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QUANTITATIVE ANALYSIS OF ANDROGRAPHOLIDE IN A STANDARDISED HERBAL EXTRACT USING THE UPLC TECHNIQUE

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ABSTRACT

The objective of this study was to establish and authenticate a UPLC method for determining the level of Andrographolide in herbal formulation that include extract of Andrographics Paniculata (Burm f.). The Ultra Performance Liquid Chromatography (UPLC) technique was developed and subsequently validated to facilitate the efficient and uncomplicated determination of Andrographolide in different extracts. The experimental setup employed for this study consisted of a high-quality Nano UPLC Waters separation module, which was equipped with a PDA detector (190-700). The column employed for the experiment was an ACQUITY UPLC Bridge (C18 180 x 130mm, 1.7 - 3.5 µm). To make mobile phase A, 40 percent acetonitrile (ACN) and 60 percent phosphoric acid (0.1 percent) were mixed. Mobile phase B, on the other hand, was made by combining 70% methanol with 0% phosphoric acid (0.1%). Adjusting the flow rate to 0.5 ml/min and keeping the detecting wavelength at 230 nm

required constant attention. The findings indicated that 0.15 ng/ml was the lower limit of detection (LOD) and that 0.27 ng/ml was the lower limit of quantitation (LOQ). A coefficient of determination (r2) of 0.987 indicated that the linearity of the calibration curve was in the range of 0.01-0.1 g/mL. The approach has a relative standard deviation (RSD) of 1.34% and an accuracy of 96.89% to 100.2%. We calculated an intra-day accuracy of 6.15% and an inter-day precision of 6.53%. The currently-being-developed herbal Nano emulsion is anticipated to be standardised using the validated approach.

KEYWORDS: Andrographics Paniculata, validation, UPLC, Andrographolide,

INTRODUCTION

Individuals diagnosed with diabetes mellitus have been found to exhibit heightened levels of Reactive Oxygen Species (ROS), which may have the potential to compromise the functionality of the cellular antioxidant system. This condition possesses the capacity to give rise to complications linked to diabetes mellitus. Prior research has demonstrated that the concurrent administration of an oral antidiabetic agent alongside an antioxidant holds promise in the treatment of diabetes mellitus and its related complications. Numerous studies have provided evidence regarding the effectiveness of Andrographics Paniculata as a therapeutic agent for diabetes, supported by references.[1,5] Additionally, its antioxidant properties have been well-documented. The herb Meniran, also referred to as Phyllanthus niruri in scientific literature, is a botanical species recognised for its medicinal attributes. It has a long-standing history of traditional usage in countries such as India, China, and Indonesia, where it is valued for its therapeutic properties. Phyllanthus niruri has been employed for its therapeutic attributes, encompassing its potential as a laxative, capacity to dissolve kidney stones, diuretic properties, treatment of hepatitis, management of dysentery, and alleviation of rheumatism. The scientific community has provided evidence of the antioxidant properties of Phyllanthus niruri, along with its documented antibacterial, antidiabetic, and anticancer activities.[6],[7] A recent accomplishment herbal commercialisation industries has been made in the formulation of a self-nanoemulsifying drug delivery system (SNEDDS) incorporating a blend of A. Paniculata extract (APE) and P. Niruri extract (PNE). The utilisation of a Nano emulsion system has yielded numerous favourable consequences. Initially, the extract's necessary dosage has been significantly decreased. Moreover, it has augmented the efficacy of the extract as both an antidiabetic and antioxidant agent. Furthermore, this system has played a significant role in enhancing patient receptiveness

