

DEVELOPMENT OF METHOD FOR EXTRACTION AND VALIDATION CRUDE DRUGS FROM PLANT EXTRACT**Apurva Darwai*, Neelam Jain****Department of Pharmaceutics, Faculty of Pharmacy, Oriental University, Indore-453555, Madhya Pradesh, India***Corresponding Author's E mail: apurwa0409@gmail.com

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<https://dx.doi.org/10.38164/AJPER/11.3.2022.47-67>**ABSTRACT**

This research is focused on development of a modified, simple, accurate, and economical HPLC method for quantitative determination of selected herbal drugs. Use of herbal drug for the treatment of various diseases have intensively increasing due to its economical nature as well as less side effects as compared those seen in other conventional medications. With increasing use of these drugs, a define method is been required for standardization of these Polyherbal medication to maintain its quality, safety, and efficacy. Method development for proper standardization of various phytochemical analysis along with HPLC method development was conducted to analyse the extract obtained for the plant. Method validation was conducted by analysing the method for its linearity, recovery, method and system precision, limit of detection (LOD), limit of quantification (LOQ) and robustness in accordance with ICH guidelines Q2 (R1). Percentage purity of the extract obtained was found to be 98% with presence of 23.86 mg of extract. Linearity study followed a range of 2 - 12 µg/ml with correlation coefficient 0.9996. Precision study conducted for both inter-day and intra-day depicted a % RSD of 0.56% and 0.171% respectively indicating that the proposed method was more accurate than the existing methods. The LOD and LOQ value for Piperine was found to be 0.126 µg/ml, 0.384 µg/ml, respectively. Thus resembles that the proposed method was sensitive.

Keywords: Validation, Precision, Polyherbal Formulation, Precision, Accuracy, LOD, LOQ.**INTRODUCTION**

Use of plant-derived products has been becoming increasingly popular as medicinal products, nutraceuticals, and cosmetics in recent years, and are now available over the counter in health food stores and pharmacies as self-medication or as drugs prescribed in non-allopathic systems¹. Herbal medicines, which are widely used in health care in both developed and developing countries, are complex chemical mixtures made from plants that are limited in their effectiveness because they are poorly absorbed when taken orally². Herbal drugs have been used as

medicines for various diseases since ancient times. Medicinal plants have played an important role in global health. Despite significant advances in modern medicine in recent decades, plants continue to play an important role in health care³. Natural products have proven to be the most effective source of medicines. Each plant functions as a factory, capable of producing an infinite number of highly complex and unusual chemical substances, the structures of which would otherwise escape the imagination⁴.

Herbal formulations are widely used as therapeutic agents for diabetics, arthritics, liver diseases, cough remedies, memory enhancers, and adaptogens. Herbal medicines are classified into three types, according to the WHO: raw plant material, processed plant material, and medicinal herbal products. Herbal drugs are labelled finished products that contain active ingredients such as aerial or underground plant parts, other plant material, or a combination of these, whether in their raw form or as plant preparations.

Herbal preparations that are used for the preparation of complete herbal medicines and might comprise comminuted or powdered herbal ingredients, as well as extracts, tinctures, and fatty oils of herbal materials. They are created through extraction, fractionation, purification, concentration, or other physical or biological processes. Herbal preparations created from one or more herbs are considered finished herbal goods. When more than one herb is utilized, the term "mixture herbal product" can also be used. Excipients, in addition to the active components, may be present in finished herbal products and herbal mixtures.

As the use of herbal therapy has been growing commercially, ensuring the safety, purity, and efficacy of medicinal plants and herbal products has become increasingly crucial. Although, Herbs have historically been thought to be innocuous, and they are increasingly being used by people who do not have a prescription. However, some can be harmful to one's health, while others are ineffective and may interact with other medications⁵. The identity of the plants and seasonal variation (which affects the time of collection), ecotypic, genotypic, and chemotypic variations, drying and storage conditions, and the presence of xenobiotics are all essential elements in the variation of herbal raw material. Standardization of herbal formulations is required in order to determine medicine quality based on the concentration of active principles⁶.

Standardization of herbal drugs

According to WHO, Standardization and quality control of herbs is a process involving physicochemical evaluation of a raw drug, which includes aspects such as raw material acquisition and processing, safety assessment, potency and stability of the finished product, safety and risk documentation based on experience, providing information about the product to the consumer, and its promotion. Standardization assures that each dose contains a predetermined amount of quantity, quality, and therapeutic impact of substances. Herbal products cannot be considered scientifically legitimate unless the medicine tested has been authenticated and described to assure reproducibility in product manufacturing⁷.

