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ISOLATION, IDENTIFICATION AND QUANTITATIVE ANALYSIS OF PIPERINE FROM PIPER NIGRUM LINN. OF VARIOUS REGIONS OF KERALA BY RP-HPLC METHOD

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ABSTRACT

Piperine is the naturally occurring alkaloid that gives the spice; Piperine is extracted from *Piper nigrum*, *Linn*. It is medicinally used as Anti-inflammatory, Anti-malarial, Cure for Stomach Ache, Weight Loss, Anti-leukemia, Fever prevention and reducer, Treatment for Snake Venom Poisoning and Anti-epileptic, also helpful in increasing the absorption of certain vitamins such as Selenium, Vitamin B and Beta-Carotene. Extraction and Standardization of piperine is performed. In Standardization, Solubility, Total ash content, Acid insoluble ash, TLC, UV Spectroscopy, HPLC were performed. Piperine was extracted successfully, it was found to be soluble in ethanol and chloroform. Piperin extracted from *Piper nigrum Linn.* By refluxing 32 hrs with chloroform. Black pepper is collected from various regions of kerala at the month of august and january. The various geographical sources of black pepper are Wayanad, Idukki, Kozhikode, Alappuzha, Palakkad and Thiruvananthapuram district. Six pepper samples are collected from above six different district and extracted. A simple, rapid and precise RP-HPLC method has been developed for the quantitative determination of Piperine from *Piper nigrum Linn.* (Black pepper). Chromatographic analysis was carried out on Shimadzu phenomenex C18 column (250mm x 4.6mm, 5µm particle size). Quantitation was performed using a UV detector at 343 nm. RP-HPLC was performed by using ACN and aqueous acetic acid 1as mobile phase. The method was validated for linearity, precision, accuracy and can be effectively used to evaluate quality of black pepper. Quantitation, based on peak areas, achieved by reference to purified piperine as external standard.

KEYWORDS: Back pepper, Piperine, Isolation, Chloroform extract, TLC, RP-HPLC, UV spectrum, IR spectrum.

INTRODUCTION

In pharmaceutical industry, Herbal medicines have very important role because of there therapeutic value and 80% of world population depends upon traditional medicines. With an ever-increasing global inclination towards herbal medicine, there is an obligatory demand for a huge raw material of medicinal plants. Medicinal herbs are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals.

Analytical chemistry, which is both theoretical and a practical science is practiced in large number of laboratories in many diverse ways. Methods of Herbal analysis are routinely developed, improved, validated, collaboratively studied and applied. The use of analytical science in the discovery development and manufacture of herbal medicines are wide ranging. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw material used and the final product obtained meets the required specification.

The herbal drug contain number of active constituents and there dosage forms prove to be effective due to the combined mode of action on the body and less side effects. The complexity of crude drug dosage forms including the presence of multiple active constituents entities posses considerable challenge to the analytical chemist during the development of assay procedure and quantitative analysis. the crude druge also contain impurities from atmosphere. The estimation of individual drugs in these herbal drug forms becomes difficult due to cumbersome extraction or isolation procedures.

For the quantitative analysis of herbal medicines mostly HPLC and UV-spectro- photometric methods are used, due to the merits of high sensitivity, less time consuming, ruggedness of HPLC and more economical process of UV- spectrophoto- metric method than other methods. For the present study *Piper nigrum linn.* (Black pepper) were selected from various regions of kerala for quantitative estimation piperine. The extensive literature survey carried out revealed that some methods have been reported for the estimation of piperine from piper nigrum. However there is no method reported for the piperine estimation using chloroform extract by using RP-HPLC method.and it is also a comparative study of piperine content present in black pepper from different regions of kerala.

Hence, present study aims to develop simple, rapid, accurate, precise and validated methods for the isolation, identification and quantitative analysis of piperine from *piper nigrum linn.*from various regions of kerala by RP-HPLC methode. The plan of the work for the aimed study was designed as, Phyto chemical screening of crude drug, Extraction of crude drug, Isolation of piperin, Identification by using TLC, Spectrophotometric analysis of piperin by UV-visible spectrophotometry and Quantitative analysis of piperine by HPLC..

MATERIALS AND METHODS

COLLECTION OF THE PLANT

Samples of different piper nigrum (black pepper) cultivars were collected from six major black pepper growing areas in kerala including Wayanad, Idukki, Kozhikode, Alappuza, Palakkad and Thiruvanandhapuram. Collection was done at the month of October, The plucking season of black pepper is october. To obtain black pepper, fruiting spikes are harvested when fruits are fully grown but still green and shiny. Fruit spikes are left in heaps overnight for brief fermentation. The next morning, the mass of spikes are usually spread out on bamboo mats or concrete floors to dry in the sun for about 4 to 5 days. Plant Material were dried and powdered in a cross beater mill.[1,2]

EXTRACTION

Plant Material were dried and powdered in a cross beater mill. Placed 150 gm of ground black pepper in a 250 ml Soxhelt apparatus, added 150 ml of chloroform and 5 boiling chips, and heated at reflux for 20 hours. Filtered the mixture by suction filtration & then concentrated by simple distillation or by use of a rotary evaporator.[3]