towards the utilisation of traditional medicine. According to the regulations established by the Indian government, the term Standardised Herbal Medicine (SHM) pertains to a formulation consisting of natural substances that have undergone preclinical trials in order to determine their safety and effectiveness. Furthermore, the raw materials employed in the production of SHM have undergone a rigorous standardisation procedure. The incorporation of traditional medicine formulations, such as SHM, holds promise for augmenting the calibre and efficacy of traditional medicinal products currently accessible in the marketplace. Furthermore, it has the potential to enhance the perception and overall portrayal of traditional medicine, thereby promoting patient adherence. This regulation is in accordance with the guidelines established by the World Health Organisation (WHO), which mandate that herbal products must undergo standardisation prior to their introduction into the market. This measure is of utmost importance in ensuring the safety and efficacy of the product. The standardisation of raw materials within the domain of herbal medicine plays a pivotal role. Its purpose is to guarantee a uniform biological activity and chemical composition of the product. Moreover, it functions as a significant instrument for ensuring quality control in the production and manufacturing procedures of herbal pharmaceuticals. Numerous methodologies have been devised for the quantitative assessment of Andrographolide. Maiti has incorporated the application of spectrophotometry to quantify the concentration of Andrographolide in the extract derived from A. Paniculata.[8],[9] The procedure is predicated on the visual detection of a crimson hue that arises from the creation of a chemical compound following the introduction of sodium hydroxide. Nevertheless, it is important to acknowledge that this phenomenon of colour complexity is intrinsically unstable and susceptible to swift dissipation. The quantification of Andrographolide can be achieved using the gravimetric method as described in the Pharmacopoeia of India. Furthermore, the implementation of lactone titration, as proposed by Aromdee, represents an alternative and feasible method. Nevertheless, it is crucial to acknowledge that this approach is recognised for its propensity to consume a significant amount of time. Two additional methodologies were introduced, specifically introduced two supplementary methodologies, namely High-Performing thin-layered chromatography (HPTLC) and to reverse Phase high-performance liquid chromatography (RP-HPLC), which employed a gradient elution system.[10] The HPTLC method utilised a solution containing 1% formic acid and methanol, while the RP-HPLC method employed a mixture of ACN: methanol and 0.1% phosphoric acid with a gradient elution.[12] The study titled "Pharmacokinetic and Pharmacodynamics Herb–Drug Interaction of Andrographics Paniculata (Knees) Extract and Andrographolide with Etoricoxib after Oral

Administration in Rats, investigates the potential interactions between Andrographics Paniculata (Knees) Extract, Andrographolide, and Etoricoxib in rats, focusing on their pharmacokinetic and pharmacodynamics properties.[13] The current study focused on the analysis of Andrographolide using a mobile phase consisting of solvent A. Solvent A was composed of a mixture of acetonitrile and 0.1% phosphoric acid in a ratio of 40:60. Furthermore, solvent B was utilised, comprising a blend of methanol and 0.1% phosphoric acid in a proportion of 70:30. A flow rate of 0.5 ml/min was employed, and the analysis was observed at a wavelength of 230 nm. The aim of this study was to develop a reliable methodology for the measurement of Andrographolide in herbal extracts using Ultra-Performance Liquid Chromatography (UPLC).

MATERIALS AND METHODS

Chemical substances and reagents The experimental materials used in this study included Andrographolide with a purity greater than 98%, Gallic acid with a purity greater than 98% which was used as an internal standard (IS), acetonitrile of gradient grade for liquid chromatography from (Merck), methanol of gradient grade for liquid chromatography from (Merck), 85% phosphoric acid from (Merck). The herbs A. Paniculata and P. Niruri were procured from the market located in Bengaluru, India.

THE PROCESS OF EXTRACTING ANDROGRAPHOLIDE FROM HERBAL SOURCES

A total of 100grammes of dry powder containing extracts of A. Paniculata (APE), P. Niruri (PNE), and Andrographolide herbal tablet extract were subjected to maceration using petroleum ether in a ratio of 5:1 with respect to the weight of the crude herbs.[14] The dried pulp was subsequently subjected to maceration using 70% ethanol in a ratio of 10 times the weight of the crude herbs. This process lasted for a duration of 5 days, with occasional stirring. The filtrate underwent evaporation using a vacuum rotary evaporator in order to obtain a substance with a high viscosity known as an extract.