The phytochemical ingredients of herbal preparations determine their therapeutic activity. Scientists face a significant problem in developing legitimate analytical methods that can reliably profile the phytochemical composition, including quantitative studies of marker/bioactive chemicals and other key ingredients. In light of the foregoing, standardization is a key step in establishing a consistent biological activity, a consistent chemical profile, or simply a quality assurance programme for the development and manufacturing of a herbal drug ⁸. Authenticating herbal medications and distinguishing adulterants from genuine medicinal herbs are critical for both pharmaceutical firms and public health, as well as ensuring the reproducible quality of herbal medicine ⁹.

Phytotherapeutic agents, often known as phytomedicines, are standardized herbal preparations made up of complex mixes of one or more plants that are used in most countries to treat a variety of disorders. Standardization of herbal plants includes various analysis of different phytochemical profiles³. The phytochemical profile is especially important because it has a direct impact on the activity of herbal medications. The fingerprint profiles serve as a guideline to the drug's phytochemical profile in guaranteeing quality, while quantification of the marker compound/s would act as an additional metric in assessing sample quality. Phytochemical standardization includes all information generated about the chemical constituents included in a herbal drug.

Each traditional medical system has its own technique of standardization for ensuring quality, most notably in human linguistic terms. This method of evaluation must be considered while standardizing herbal medication ¹⁰. For quality control and standardization, most regulatory recommendations and pharmacopeias recommend macroscopic and microscopic evaluation, as well as chemical profiling of botanical materials ¹¹. In this regard, the Department of AYUSH of the Government of India established some parameters for Drug Development, Standardization, and Quality of Ayurvedic, Siddha, and Unani drugs, which include five protocols: Protocol-I (Standardization of Single Plant Material), Protocol-II (SOP of Extract Preparation), Protocol-III (Standardization of Plant Extract), Protocol-IV (SOP of Finished Product), and Protocol-V (SOP of Finished Product) (Standardization of Formulations) ¹².

Standardization of herbal products involves the use of Good Manufacturing Practices (GMP) ¹³. Furthermore, the investigation of different factors such as pharmacodynamics, pharmacokinetics, dose, stability, self-life, toxicity evaluation, and chemical profiling of herbal formulations is deemed necessary ¹⁴. Other considerations include pesticide residue, aflatoxins concentration, heavy metal contamination, and Good Agricultural Practices (GAP) in herbal drug standardization⁴⁰. Standardization of herbal raw medications includes passport data of raw plant drugs, botanical authentication, microscopic and molecular investigation, chemical composition identification using various chromatographic techniques, and biological activity of the entire plant ¹⁵. For quality control and standardization, macroscopic and microscopic evaluation, as well as chemical profiling of herbal materials, have been used ¹⁶. The macroscopic identity of medicinal plant materials is based on sensory evaluation factors such as shape, size, color, texture, odor, and taste, whereas microscopy comprises a comparative microscopic examination of powdered herbal medication.

WHO Guidelines for Herbal Drugs Standardization and Evaluation

1. Preliminary Evaluation- Sampling, foreign determination, and total fiber determination
Color, odor, taste, size, shape, and other traits are all evaluated morphologically.
3. Microscopical evaluation- Qualitative histological assessment of tissue kinds, quantitative assessment of palisade ratio, vein-islet, vein termination, stomatal index, stomatal number, and Lycopodium spore technique
4. Physical qualitative evaluation- Solubility, refractive index, optical rotation, melting and boiling points, density, viscosity, chromatographic and spectroscopic evaluation:
5. Physical quantitative evaluation- assessment of ash values, extractive values, moisture content, and volatile oil.
6. Chemical Evaluation-To detect various kinds of phytochemicals, quantitative determination of phytochemicals, and assay
7. Biological Evaluation: Swelling Index, Hemolytic Index, Bitterness Value, Foaming Index, Total Tannins Value
8. Toxicological Evaluation- Pesticide determination, arsenic, and heavy metal determination, radioactive contamination determination, aflatoxins determination
9. Analytical Evaluation- Chromatographic-TLC, Paper, HPTLC, HPLC AND GC, and spectroscopic evaluation.

MATERIALS AND METHOD

Collection and authentication of plant

The plant material employed for method development and validation is dried fruits obtained from the local market. Post collection, it was authenticated by. They are dried for 15 days in the shade, separately powdered, and passed through sieve no.40 before being kept in well-closed airtight containers for future use. The powder was branded after being produced from plant parts acquired from a local market in New Delhi. The plant powder purchased from the supplier was marked as powder ¹⁷.

Extraction

The shade dried coarsely powdered plant portion was extracted with 80% aqueous methanol by maceration at room temperature for 72 hours. Following the end of the extraction, the extract was filtered and concentrated to dryness in a rotavapor at decreased pressure and controlled temperature (40-50°C). The residue was then placed in a desiccator. Hydroalcoholic extract prepared from plant components was labeled as sample extract, while extract obtained from a source was labeled as a standard extract ¹⁷.