PHYTO CHEMICAL SCREENING

Phytochemical screening of extracts used for the identification of alkaloids and other active constituents present in piper nigrum. The chemical tests are carried out by using chloroform extract.[4,5]

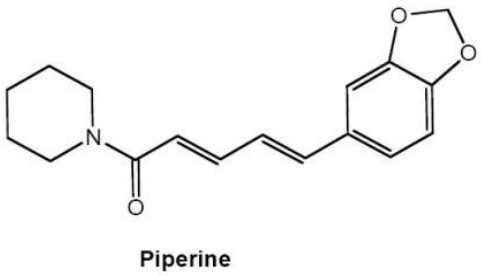
Preparation of standard solution (Solution A):- Prepare a standard solution by dissolving 50 mg of chloroform extract in 50 ml of 95% ethanol**,** and shake well.

Test for alkaloids.[6]

The above extracts were screened for various secondary metabolites by Mayer's Test, Dragendroff's Reagent, Hager's Test and Wagner's Test.[7,8]

ISOLATION OF PIPERIN

Piperine can be isolated from ground black pepper, which is made up of 8-10% of alkaloids that also include piperidine, piperettine and piperanine and comes from the dried fruit of piper nigrum. The common procedure for the isolation of piperine involves extraction using ethanol (95%) and KOH; however, the procedure below involving refluxing with chloroform also gives good yield of piperine. Because of the widespread uses of pepper, many synthetic approaches have also been designed for commercial production.[9]



Piperine

Fig. 1: chemical struture of piperine.

To 10mL of a 10% solution of KOH in 95% ethanol contained in a 125Ml Erlenmeyer flask added the concentrated pepper chloroform extract. Heated the resulting solution and add water drop wise. A yellow precipitate formed. Added water until no more solid appears to form and then allowed the mixture to stand at least overnight collect the solid by suction filtration & recrystallized it with 10-20mL of acetone. Piperine should precipitate out; if it does not, repeat the above procedure. Using the Hirsch funnel, vacuum filter the yellow piperine crystals. Wash them with cold ether (2x4 mL) and save ~5 mg of crystals for TLC analysis. To recrystallize, place the piperine in a test tube and dissolve it in ~5 mL of a hot 3:2 acetone: hexane solution. Let sit for 15 min at room temperature and then 30 min in an ice bath. Vacuum filter the crystal using a Hirsch funnel and wash with 4 mL of cold ether. It may be necessary to get a second crop of crystals from the mother liquor to improve yield. After dried, take a melting point of the crystals.[10]

IDENTIFICATION OF ISOLATED PIPERINE

THIN LAYER CHROMATOGRAPHY

Stationary phase - Silica gel 60-120 mesh size,

Mobile phase - Toluene : Ethyl acetate (70:30).

Detecting agent - Vanillin Sulphuric acid reagent.

Identification mark - Yellow spots

Rf value was calculated. The sample spots are also visualized in suitable UV light chamber.[11,12,13]

CHARACTERISATION OF PURE PIPERINE USING FTIR SPECTROSCOPY STUDIES

Materials required

Soxhlet extractor**,** Black pepper (Generic pepper does not work as well as major brands.)**,** Chloroform**,** 95% ethanol**,** 10% alcoholic potassium hydroxide (KOH) in 95% ethanol**,** Whatman #1 filter paper**,** Agate mortar and pestle**,** Drying oven**,** Desiccator**,** IR-grade potassium bromide (KBr)**,** Wig-L-bug and accessories (stainless steel mixing vial and mixing ball)**,** KBr die**,** Hydraulic lab press**,** KBr pellet holder**,** IR spectro photometer and 50°C water bath.[14,15]

Procedure

- Grind 150 g black pepper to a fine powder with a mortar and pestle.
- Extract the finely ground pepper with 150 mL 95% chloroform in a soxhlet extraction apparatus for4 hours. (The ground pepper is placed in the thimble and the chloroform in the round -bottomed flask.)
- Allow the solution to cool and filter through Whatman #1 filter paper.
- Concentrate the solution *in vacuo* on a 50°C water bath to remove most of the chloroform solvent.
- The final volume should be about 5 mL.
- Add 10 mL 10% alcoholic KOH to the residue and let stand 1 hour.
- Decant the solution from the insoluble residue.
- Allow the alcoholic solution to stand undisturbed overnight; long yellow needles of piperine will be deposited. (The crystals may take 24–48 hours to form.) The yield is approximately 0.3 g.
- Collect the yellow needles by vacuum filtration and wash with a minimum volume of 95% ethanol.
- Allow the crystals to air dry. Weigh them and determine the melting point.
- It should be 125–126°C.