THE PROCESS OF PREPARING A SAMPLE FOR ANALYSIS OR EXPERIMENTATION

A precise measurement of 100.0 mg of Andrographolide extract was conducted using a weighing bottle, followed by dilution with methanol to a final volume of 5.0 ml. The solution underwent a 10-minute ultrasonic treatment in order to facilitate degassing and enhance dilution. The sample underwent filtration, and subsequently, the resulting filtrate was passed

through a micropore syringe filter with a pore size of 0.25µm. A 50µl portion was extracted and subsequently supplemented with methanol to reach a final volume of 1000µl. The prepared solution was poised for introduction into the separation system.

THE PROCESS OF GENERATING A STANDARD CURVE AND PREPARING QUALITY CONTROL (QC) SAMPLES

1.1 Preparation of a standard stock solution of Andrographolide at a concentration of 1mg/ml.

A precise measurement of 10mg of gemcitabine, which conforms to the API Standard, was performed and subsequently transferred into a desiccated 10ml volumetric flask. The gemcitabine working standard solutions were prepared by diluting with a small amount of acetonitrile and making up to water, followed by sonication for 10 minutes.

1.2 Preparation of the internal standard stock solution The concentration of Gallic acid in the solution is 1 milligram per millilitre

The API Standardised Gallic acid was precisely weighed at a quantity of 10mg and subsequently transferred into a 10ml volumetric flask that had been previously dried. The working standard solutions of Gallic acid were prepared by diluting a small amount of the compound with the mobile phase and adjusting the volume accordingly. The resulting mixture was then subjected to sonication for a duration of 10 minutes.

The parameters pertaining to the chromatographic system

A solution A with a volume of 100 µL was introduced into the vial, and subsequently, 900 µL of methanol was added to attain a final volume of 1.0 mL. A volume of 20 µL was introduced into the system and subsequently separated using a mobile phase composed of acetonitrile and 0.1% phosphoric acid in a proportion of 40:60. The elution process was conducted using an isocratic system at a flow rate of 0.5 mL/min. The detection was observed at a wavelength of 230 nm. The optimisation procedure resulted in (table:1) an elution time of 20 minutes.

The procedure for validating the dependability and precision of an analytical technique

The chromatographic method underwent comprehensive validation in accordance with the protocols specified in the International Council for Harmonisation (ICH) guidelines for the testing of analytical methods. ICH 2008.[15]

Specificity

The concept of specificity refers to the ability to accurately identify and analyse a particular analyte, even in the presence of other components that are expected to be present. In order to assess the specificity of the method, two samples were prepared. One of the samples consisted of a placebo formulation without any added substances (referred to as the unspiked sample), while the other sample contained the placebo formulation spiked with a single phytomarker. The level of specificity was assessed by conducting a comparison between the chromatograms acquired from unspiked samples and those obtained from samples that were spiked with individual standards.

Linearity,

The Andrographolide stock solution with a standard concentration of 100µg/ml was appropriately diluted using the mobile phase to obtain concentrations of 1.5, 3.125, 6.25, 12.5, 25, 50, and 100µg/ml. The concentrations were meticulously prepared and subsequently injected into the chromatograph for replication. Linearity is assessed through the utilisation of the linear least-squares regression model, incorporating the weight factor x. The present study employs statistical analysis utilising prism 9.0. A significance level of 5% was employed for the purpose of evaluation.

The concepts of limit of detection (LOD) and limit of quantification (LOQ) refer to the lowest concentration or amount of a substance that can be reliably detected or quantified using a given analytical method.

The evaluation of this method was conducted by determining the correlation coefficient and intercept values. The limits of detection (LOD) and limits of quantification (LOQ) were calculated using a formula that relies on the standard deviation of the intercept and the slope denoted as S. The limit of detection (LOD) can be calculated using the formula LOD = 3.3 x σ (n-1)/slope, where σ represents the standard deviation and n represents the sample size. Similarly, the limit of quantification (LOQ) can be calculated using the formula LOQ = 10 x σ (n-1)/slope. The standard deviation, denoted as σ, was utilised in the following equations to determine the limit of detection (LOD) and the limit of quantification (LOQ).