PHYSICOCHEMICAL PARAMETERS:

Ash value:

Ash values can be used to determine the quality and purity of a sample. As a result, this test is extremely important in the evaluation of raw medicines. Total ash and acid insoluble ash are the two types of ash values. The total ash is largely composed of inorganic components such as carbonates, sulfates, and potassium, sodium, and magnesium silicates. Sulfated ash is produced by treating a plant sample with concentrated sulphuric acid and filtering the solution. However, the majority of the variables are soluble in concentrated sulfuric acid. To address the issue of soluble ash, acid insoluble ash is being developed. All powdered plant materials were passed through sieve no 40 to determine various ash values such as total ash and acid-insoluble ash.

Total ash:

1gram of the powdered drug was weighed and taken in a tarred silica crucible and weighed. The powdered drug was incinerated gradually by increasing the heat not exceeding dull red heat, cooled, and weighed. The obtained charred mass was exhausted with hot water and the residue was collected on ashless filter paper. The process was repeated until the successive weights differed not more than 0.5 mg (ignition to constant weight). The percentage of ash with reference to the air-dried drug was calculated and tabulated ¹⁸.

Acid insoluble ash:

The total ash was boiled for 5 minutes with 25 ml of dilute hydrochloric acid and the insoluble matter was collected on an ash-less filter paper wet with hot water ignited and weighed. The percentage of acid-insoluble ash was calculated with reference to air-dried drugs and tabulated ¹⁸.

A) Loss on drying:

- A glass stoppered shallow bottle was weighed that had been dried in the same conditions to be employed in the determination.
- Nearly 1 gm of the sample was transferred to the bottle, closed, and accurately weighed. The samples were distributed as evenly as practicably gently side-wise shaking to a depth not exceeding 10 mm.
- The sample-loaded bottle was placed in a drying chamber by removing the stopper. The sample was dried to a constant weight, the drying chamber was opened and the bottle was closed and allowed to

cool.

- The bottle and the contents were weighed. The process was repeated until the successive weights differed not more than 0.5 mg (Drying to constant weight). The percentage loss of weight was calculated and tabulated ¹⁹.

Extractive value: Alcohol soluble extractive

About 5 grams of dried coarse powder with 100 ml of 90 % methanol was kept in a closed flask for 24 hrs, shaken frequently for 6 hours, and allowed to stand for 18 hours. Then it was filtered immediately to prevent loss of methanol. 25 ml of the filtrate was evaporated to dryness in a tarred shallow dish. The residue was dried at 105 °C and weighed. The percentage of alcohol-soluble extractives was calculated with reference to air-dried drugs and tabulated ¹⁹.

Water-soluble extractive

6gm of coarse powder of sample weighed and dissolved in 100 ml of water in a stoppered flask, heated at 80 °C, shaken well and allow to stand for 10 minutes, cool and add 2 gm of kieselguhr, filtered and transfer 5 ml of the filtrate to a tarred evaporating dish, evaporate the solvent on a water bath and the residue was weighed. The percentage of water-soluble extractives was calculated with reference to air-dried drugs and tabulated.

pH

A 10% w/v solution is prepared using water as a solvent. The values for both the extract and powders were determined and tabulated.

Qualitative analysis of phytochemical parameters:

The plant extract was subjected to the following tests for qualitative determination of various phytochemical constituents.

Test for carbohydrates:

A) Molisch's test

The extract was dissolved in 4 ml of distilled water and filtered. The filtrate was treated with 2-3 drops of 1 % alcoholic α -naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Appearance of brown ring at the junction of two liquids indicated the presence of carbohydrates.

B) Test for glycosides:

Small portion of extract was treated with dilute hydrochloric acid for few hours on a water bath and to the hydrolysate 1ml of pyridine and few drops of sodium nitroprusside were added and sodium hydroxide solution to make it alkaline. Presence of pink to red colour shows the presence of glycosides. This test is known as Legal's test.

C) Detection of fixed oils and fats:

Few drops of 0.5 N alcoholic potassium hydroxide was added to small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Formation of soap indicates the presence of fixed oils and fats.

D) Test for proteins and aminoacids: Ninhydrine test

The extract was treated with Ninhydrine reagent. Purple colour produced shows the presence of proteins and free amino acids.

E) Test for phenolic compounds and tannins:

Small quantity of the extract was taken in water and test for the presence of phenolic compounds and tannins was carried out with 5% Ferric chloride solution. Formation of violet colour shows the presence of tannins and phenolic compounds.

F) Test for alkaloids:

Small quantity of extract was separately treated with few drops of dilute hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. Formation of reddish brown precipitate shows the presence of alkaloids.

G) Test for flavonoids:

Small quantity of extract was dissolved in aqueous sodium hydroxide. Appearance of yellow colour indicates the presence of flavonoids.