Preparation of KBr Pellet for IR Analysis (Pressed pellet technique)

- Dry IR-grade KBr in a drying oven for at least 1 hour.
- Grind approximately 15 mg of the piperine needles in an agate mortar and pestle for 20 minutes.
- Make sure the crystals are kept dry and are thoroughly ground to remove any reflective surfaces
  and reduce particle size.
- Allow the KBr to cool in a desiccator. Weigh out 300 mg and place in a stainless steel mixing
  vial with a stainless steel mixing ball.
- Carefully weigh 3 mg of the finely ground piperine and place in the vial with the KBr.
- Shake the vial on a Wig-L-bug for 60 seconds to thoroughly mix.
- Place the KBr-piperine mixture into an evacuable die on a hydraulic laboratory press. Press *in vacuo* at 15,000 pounds of pressure for 6 minutes.
- Release the pressure, remove the die from the press, disassemble the die, and remove the KBr pellet.
- Place the KBr pellet in a pellet holder and put it into the sample beam of an IR spectrophotometer.
- Run the spectrum of the pellet from 4,000–600 cm-1.
- Using the following table of expected absorption bands, look for the corresponding bands on the IR spectrum of isolated piperine.[16]

Table No.1: Expected Absorption Bands for Piperine in KBr.

S.No	Types of phenomenon	Wave number (cm-1)
1	Aromatic C-H stretching	3,000
2	Symmetric and asymmetric stretching of C=C (diene) 1	1635; 1608
3	Aromatic stretching of C=C (benzene ring) 1	1608; 1580; 1495
4	Stretching of -CO-N	1635
5	Asymmetric and symmetric CH2 stretching, aliphatic C-H stretching	2925; 2840
6	CH2 bending	1450
7	Asymmetrical stretching =C-O-C	1250; 1190
8	symmetrical stretching =C-O-C	1030
9	Out-of-plane C-H bending 1,2,4-trisubstituted phenyl	850; 830; 805
10	C-H bending of *trans*-CH=CH-	995
11	in-plane bending of phenyl C-H	1132
12	C-O stretching (most characteristic)	930

UV SPECTROSCOPY STUDIES

This UV spectroscopic studies reports on instrumental methods for ensuring the Identity, Potency, Purity, Safety and efficacy of herbal drug. This method includes the investigation of establishment of UV-spectrophotometric analysis methods for Samples of different piper nigrum (black pepper) cultivars were collected from six major black pepper growing areas in kerala including Wayanad, Idukki, Kozhikode, Alappuza, Palakkad, And Thiruvananthapuram.[17]

MATERIALS AND INSTRUMENTS

Chemicals and Solvents Used For Estimation

Deionized water, Methanol

Instruments used

Electronic balance AY 220, Sonica Ultrasonic cleaner, Elico UV-Spectrophotometer – 1800 A, Cuvettes (quartz cells).

Preparation of standard solution of piperine

An accurately weighed standard pure piperine(100mg) was dissolved in methanol and volume was made up to 100ml with methanol in volumetric flask. Shake well and dissolve the standard piperine in methanol. From this take two ml of this solution was diluted with methanol up to 100ml in volumetric flask to give 20µg/ml piperine solution.[18]

Preparation of sample solution

Prepare sample solutions of each black pepper collected from Wayanad, Idukki, Kozhikode, Alappuza, Palakkad, and Thiruvananthapuram. Take 5.0ml of isolated (six samples) was exactly pipetted out into a separate 100ml volumetric flask. The volume was made up to the mark with alcohol and shaken well to make the contents homogenous. These solutions were further used for spectrophotometry.[19]

Experimental

Calibration curve from standard solution of piperine was prepared and with the help of this curve the content of piperine from piper nigrum was estimated. The method was validated for precision and accuracy.[20]

Estimation of piperine in piper nigrum

The appropriate aliquots from piperine extract of each six samples from various places withdrawn in 10ml volumetric flask separately absorbance for aliquots of each was noted at 343 nm. the corresponding concentration of piperine against respective absorbance value was determines using the piperine calibration curve. The statistical analysis for checking uniformity in all samples is also performed.

Procedure

Dissolved 100mg of the isolated Piperine in 100ml volumetric flask and made the volume up to the mark with ethanolic HCl (1:1) (100µg/ml). From this solution 5ml is taken to 100ml volumetric flask and made up the volume (50µg/ml). The sample is scanned in UV Visible spectrophotometer. The peaks obtained in each spectrum were compared.

RP-HPLC METHOD DEVELOPMENT AND VALIDATION

EXPERIMENTAL METHODS

Materials and Instruments

Drug samples

Standard pure piperin and isolated sample solutions of each black pepper collected from various part regions of kerala including Wayanad, Idukki, Kozhikode,Alappuza, Palakkad, and Thiruvananthapuram.

Chemicals and Solvents used for estimation

Water for HPLC, Acetonitrile HPLC grade, Methanol HPLC grade, Water HPLC grade, Acetic acid (AR)s.

Instruments used

pH meter LI 127, Elico Spectrophotometer 1800 A, Sonica Ultrasonic cleaner, Electronic balance AY 220.Shimadzu LC-20 AT HPLC [Pump-Prominence LC 20 AT (double pump), Column - Phenomex Luna 5µ C_{18} (12) 100A, (250 × 4.6m.m × i.d, 5µ), Injector - Hamilton type injector with 20µl loop capacity, Detector - UV visible spectrophotometric, Diode Array Detector], Computer and Printer.