Precision

The precision of the experiment was assessed by conducting triplicate measurements on quality control samples containing concentrations of 1, 10, 20, 50, and 100 µg/ml of Andrographolide. These measurements were performed at various time intervals (2, 4, and 6 hours) and on different days. The method was assessed based on its intraday precision,

specifically the relative standard deviation (RSD) of the method. The estimation of intermediate precision was conducted by calculating the relative standard deviation (RSD) of the analysis of samples that were prepared at the same concentration but analysed at different times and on different days, at various concentration levels. Intraday precision, on the other hand, was determined by calculating the RSD of the analysis conducted at the same concentration levels within the same day, but at different time intervals.

Accuracy

The accuracy of the method was assessed by the addition of a predetermined quantity of Andrographolide, with a sample size of 5. The recovery studies were conducted using the standard addition method, wherein the sample solution of known concentration (formulation) was subjected to varying concentrations (60%, 80%, and 120%). The solutions were prepared and subsequently subjected to analysis, resulting in the calculation of the total amount recovered. The acceptance criterion for the mean recovery of the target concentration is set at 100 ± 5%.

Evaluation of Robustness

The evaluation of the method's robustness involved an analysis of the system suitability parameters subsequent to variations in the pH of the mobile phase (± 5%), organic solvent content (± 5%), flow rate (± 5%), and wavelength (± 5%). None of these modifications resulted in any significant changes in peak area or retention time. This study aims to investigate the robustness of the method. A factorial design with 22 factors is employed to assess the robustness of chromatographic separation. The experimental design employed in this investigation aimed to minimise the number of experiments while examining multiple parameters. The investigation focused on the acetonitrile contents of the mobile phase, pH, column temperature, and flow rate. The gemcitabine samples are run in a random manner. The chosen response was resolution (Rs) and the tailing factor (TfL-D). The data presented in brackets indicate.

RESULTS AND DISCUSSION

OPTIMISATION OF CHROMATOGRAPHIC METHODS AND QUANTIFICATION OF ANDROGRAPHOLIDE

Preliminary experiments were performed utilising different combinations of methanol, water, and acetonitrile as mobile phase constituents. However, satisfactory separation was not attained. Several gradient methods were also attempted using these solvents, however, the

desired separation with peak shape was not successfully achieved. Subsequently, a series of experiments were conducted to evaluate the efficacy of various ratios of methanol and acetonitrile with 1% orthophosphoric acid buffer at different pH levels in order to enhance the resolution of phytomarker. The table below displays the completed gradient mobile phase programme (Table 2). The sample solutions were analysed using optimised chromatographic conditions to quantify both the phytomarker present in the formulation and a mixture of standards the API samples chromatograms as show in (Figs. 1a and b). The quantification of individual raw materials utilised in the formulation preparation, namely A. Paniculata (APE) dry extract, P. Niruri (PNE) dry extract, and Andrographolide herbal tablet extract, was also conducted using specific phytomarker (refer to Figs. 2a and 2b). The results obtained from the calibration curve of Andrographolide are presented in (figs:3).

Validation of the analytical method that has been developed

Specificity

The demonstration of specificity involved the comparison of chromatograms obtained from standard and sample solutions, as shown in (Fig 1). Based on the findings of this study, it is evident that both phytomarker were successfully resolved without any interference from the sample matrix.

System suitability refers to the assessment and evaluation of a system's ability to meet specific requirements and perform effectively within its intended environment.

The system suitability of the developed method was verified by calculating several chromatographic parameters, including the number of theoretical plates (N), resolution (Rs), tailing factor (T_f), and capacity factor (k′), using the chromatogram of standard solutions. The obtained results are presented in Table 4. The system suitability parameters have been verified, confirming that the provided chromatographic conditions are suitable for both method development and validation.

