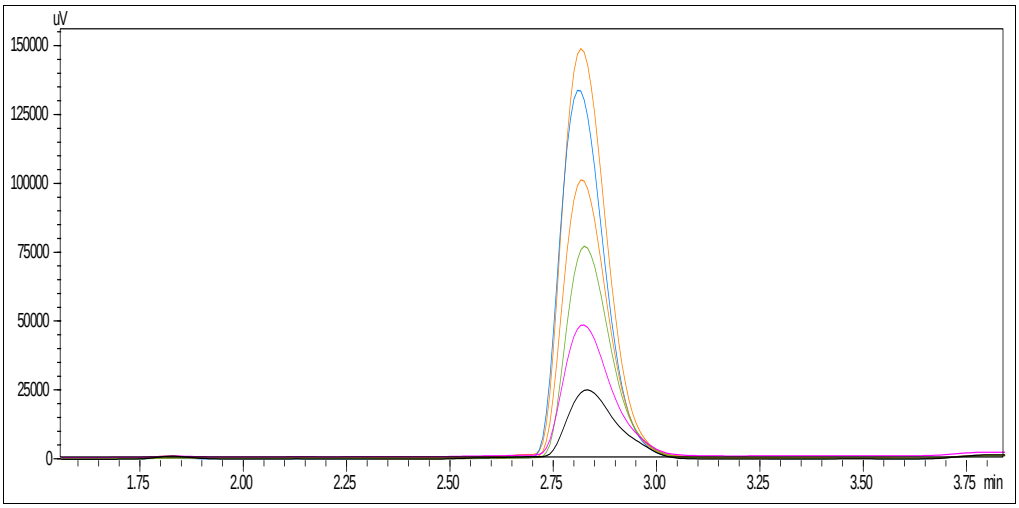


Fig 4: Overlay HPLC Chromatogram for different linearity concentration for Gallic Acid

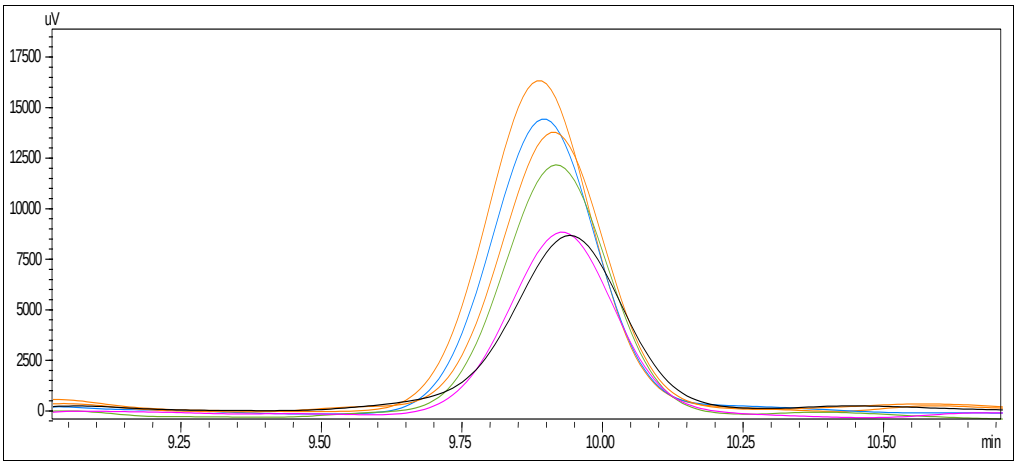


Fig 5: Overlay HPLC Chromatogram for different linearity concentration for Oleanolic Acid

Table 7: Peak area of GA

Concentration of GA in µg/ml	Avg. Area of Gallic acid
1	204339
2	379961
3	597321
4	775443
5	984878
6	1128298

Table 8: Peak area of OA

Concentration of OA in µg/ml	Avg. Area of Oleanolic acid
50	118299
60	140246
70	163724
80	189978
90	209210
100	229411