Method Development and Optimization

Development of RP-HPLC method for quantitative estimation of piperin from piper nigrum (black pepper) collected from various regions of kerala including Wayanad, Idukki,

Kozhikode, Alappuzha, Palakkad and Thiruvananthapuram.

Selection of Wavelength

The wavelength for the analysis of piperin was selected from the UV spectrum of piperin by scanning in the range of 200-400 nm. From this, the wavelength of 343 nm was selected for the final method since these drug has shown good absorbance at 343 nm.[21]

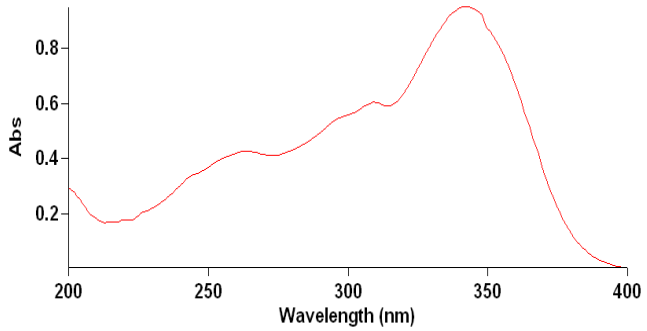


Fig. 2: UV absorption spectra of Piperine.

Selection of mode of separation

The selection of method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence RP-HPLC method was preferred because of its simplicity and suitability. HPLC is a marked difference over liquid chromatography which uses gravity instead of a high speed pump to force compounds through the densely packed tubing. The results produced are of a high resolution and are easy to read, and the tests are easily reproduced via the automated process.[24]

Initial Chromatographic conditions

The following chromatographic conditions were used:

Stationary phase : Phenomenex C18 column (250 mm x 4.6 mm i.d, 5µm)

Mobile phase : ACN: 1% Acetic acid

Solvent ratio : 70:30

Detection wavelength : 343 nm

Flow rate : 1 ml/min

Temperature : Room temperature of 25 ± 2^0 C

Preparation of Standard Solution

- 5mg of Piperin was taken in a 10 mL standard flask. To this 5 mL of methanol was added

for dissolving the drug. The contents were shaken for 1 min to get a clear solution and the volume was make up to 10 ml with methanol (Stock solution A).

- The final standard solution was prepared in such a way that each standard flask contains 10, 20, 30, 40, 50µgmL^{-1} concentration of piperin.

Preparation of sample solutions

Take piperin extract collected from various regions of kerala including Wayanad, Idukki, Kozhikode, Alappuza, Palakkad, Thiruvanandhapuram, 1gm of each extract was taken in a 10 mL standard flask. To this 5 mL of ACN:1% Acetic acid mixture was added for dissolving the drug. The contents were shaken for 1 min and the volume was make up to 10 ml with ACN:1% Acetic acid mixture. The final sample solution was prepared in such a way that each standard flask contains 10, 20, 30, 40, 50µgmL^{-1} concentration of piperin.[23]

Method or Recording of chromatogram

With the optimized chromatographic conditions mentioned above, a steady baseline for about 20 min. After the stabilization of the baseline for about 30 min., a diluent injection of the solvents used for solubilizing drug was given in duplicate. The chromatogram was recorded. Then the standard solution were injected and chromatograms were recorded until the reproducibility of the peak areas were found satisfactory and finally 20 µL of the standard solution of the individual sample of piperin were injected and the chromatograms were recorded. Successive aliquots of 20µL of mixed standard solutions were injected and the chromatograms were recorded.[24]

The procedure was repeated using the sample solution to be estimated and the chromatogram was shown in chromatogram the peak areas were noted. The elution order of mixture was found as piperin(Retention time 12.653).

RESULTS AND DISCUSSION

PHYTOCHEMICAL EVALUATION FOR ALKALOIDS

The chemical tests gives positive results for the identification of alkaloid present in the black pepper extract. Piperine is a alkaloid that give specific colour when react with Mayer's, Hager's, Wagner's and Dragendroff's reagents.

- **Dragendroff's reagent** give an orange red precipitate indicates presence of alkaloid
- **Wagner's reagent** give a reddish brown precipitate indicates presence of alkaloid

- **Hager's reagent** give a yellow precipitate indicates presence of alkaloid.
- **Mayer's reagent** give a dull white precipitate indicates presence of alkaloid.

ISOLATION OF PIPERINE

The Piperine was successfully isolated from the pipper nigrum fruits. Product was small yellow crystals with a melting point of 132^0C – 134^0C and the percentage yield of Piperine from black pepper powder was found to be.

Table No. 2: isolation results.

S.No	SAMPLES	%YIELD
1	Wayanad	1.9%
2	Idukki	2.8%
3	Kozhikode	2.4%
4	Alappuzha	3.1%
5	Palakkad	2.2%
6	Thiruvananthapuram	2.5%

The result shows, the yield differs from location to location and the % yield lays between 1.9 % and 3.1%. The sample collected from Alappuzha gives high yield (3.1%) and sample collected from Wayanad gives low % yield (1.9%).

IDENTIFICATION OF ISOLATED PIPERINE

THIN LAYER CHROMATOGRAPHY

After isolation, Piperine is identified by TLC. The standard Rf- value of Piperine was 0.25. The Rf- value of purified Piperine from TLC was found to be.