METHOD DEVELOPMENT

A) Apparatus and chromatographic conditions:

Analysis using UV was performed on a Perkin-Elmer UV-VIS Double Beam Spectrophotometer. Data acquisition was made with software named as lambda 25 and 343 nm is used as wavelength for the study. HPLC analysis was performed on a chromatographic system of **Waters 2695** equipped with an auto injector with UV/Visible detector (UV-2489). A chromatographic separation was achieved on merck C18 analytical column (5 μ m, 250 mm x 4.6 mm, i.d). Data acquisition was made with Empower 3

software. Analytical Balance (BSA224S-CW, Sartorius), Ultra Sonicator (Fast Clean) from Shimadzu were used for the study.

B) Preparation of standard and sample solutions:

As the compound is freely soluble in ethanol standard stock solutions of standard were prepared in ethanol at a concentration of 100µg/ml. The aliquots of standard were prepared by using mobile phase.

Accurately weighed and transferred 100mg of the extract into a 100ml clean dry volumetric flask containing 10ml ethanol. The solution was sonicated for about 10 mins and then made up to volume with mobile phase. 10ml was pipette out from stock solution into separate 100ml volumetric flask and made the volume up to the mark with mobile phase.

C) Selection of mobile phase:

The method development and validation of Piperine extract (PE) requires greater resolution. Hence different solvent systems were tried.

D) Selection of flow rate: The flow rate of plant extract was tried from 0.8 ml to 1 ml.

Trial-1

Preparation of mobile phase: Mixture of water 300ml and 700ml of methanol was taken and degassed in ultrasonic water bath for 5 minutes. The solution was filtered through 0.45 µ filter under vacuum filtration.

Preparation of Standard Solution: About 25mg of PE working standard was weighed and transferred into a 25ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 10mins and then made up to volume with mobile phase. From this 0.5ml of PE was taken into separate 10ml volumetric flask and made up the volume to the mark with diluent.

Trail II

Preparation of mobile phase: A mixture of water 50ml and 950ml of methanol was taken and degassed in an ultrasonic water bath for 5 minutes. The solution was filtered through a 0.45 µ filter under vacuum filtration.

Preparation of Standard Solution: About 25mg of PE was weighed and transferred into a 25ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 20mins and then made up to volume with mobile phase. From this 0.5ml of PE was taken into separate 10ml volumetric flask and made up the volume to the mark with diluent.

METHOD VALIDATION:

The chromatographic conditions were validated by evaluating linearity, recovery, method and system precision, limit of detection (LOD), limit of quantification (LOQ), and robustness in accordance with ICH guidelines Q2 (R1).

Linearity:

Preparation of Standard Stock Solution: Accurately weighed and transferred 25mg of Plant extract working standard into a 25ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 10mins and then made upto volume with mobile phase. 10ml from the stock solution was taken in a 100ml volumetric flask and made upto volume with mobile phase, which contains 100ppm.

Preparation of Level: I

0.2ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with mobile phase.

Preparation of Level: II

0.4ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with mobile phase.

Preparation of Level: III

0.6ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with mobile phase.

Preparation of Level: IV

0.8ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with mobile phase.

Preparation of Level: V

1ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with mobile phase.

Preparation of Level: VI

1.2ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with mobile phase.

Procedure: Twenty microlitres of the above-prepared solution was injected through an auto-injector and the chromatogram was developed using Methanol: Water (95:5), as a mobile phase. The flow rate was maintained at 0.8ml/min and the column temperature was maintained at 25°C. Total run time was fixed as 20 minutes. The Detector wavelength was fixed at 343nm. Each level was injected into the chromatographic system and the peak area was measured. A calibration curve was plotted for the concentration v/s peak area and the correlation coefficient was calculated ²⁰.

Accuracy:

Preparation of 50% solution: Accurately weighed and transferred amount equivalent to 25mg of Plant extract working standard into a 25ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 10mins and then made up to volume with the mobile phase. 0.5 ml of the above stock solution was pipette out into 10ml of volumetric flask and diluted up to the mark with mobile phase this is 50ppm. Then 1ml of the above stock solution was pipette out into 10ml of volumetric flask and 1 ml of above standard stock solution of 50ppm concentration was added and diluted up to the mark with the mobile phase.

Preparation of 100% solution: Accurately weighed and transferred amount equivalent to 25mg of Plant extract working standard into a 25ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 10mins and then made up to volume with the mobile phase. 0.5 ml of the above stock solution was pipette out into 10ml of volumetric flask and diluted up to the mark with mobile phase this is 50 ppm. Then 2ml of the above stock solution was pipette out into 10ml of volumetric flask and 1 ml of above standard stock solution of 50ppm concentration was added and diluted up to the mark with the mobile phase²⁰.

Procedure: Twenty microliters of the above-prepared solution were injected through an auto-injector and the chromatogram was developed using methanol: water (95:5), as a mobile phase. The flow rate was maintained at 0.8ml/min and the column temperature was maintained at 25°C. The total run time was fixed as 20 minutes. The detector wavelength was fixed at 343nm. The standard solution 50%, 100%, and 150% solutions were injected. Individual recovery and mean recovery values were calculated.