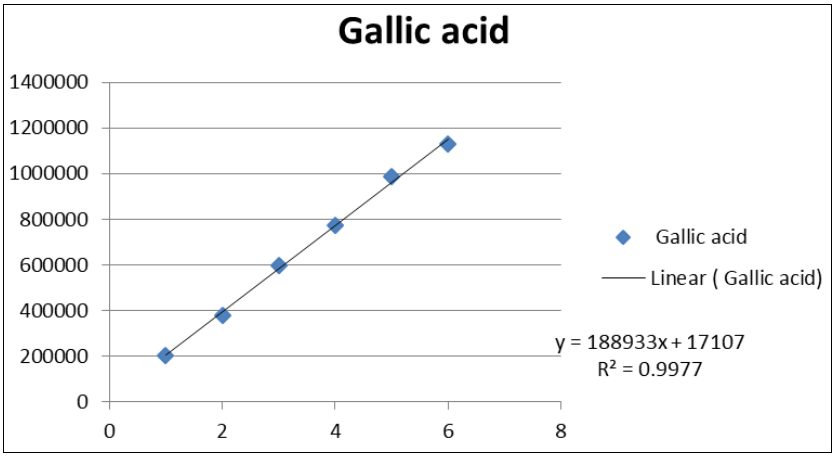


Fig 6: Calibration curve between Area of peak GA verses its Concentration

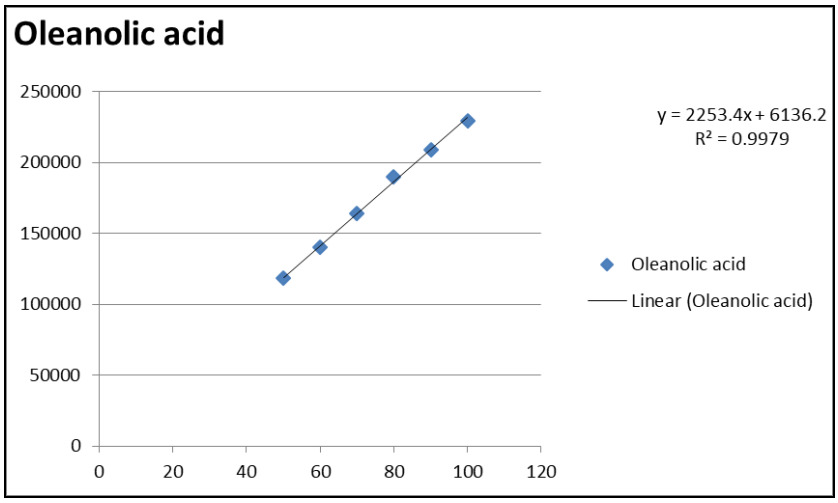


Fig 7: Calibration curve between Area of peak OA verses Concentration.

4.3.2 Precision data

Table 9: Interday and Intraday precision data.

Marker	Concentration (µg/ml)	Intraday (n=3)			Interday (n=3)		
		Area ± SD	% RSD	%RSD of RT	Area ± SD	% RSD	%RSD of RT
Gallic acid	1	252159 ± 3911.02	1.5510	0.248	272457 ± 5289.85	1.9415	0.582
	4	717412.7 ±7355.19	1.025	0.102	723515 ± 7016.34	0.9698	1.388
	6	1239953 ±27936.93	2.2530	0.529	1082755 ± 41290	3.8134	0.600
Oleanolic acid	50	104470 ± 2265.33	1.2174	0.768	122503 ± 1728.42	1.4109	1.0217
	80	155964± 8882.91	1.5507	0.566	195600 ± 2910.17	1.4878	0.181
	100	245101.3 ± 16488.8	0.7220	0.256	228147 ± 5850.26	2.564	0.117

Limit: % RSD of RT should be less than 2.0 and for area NMT 5.0

Here both the markers in combination mixture at lower, middle and higher concentration range showed %RSD of Retention time and Peak area in limit specified in ICH guideline.

4.3.3 Accuracy

Accuracy was performed by recovery study where a known concentration of markers were to be added and calculated the amount to be recovered which shown in following table.

Table 10: Recovery study of HPLC method.

Markers	Initial Amount (A)	Addition of known quantity (B)		A+B	Amount recovered (mg)	% Recovery	Accepted % Limit for Recovery
Gallic acid	0.031	80%	0.025	0.0558	0.0561	100.54	98-102%
		100%	0.031	0.062	0.0619	99.84	
		120%	0.0372	0.0682	0.0689	101.03	
Oleanolic acid	0.01	80%	0.008	0.018	0.0182	101.1	
		100%	0.01	0.02	0.0198	99	
		120%	0.012	0.022	0.0219	99.54	

4.3.4. Robustness data

Table 11: Robustness data for method validation.