Table No. 3: Rf value of piperin standard and sample.

S.No.	SAMPLES	R_f VALUE
1	Pure Piperine standard	0.250
2	Wayanad	0.235
3	Idukki	0.244
4	Kozhikode	0.240
5	Alappuzha	0.269
6	Palakkad	0.254
7	Thiruvananthapuram	0.246

CALCULATION OF STANDARD PIPERINE

$$R_f = \frac{\text{Distance from start to center of substance spot}}{\text{Distance from start to solvent front}}$$

Distance travelled by solute -1.3cm

Distance travelled by solvent front - 5.2 cm

R_f value of pure piperine standard -0.250

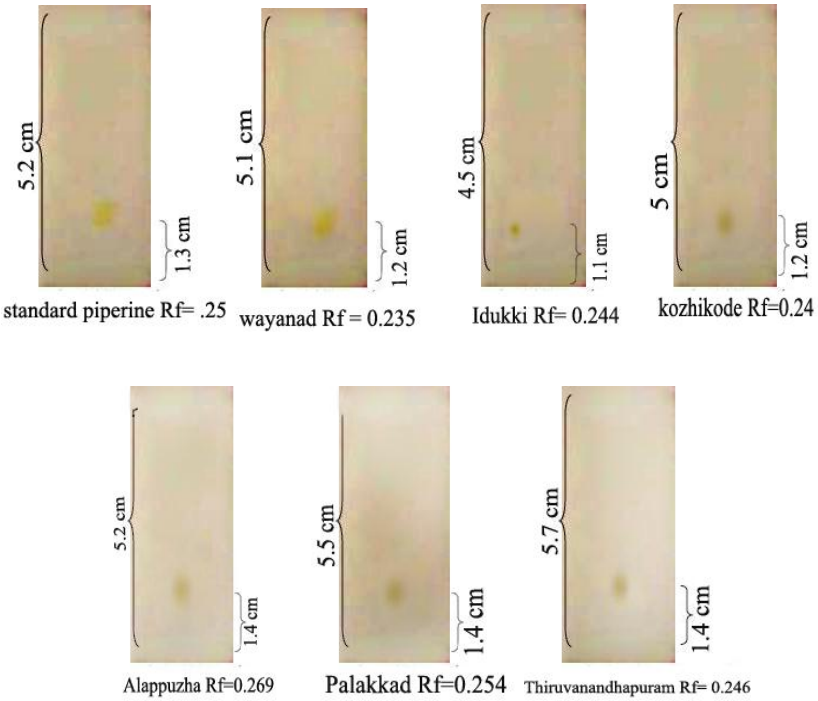


Fig. 3: Rf value using TLC plate.

The result shows, Rf values of piperine present in samples collected from different places give similar Rf value. The Rf value lays between 0.235 and 0.269.

UV SPECTROSCOPY STUDIES

The UV absorption spectra shows λ-max of piperine at 343 nm**.** The method involves measurement of UV absorbance at 343nm for different sample of piperine corresponding to the absorption maxima of the pure piperine standard.

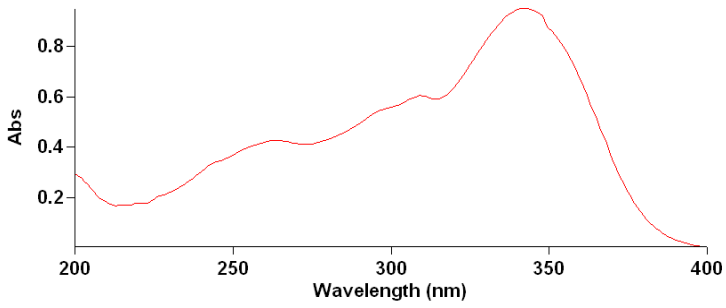


Fig. 4: UV absorption spectra of piperine.

Validation parameters of piperine

The absorbance characteristics showed that piperine obeys Beer Lambert's law within the concentration range 2-20 µg/ml at the λ-max of 343 nm with the regression value of 0.9956 and calibration equation Y = 0.07733*X -0.04974.

Table No. 4: Validation parameters of piperine (Mean% ± SD,n=6).

S. No.	Parameters	Value
1	Absorption maxima	343nm
2	Beer's law limit	10-50ug/ml
3	Regression equation(y=bx+a)	0.013x+0
4	Intercept(a)	0
5	Slope(b)	0.013
6	Correlation coefficients(r2)	0.9961
7	Precision (n=6, % RSD)	2.24
8	Accuracy(%)	98.99

Calibration curve of piperine

A series of calibrated 10ml volumetric flask were taken and appropriate aliquots of the working standard solution of piperine were withdrawn and diluted up to 10ml with methanol.The absorbance was measured at absorption maxima(λmax) 343 nm, against the reagent blank prepared in similar manner without the piperine. The absorption maxima and Beer's law limit were recorded and data that prove the linerity and obey Bee'r law limit were noted. The linear correlation between these concentrations (x-axis) and absorbance(y-axis) were graphically presented and slope(b), intercept(a), and correlation conffiçiant (r2) were calculated for the linear equation (Y=bx+a) by regression using the methanol of the least square.