Linearity

The calibration curve had a good linearity in the concentration range of 1–2000 ng/ml. The linear regression equation for Andrographolide was: Y= 0.0150887 × X - 0.0132206 (r 2 = 0.987). data as show in (Figure:2b)

Limit of detection and limit of quantification

The LOD and LOQ values were determined and results obtained are shown in Table 6.

Precision

The intra-day precisions were assessed six times per day, yielding a % relative standard deviation (RSD) ranging from 0.6078% to 1.5111%. Similarly, the inter-day precisions were evaluated over six consecutive days, resulting in a % RSD ranging from 0.5602% to 1.1906%. The precision study yielded results that are presented in Table 6.

Accuracy

The assay method demonstrated a high level of accuracy, as indicated by the relative recovery measurements at three different concentration levels, which ranged from 98.96% to 101.39%. Additionally, all values for the relative standard deviation (RSD) were found to be equal to or less than 2%. The results of the recovery study are presented in Table 7.

Robustness

The robustness of the developed method was assessed by intentionally modifying the experimental conditions. Specifically, the flow rate was adjusted to 0.9 mL/min and 1.1 mL/min, while the column temperature was varied between 38 °C and 42 °C. The results obtained from the robustness study are presented in Table 8.

Tables

Table: 1 chromatographic conditions for development method.

Parameters	Values
Mobile phase	Methanol, Acetonitrile: 0.1% OPA (30:70) pH- 5.0 with OPA
Detection Wavelength	230nm
Flow rate	0.7ml/min
Sample Injection volume	20µl
Temperature	40^{o}C
Needle wash	Water HPLC grade
Run time	15min

Table: 2 Optimised gradient mobile phase.

S.no	(%) ratio of mobile phase		Time (minute)
	Mobile phase A (ACN and Methanol)	Mobile phase B (0.1%OPA)	
1	25	75	0
2	40	60	5
3	40	60	8
4	30	70	12
5	50	50	15
6	50	50	20

Table: 3 Applications of development methods for polyherbal formulations.

Name of extract	Assay %(w/w) Andrographolide
A. Paniculata (APE)	
P. Niruri (PNE)	
Andrographics tablet	

Table: 4 System suitability factors of the Andrographolide extracts.

S.no	Parameters	Andrographolide
1	RT	3.140
2	(N) theoretical plate	920875
3	Resolution	1.625
4	Capacity	3.391
5	Tailing factor	1.0814

Table: 5 Results of LOD and LOQ for developed method.

S.no	Parameters	Andrographolide
1	LOD	0.15µg/ml
2	LOQ	0.27 µg/ml

Table: 6 precision Intra-day and inter-day results of developed method.

	Intra-day (n=5)				Inter-day(n=5)			
Level	Concentration added (ng/ml)	mean concentration (ng/ml)	RE %	RSD%	mean concentration (ng/ml)	RE %	RSD%	Recovery (%)
LOQ	250	254.67	2.31	6.24	255.48	2.23	5.46	80.45
MOQ	3000	2984.36	-3.2	5.91	2952.56	-4.6	7.6	82.21
HOQ	8000	8141.76	4.7	6.51	8080.48	2.36	5.86	76.35
IS	_							80.75

Table: 7 percentage of recovery for Andrographolide.

Level of concentrations (µg/ml)	Amount found (µg/ml)	(%) of recovery	RSD (%)
6.25	6.21±05	99.36	0.15
12.5	11.93±67	95.44	1.52
25	24.85±22	99.4	0.54
50	49.71±11	99.42	0.49
100	99.53±94	99.53	0.42

Table: 8 Robustness results of developed method.