Precision:

Preparation of Sample stock solution: Accurately weighed and transferred amount equivalent to 25mg of Plant extract working standard into a 25ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 10mins and then made upto volume with the mobile phase. 0.5 ml of the above stock solution was pipette out into 10ml of volumetric flask and diluted up to the mark with mobile phase this contains 50 ppm. 10 ml of the above stock solution was pipette out into 50ml of volumetric flask and diluted up to the mark with mobile phase this contains 10 ppm.

Procedure: Twenty microliters of the above-prepared solution were injected through an auto-injector and the chromatogram was developed using methanol: water (95:5), as a mobile phase. The flow rate was maintained at 0.8ml/min and the column temperature was maintained at 25°C. The total run time was fixed as 20 minutes. The detector wavelength was fixed at 343nm. The standard solution was injected

five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits ²¹.

Robustness:

Effect of variation of Flow rate

A study was conducted to determine the effect of variation in flow rate by injecting 0.6 and 1ml/min. A standard solution was prepared and injected into the HPLC system. The retention time values were measured.

Effect of variation of wavelength:

A study was conducted to determine the effect of variation in wavelength. The standard solution was prepared and injected into the HPLC system at 341nm and 345nm. The effect of variation in wavelength was evaluated

Ruggedness:

A stock solution was prepared by adding 25mg of plant extract of working standard to the mobile phase in a 25ml volumetric flask. Then the solution was sonicated for about 10 mins and the volume made upto 10ml with the mobile phase. From the above-prepared solution 0.5ml of the sample was withdrawn and the volume was made upto 10 ml with the mobile phase.

Procedure: 20µl of the sample was run through HPLC using methanol: water (95:5), as the mobile phase. The flow rate was maintained at 0.8ml/min and the column temperature was maintained at 25°C. The sample was run for 20 minutes and analyzed at a fixed wavelength of 343 nm. The standard solution (50ppm) was injected by different analysts and the area for injections in HPLC was measured. The % RSD for the area of replicate injections was found to be within the specified limits.

Limit of detection (LOD) & limit of quantification (LOQ):

The Limit of Detection (LOD) can be defined as the smallest level of analyte that gives a measurable response. The Limit of Quantification (LOQ) can be defined as the smallest concentration of analyte which gives a response that can be accurately quantified. The LOD & LOQ was determined by the formula:

$$\text{LOD} = \frac{3.3 \sigma}{S}$$

$$\text{LOQ} = \frac{10 \sigma}{S}$$

The values of LOD & LOQ were found to be 0.1269ppm and 0.3847ppm respectively.

Specificity:

It is evaluated by injecting the blank, placebo, and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of plant extract.

RESULT & DISCUSSION:**Physico-Chemical Parameters of Piperine****Table 1: Physico-Chemical Parameters of Piperine**

S.No	Parameters	Powder (%w/w)*		Extract (%w/w)*	
		1	2	1	2
1	Total Ash	1.54	1.03	1.15	1.01
2	Acid Insoluble Ash	0.13	0.28	0.10	0.15
3	LOD	3.81	4.29	6.18	5.06
4	Alcohol soluble extractive	13.36	10.75	19.97	17.61
5	Water soluble extractive	12.72	11.45	17.78	14.98
6	pH	7.21	5.19	5.31	4.67

QUALITATIVE ANALYSIS OF PHYTOCHEMICAL PARAMETERS:**Table 2: Qualitative Analysis of Phytochemical Parameters**

S.No	Parameters	Present	Absent
1	Alkaloids	√	----
2	Glycosides	----	√
3	Flavonoids	----	√
4	Tannins	√	----
5	Phenols	√	----
6	Carbohydrates	√	----
7	Amino-acids	√	----
8	Fixed Oils & Fats	√	----

Microbial Analysis

Microbial analysis was performed to determine the level of contamination in the samples. This occurs mainly during the collection of samples or during their storage. Hence it is important to determine the values and find out whether they fall within the limit or not according to WHO. The results obtained were tabulated as follows.

Table 3: Microbial Analysis

S.No	Microbial Analysis	Powder (%w/w)		Extract (%w/w)	
		1	2	1	2
1	Bacteria	120	132	128	140
2	Yeast & Mould	32	46	29	44

Chromatographic analysis

The standard solution of plant extract prepared was analyzed through a range of 200 nm – 400 nm. The maximum absorbance was found at 343 nm. For Trail I mobile phase used was water: methanol (30:70) and the flow rate was 1ml/ min at a column temperature of 25°C. The total run time was fixed as 20 minutes. The resultant chromatogram as shown in **figure 1**