Param eters	Changes	Concentration in µg/ml		Retention time(RT) in minute		RSD of RT		Area Under Peak		RSD of Area	
		GA	OA	GA	OA	GA	OA	GA	OA	GA	OA
Flow rate	0.9 ml	4	80	3.136	10.987	0.08	0.05	1007007	234933	0.15	1.40
	1 ml			2.827	9.93	0.10	0.41	769777	191644	1.11	0.62
	1.1 ml			2.56	9.020	0.11	0.07	828029	193256	0.23	0.6
Detection wavelength	221 nm			2.827	9.805	0.089	0.76	839239	230555	2.285	0.76
	222 nm			2.835	9.687	0.058	0.26	775443	189978	1.154	0.12
	223 nm			2.817	9.751	0.23	0.45	725557	156890	1.852	0.25
Mobile phase composition	90: 10			2.829	19.3	0.35	0.21	243330	137715	2.012	1.87
	98 : 2			2.804	7.5	0.21	0.14	238514	129056	1.478	2.45
	97 : 3			2.826	7.916	0.41	0.45	256412	156256	0.75	1.89

For changes in mobile phase combination, flow rate and detection wavelength, the results showed that the % Relative Standard Deviation of RT and Peak area passed the specified limit as per ICH Guideline. Therefore, method should be robust.

4.3.5 LOD and LOQ

Table 12: Sensitivity of method.

Parameters	Gallic acid	Oleanolic acid
LOD	0.012	1.2116
LOQ	0.039	3.6723

4.4 Quantification of Markers in developed polyherbal tablet

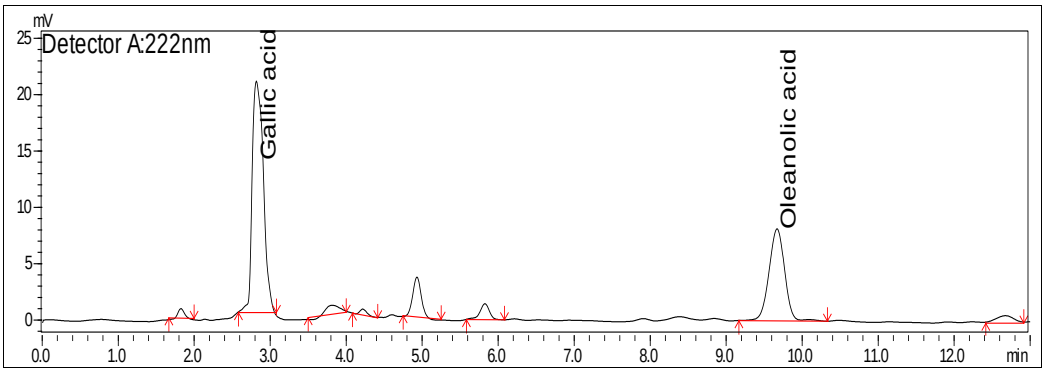


Fig 8: HPLC Chromatogram for developed polyherbal tablet.

Table 13: Quantification of markers in laboratory formulated tablet.

Sample	Amount	
	Gallic acid	Oleanolic acid
Polyherbal tablet	0.031%	0.01%

5. Summary and Conclusion

Table 14: Summary of validation data

Parameter	Gallic Acid	Oleanolic Acid
Analytical wavelength (nm)	222 nm	
Beer's range (µg/ml)	1-6	50-100
Regression equation	y=188933x + 17107, R^2=0.9977	y= 2253.4 x + 6136.2, R^2=0.9979
Intraday precision (%RSD)	0.96	1.21
Inter day precision (%RSD)	1.025	1.48
LOD	0.012	1.2116
LOQ	0.039	3.6723
Accuracy(Recovery)	99.84%	99.54%
Assay	0.03%	0.01%

The developed RP-HPLC method will assist in the standardization of Polyherbal formulation as well as quantification of markers in raw material which may prove the inferior quality of raw material in herbals. The proposed HPLC method for simultaneous estimation of Gallic acid and Oleanolic acid seems to be accurate, precise, reproducible and repeatable. The validation data indicated that the method was reliable. Here quantification of both markers were done in laboratory based formulation (tablet). With the growing demand for herbal drugs and with increased belief in the usage of herbal medicine, this standardization tool will help in maintaining the quality and batch to batch consistency of various herbal formulations.

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