Parameters	(%) Deliberate changes	(%) RSD Andrographolide
Flowrate	0.75±25	1.182
Column temperature (ºC)	25ºC±12	1.072

Figures

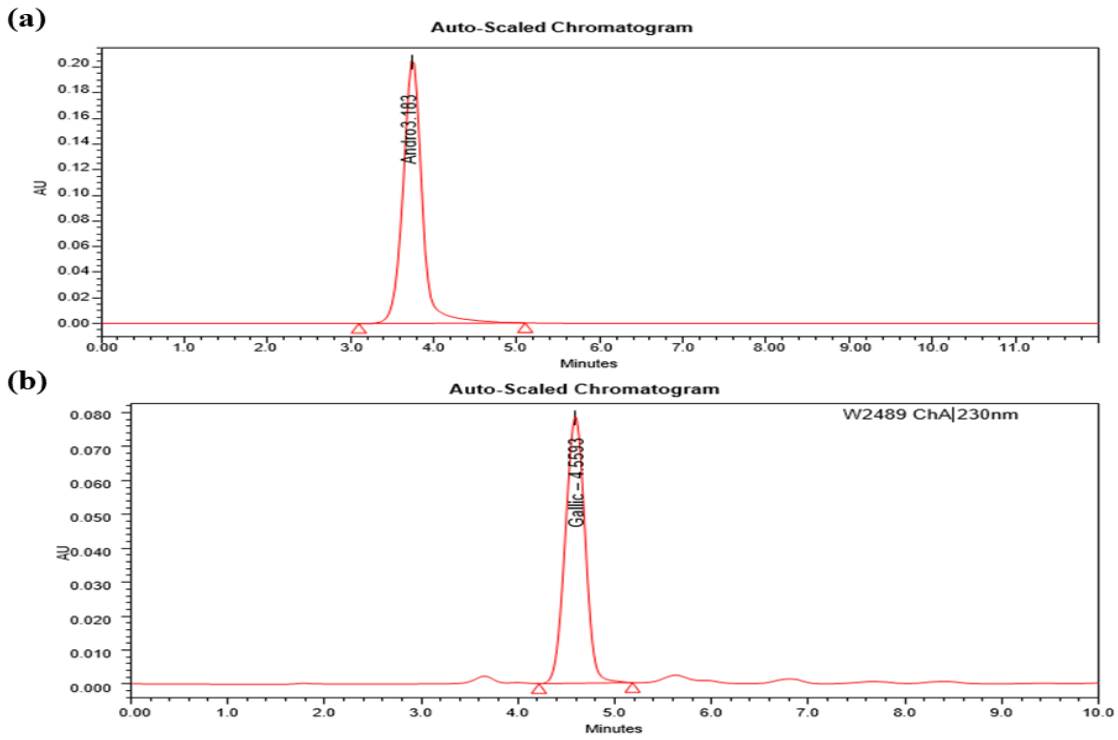


Figure: 1 Reprenststive chromatogram UPLC (a) standard Andrographolide (b) Gallic acid (IS).