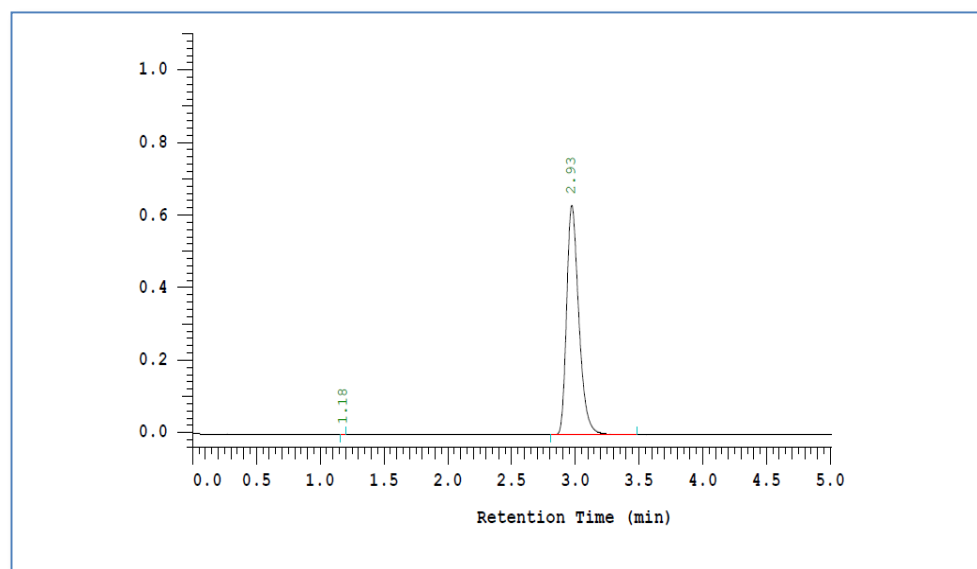


Figure 1: HPLC Chromatogram of Piperine (Trail 1)

Trial-II

20 µl solution of trail II was injected through an auto-injector and a chromatogram was developed using water: methanol (95:5) as the mobile phase. The flow rate was maintained at 0.8ml/min and the column temperature was maintained at 25°C. The total run time was fixed as 20 minutes. The detector wavelength was fixed at 343 nm. The resultant chromatogram for sample and blank were shown in figure 2.

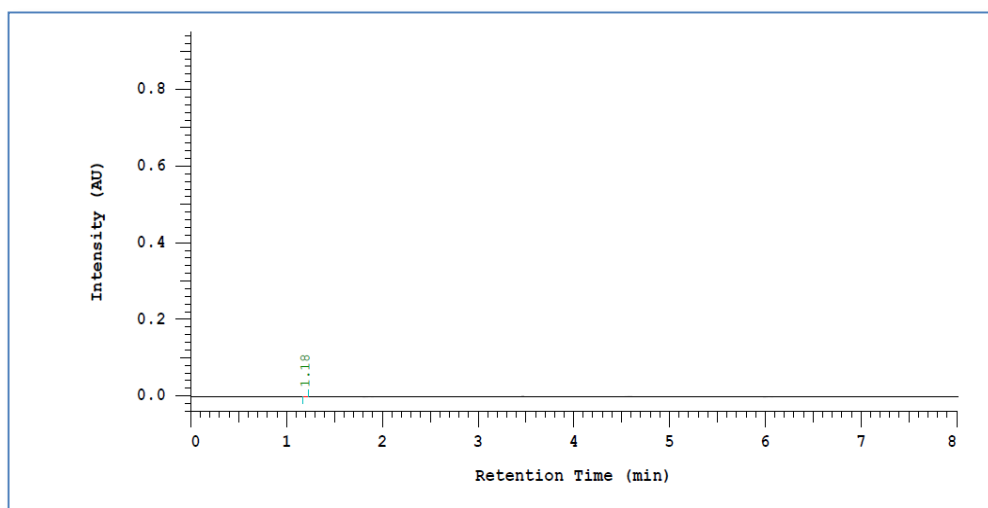


Figure 2: HPLC Chromatogram of Piperine (Trail II)