Table No. 5: UV absorbance.

S.NO.	CONCENTRATION(µgm/ml)	ABSORBANCE
1	10	0.150
2	20	0.269
3	30	0.395
4	40	0.518
5	50	0.641

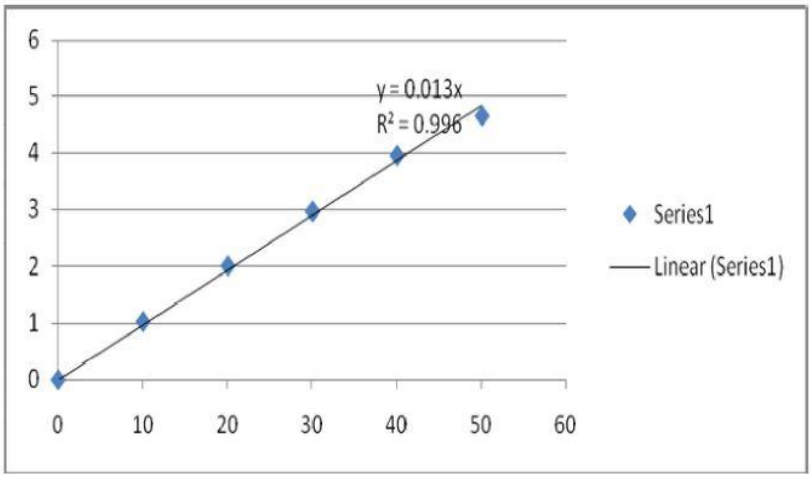


Fig. 5: Calibration curve of piperine.

UV maxima of isolated Piperine was taken in 30 parts of ethanol and UV maxima obtained at 343 nm

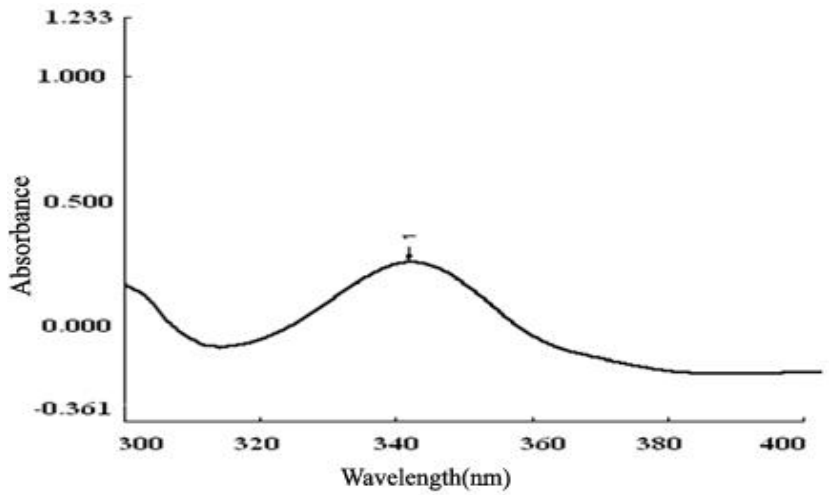


Fig. 6: uv maxima at 343 nm.

The estimation of piperine in pure piperine standard and powderd fruits of piper nigrum was carried out separately The concentration of piperine in different piper nigrum samples was found to be.

Table No. 6: The concentration of piperine in different samples.

S.No	SAMPLE	PIPERINE CONTENT %w/w
1	Wayanad	1.59±0.001
2	Idukki	1.53±0.002
3	Kozhikode	1.74±0.004
4	Alappuzha	1.78±0.002
5	Palakkad	1.69±0.002
6	Thiruvananthapuram	1.64±0.006

In order to optain precision and accuracy the recovery study were performed at three levels by adding known amount of piperine with preanalysed sample of piperine in piper nigrum. The experment was repeated Six Times at both level and result shows 99.28%, 99.43%,and 99.77% recovery of piperine at all the level with mean value 99.49%, which prove reproductibility of the result. This shows significant precision of methods at 95% confidence level. The percent relative standard deviation(%RSD) value was found to be 0.16, 0.28,and 0.08, with mean 0.17at all the level while the standard error was 0.31, 0.32 and 0.11 with Mean 0.24 respectively. From the data it is obvious that the present method of UV Spectrophotometric fingerprinting determination of Piperine is simple, precise, accurate, and suitable for routine analysis of Piperine in piper nigrum(Black pepper).

Table No. 7: Mean ± SD of six determinations, RSD= Relative standard deviation, SE=Standard error.

S.No	AMOUNT OF PIPERINE(µgm/ml)			RSD %	SE	RECOVERY %
	In sample	Added	estimated			
1	100	50	148.92±0.24	0.16	0.31	99.28
2	100	100	198.86±0.56	0.28	0.32	99.43
3	100	150	249.42±0.20	0.08	0.11	99.77
mean				0.17	0.24	99.49

FT-IR STUDIES

IR data indicates the presence of an amide (1631cm $^{-1}$) as well as aromatic and aliphatic C-H bonds, (2931cm^{-1}, 2842 cm $^{-1}$) All signals were annotated accordingly, and matched the predicted spectra for piperine, indicating that the desired product containing piperine. In this spectral analysis KBr pellet technique is used, Spectrum of the pellet is taken from 4000 cm-1 to 400 cm-1.