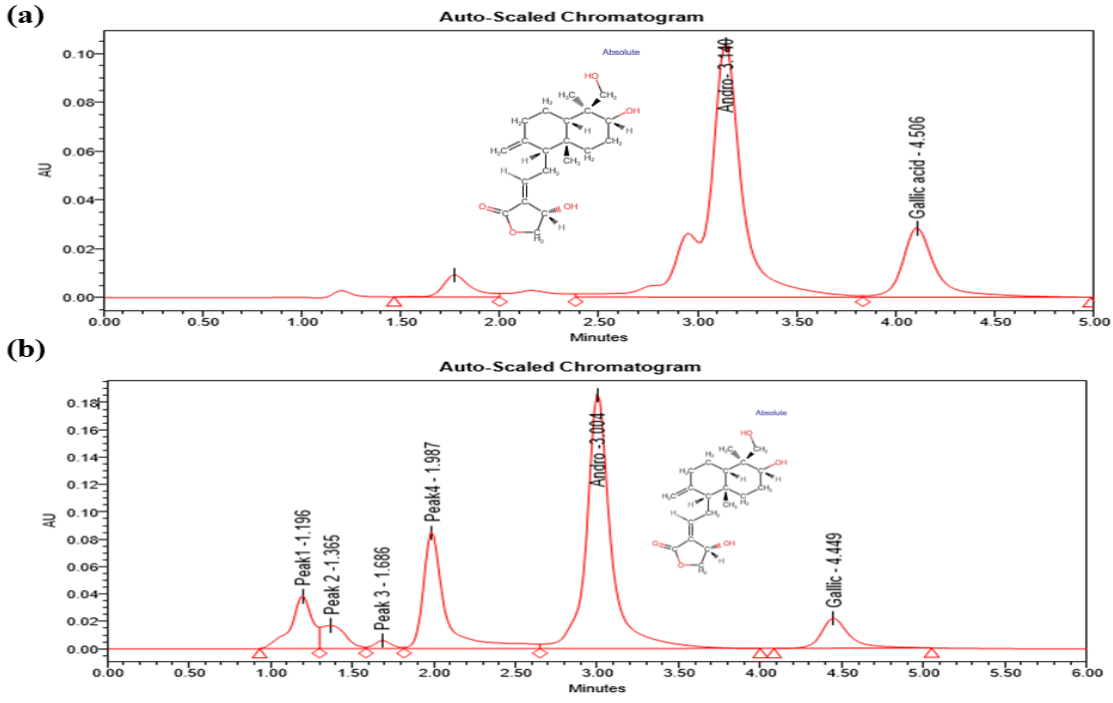
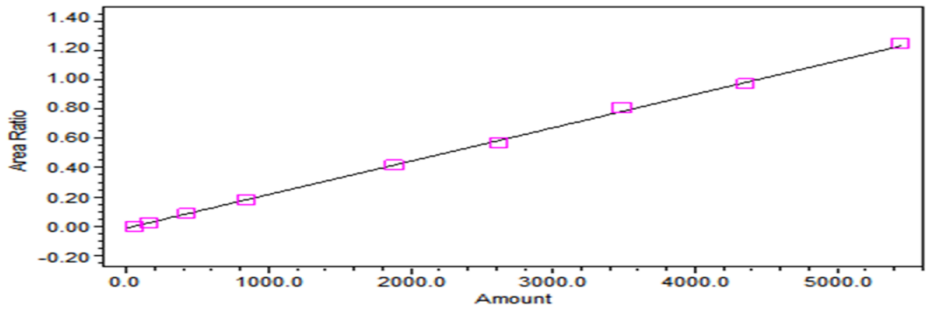


Figure: 2 Reprenststive chromatogram UPLC (a) standard Andrographolide with IS (b) herbal extract samples.



Conce (µg/ml)	Amount found (µg/ml)	(%) of recovery	RSD (%)
0.375	0.36±68	98.61	0.97
0.75	0.74±93	99.37	0.64
1.562	1.48±18	94.75	1.62
3.125	3.09±34	98.88	0.37
6.25	6.21±05	99.36	0.15
12.5	11.93±67	95.44	1.52
25	24.85±22	99.4	0.54
50	49.71±11	99.42	0.49
100	99.53±94	99.53	0.42

Figure: 3 Reprenststive chromatogram Calibration curve of Andrographolide.

CONCLUSION

In order to ensure the quality of the finished product, it is imperative to employ reliable scientific methods that have not been previously documented, given the intricate nature of the polyherbal formulation. The proposed research aims to develop and validate an analytical method for quality control of a polyherbal laxative formulation. The UPLC method that has been developed and validated will be instrumental in the quality control of polyherbal laxative formulations through the utilisation of biologically active phytomarker. The UPLC method that has been developed for the simultaneous estimation of Andrographolide demonstrates accuracy, precision, reproducibility, and repeatability. Given the escalating demand for herbal drugs and the growing confidence in their efficacy, it is imperative to establish a robust standardisation tool to ensure the consistent quality of these crucial polyherbal preparations.

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