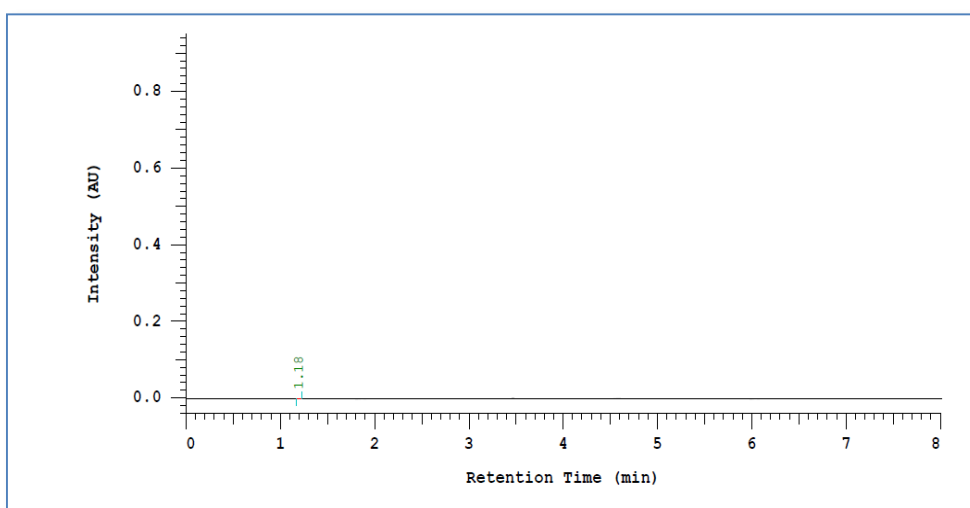


Figure 3: Blank Chromatogram of Piperine

3.4 Determination of amount present:

The total amount of active drug was measured at maximum absorbance of 343 nm and percentage purity was calculated using the given formula.

$$\text{Amount present} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \frac{\text{Standard dilution}}{\text{Sample dilution}} \times \frac{\text{Average weight}}{100} \times \text{Potency}$$

$$\text{Percentage purity} = \frac{\text{Amount present}}{\text{Label claim}} \times 100$$

3.5 METHOD VALIDATION:

The chromatographic conditions were validated by evaluating linearity, recovery, method and system precision, the limit of detection (LOD), the limit of quantification (LOQ), and robustness following ICH guidelines Q2 (R1).

3.5.1 Linearity:

The calibration curve was plotted for the different levels of the solution prepared along with the standard stock solution. The analysis was performed using a mobile phase containing Methanol: Water (95:5), as a mobile phase. The flow rate was maintained at 0.8ml/min and the column temperature was maintained at 25°C. The total run time was fixed as 20 minutes. The detector wavelength was fixed at 343nm. Each level was injected into the chromatographic system and the peak area was measured. A calibration curve was plotted for the concentration v/s peak area and the correlation coefficient was calculated which was given in Table 4.

Table 4: Linearity of Piperine

S.No.	Concentration level (%)	Conc. (ppm)	Peak Area
1	20	2	261063
2	40	4	567495
3	60	6	862877
4	80	8	1198679
5	100	10	1491611
6.	120	12	1782013

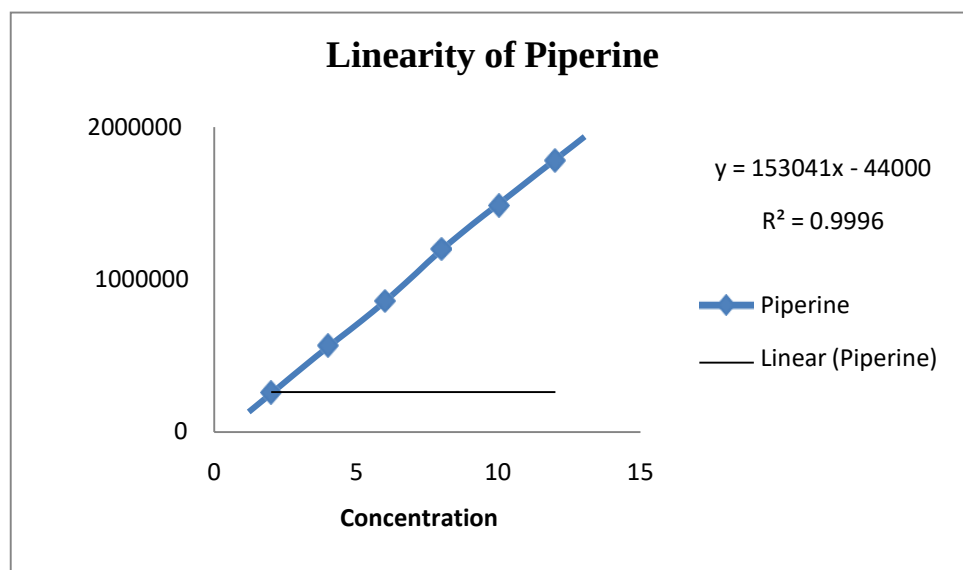


Figure 4. Calibration curve of Piperine

Accuracy:

The accuracy test was conducted with three different solutions of concentration 50%, 100%, and 150% that were made from the standard stock solution. All the three prepared solutions were run to obtain the chromatogram using mobile phase methanol: water (95:5). The flow rate was maintained at 0.8ml/min and the column temperature was maintained at 25°C. The total run time was fixed as 20 minutes. The detector wavelength was fixed at 343nm. The standard solution 50%, 100%, and 150% solutions were injected. Individual recovery and mean recovery values were calculated and the results were discussed in Table 5.