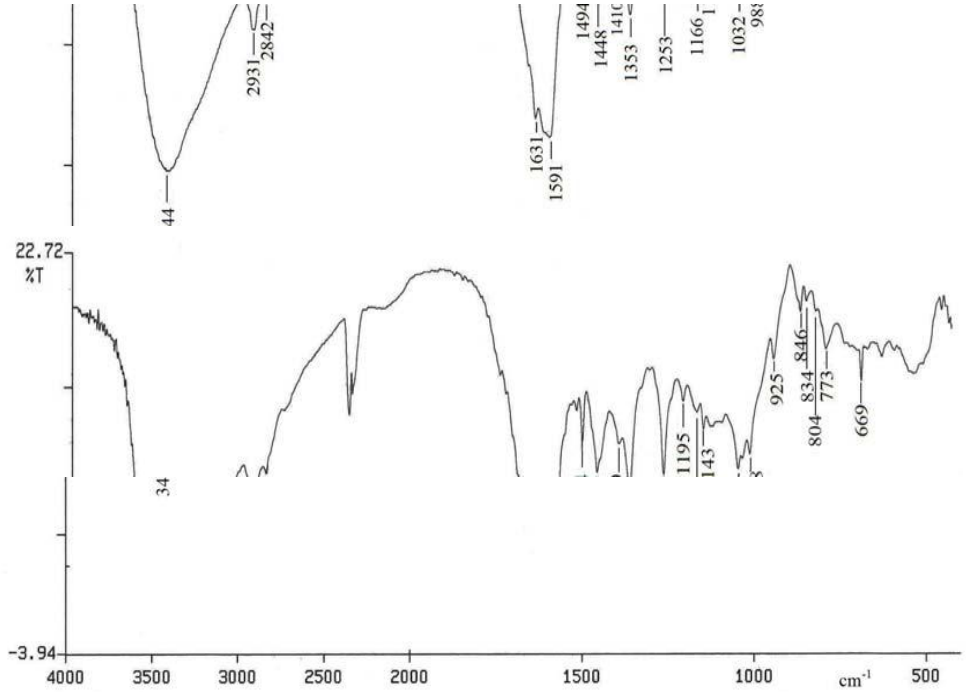


Fig. 7: FT-IR spectra of piper nigrum.

The peak obtained after the FT-IR spectra of piper nigrum compared with the standard IR absorption value of pure piperine. The values are matching with standard values indicate the presence of piperine in black pepper. The values of purified Piperine from FT-IR was found to be.

Table No. 8: The peaks obtained after taking the FTIR spectra of piper nigrum.

S.No	Types of phenomenon	Wave number (cm-1)
1	Aromatic stretching of C=C (benzene ring)	1591
2	Stretching of -CO-N	1631
3	Asymmetric and symmetric CH2 stretching, aliphatic C-H stretching	2931; 2842
4	CH_2 bending	1448
5	Asymmetrical stretching =C-O-C	1253; 1195
6	symmetrical stretching =C-O-C	1032
7	Out-of-plane C-H bending 1,2,4-trisubstituted phenyl (two adjacenthydrogen atoms)	846; 834; 804
8	C-H bending of *trans*-CH=CH-	988
9	in-plane bending of phenyl C-H	1132
10	C-O stretching (most characteristic)	925

HPLC STUDIES

Quantitative analysis of Piperine in piper nigrum fruit by High Performance Liquid Chromatography was carried out using optimized chromatographic conditions. The standard and sample solutions were prepared and chromatograms were recorded. The recorded

chromatogram of blank, standard drug and sample chromatograms are given as above figure. The linearity of the drug was determined at 6 concentration levels ranging from 200ng/ml – 3000ng/ml for piperine. The calibration curves were constructed by plotting peak areas versus concentrations, and the regression equations were calculated. The response of the drug was found to be linear in the investigation concentration range and form linear regression equation.so the unknown concentration of the formulation was found out by external standard method.

The assay procedure was repeated for 5 times and mean peak area,mean weight of standard drugs, of sample were taken and calculated. The percentages of piperine found in piper nigrum, mean and relative standard deviation in piper nigrum were calculated.

SYSTEM SUITABILITY

System suitability was determined by injecting, working standard solution of piperine (1000ng/ml) five times. The peak area values and the retention time of piperine were noted for each applied concentration of piperine. The coefficient of variation for the peak area and retention times was calculated.

Table No.9: System suitability (RSD, %).

S.No.	Method characteristic	Piperine
1	Retention Time(n=5)	1.07
2	Area(n=5)	1.50

LIMIT OF QUANTITATION (LOQ) AND LIMIT OF DETECTION (LOD)

Limit of quantitation (LOQ) was established at a signal-to-noise ratio of 1:10 and Limit of detection (LOD) was established at a signal-tonoise ratio of 1:3. The LOQ of piperine was found to be 10 ng mL-1 and LOD was 1 ng mL-1.