Table 5: Accuracy results for Piperine

Level	Amount added (ppm)	Total amount (ppm)	Amount found (ppm)	% recovery
50%	5	15	14.78	98.54
100%	10	20	19.97	99.87
150%	15	25	24.47	97.9

Precision:

A precision test was conducted by calculating the %RSD of the HPLC chromatogram obtained by running the solution through HPLC with methanol: water (95:5), as a mobile phase. The flow rate was

maintained at 0.8ml/min and the column temperature was maintained at 25°C. The total run time was fixed as 20 minutes. The detector wavelength was fixed at 343nm. The standard solution was injected five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The results were illustrated in **Table 6**.

Formula:

$$\% \text{RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Table 6 Precision results for Piperine

Parameter	Mean	SD	%RSD
Inter-day	1488066	8467.587	0.569
Intra-day	1488915	2554.798	0.171

Robustness:

A robustness study of the method was conducted by running the sample with variable flow rates and varying wavelengths of the detector. The effect of variation of wavelength and flow rate was calculated in terms of mean, standard deviation, and %RSD which are mentioned in **Table 7**.

Table 7: Robustness results for Piperine

S.No		Mean	SD	%RSD
Flow rate	0.6ml/min	1477625	1269.27	0.085
	1ml/min	1478036	1176.69	0.079
Wavelength	341nm	1477435	1492.97	0.101
	345nm	1477184	1409.28	0.095

Ruggedness:

The ruggedness of the developed method was analyzed by conducting the methods at different laboratories by different analysts for obtaining the chromatogram for the sample solution prepared for the extract. The standard solution (50ppm) was injected by different analysts and the area for injections in HPLC was measured. The %RSD for the area of replicate injections was found to be within the specified limits.

Limit of detection (LOD) & limit of quantification (LOQ):

The Limit of Detection (LOD) can be defined as the smallest level of analyte that gives a measurable response. The Limit of Quantification (LOQ) can be defined as the smallest concentration of analyte which gives a response that can be accurately quantified. The LOD & LOQ was determined by the formula:

$$\text{LOD} = 3.3 \sigma / S$$

$$\text{LOQ} = 10 \sigma / S$$

The values of LOD & LOQ were found to be 0.1269ppm and 0.3847ppm respectively.

Specificity:

It is evaluated by injecting the blank, placebo, and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of plant extract. **Figure 5** explains the specificity of Piperine.

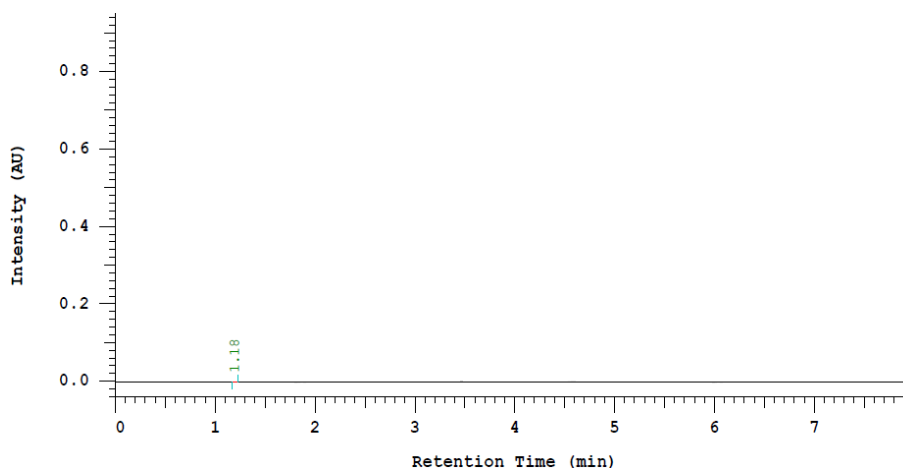


Figure 5: Specificity of Piperine

CONCLUSION

The above research conducted focused on developing a modified, simple, accurate, and economical HPLC method for the quantitative determination of selected herbal drugs. The developed method was validated for various parameters such as precision, accuracy, linearity and range, ruggedness, robustness, the limit of detection, and the limit of quantitation as per ICH guidelines. The selected herbal drug for this analysis was *Piper nigrum*. The percentage purity of the extract obtained was found to be 98% with the presence of 23.86 mg of extract. The linearity study followed a range of 2 - 12 µg/ml with a correlation coefficient of 0.9996. Precision studies conducted for both inter-day and intra-day depicted a % RSD of 0.56% and 0.171% respectively indicating that the proposed method was more accurate than the existing methods. The LOD and LOQ value for Piperine was found to be 0.126 µg/ml, and 0.384 µg/ml, respectively. This resembles that the proposed method was sensitive. The relative standard deviation for the value of Piperine obtained under deliberately modified chromatographic conditions should be less than 2%. The results obtained in the present study also indicate the method is robust. The ruggedness of the method was investigated under a variety of conditions including different analysts. The results have shown that there is no significant change which indicates that the proposed method is having ruggedness. The specificity of the proposed method was illustrated which shows that there is no interference of any peak at the retention time of Piperine in the chromatogram of blank solution. Thus the proposed method was specific and selective.

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