LINEARITY

Linearity was evaluated by analysis of working standard solutions of piperine of seven different concentrations. The peak area and concentration of piperine was subjected to regression analysis to calculate the calibration equations and correlation coefficients. The response was linear in the range of 200 ng mL-1 to 3000 ng mL-1.

Table No. 10: Summary of validation data.

S.No.	Method characteristic	Piperine
1	LOD	1 ng/ml
2	LOQ	10 ng/ml
3	Linear range (ng)	200ng/ml –3000ng/ml
4	Correlation coefficient (r)	0.9997
5	Slope	138.58
6	Intercept	1333.74

PRECISION

The precision for the method was analysed by determining intra-assay precision and intermediate precision. The Intra-Assay/within day precision was carried out on one day at three different concentration levels i.e. 250 ng/ml, 1600 ng/ml, 2500 ng/ml, with three replications of each. The Inter-day precision was carried out on multiple days. The experiment carried out for intra-day precision was repeated in the same manner on two more days by analyzing in triplicate. The method was found to be precise.

Table No. 11: Precision (RSD, %).

S.No.	Method characteristic	Piperine
1	Inter-day (n = 3)	1.16–1.44
2	Intra-day (n = 3)	1.16–1.25

ACCURACY (RECOVERY)

To check the accuracy of the developed methods and to study the interference of Other materials, recovery experiments were carried out by standard addition method.

Each sample was extracted and analysed by the developed HPLC method, and the amount of piperine recovered for each level, was calculated %Mean recovery were found to be 1.66%, 2.66%, 4.48%, 6.12%, 1.61% and 3.43% in the piper nigrum samples collected from Wayanad, Idukki, Kozhikode, Alappuza, Palakkad, and Thiruvananthapuram respectively.

HPLC chromatogram of standard piperine

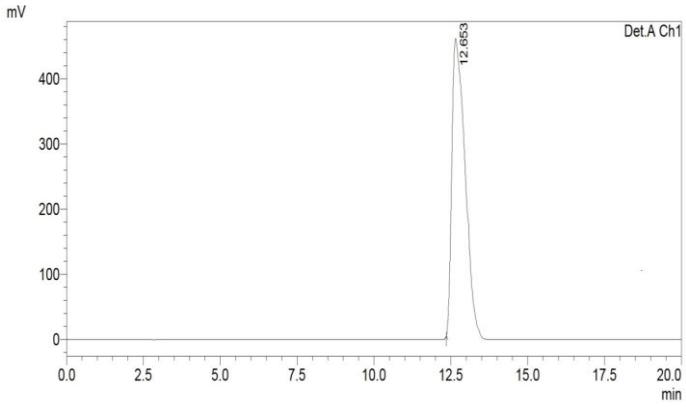


Fig. 8: chromatogram of standard piperine.

PeakTable

Detector A Ch1 343nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	12.653	9057207	392468	100.000	100.000
Total		9057207	392468	100.000	100.000

HPLC chromatogram of sample 1(Wayanad) HPLC chromatogram of sample 2 (Idukki)

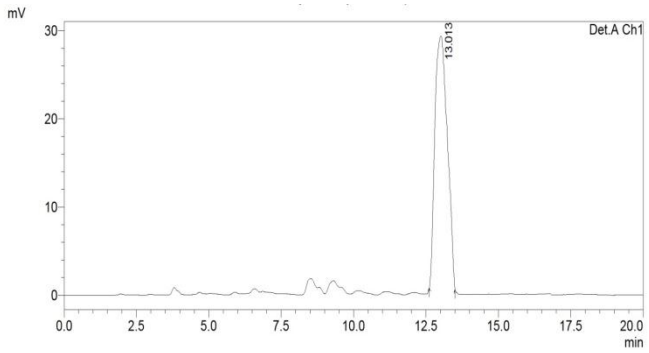


Fig. 9: chromatogram of wayanad sample.

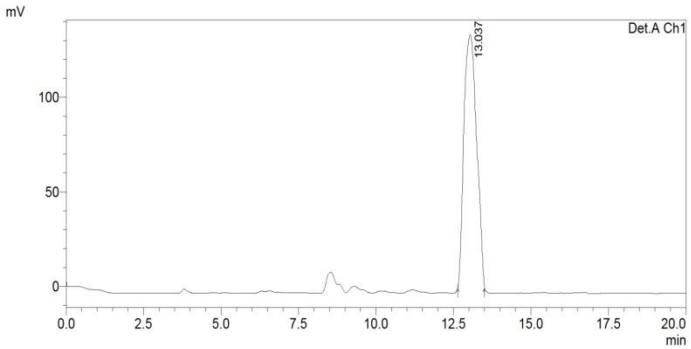


Fig. 10: chromatogram of idukki sample.

PeakTable

Detector A Ch1 343nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	13.013	1506876	20961	100.000	100.000
Total		1506876	20961	100.000	100.000

PeakTable

Detector A Ch1 343nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	13.037	2411256	101293	100.000	100.000
Total		2411256	101293	100.000	100.000

HPLC chromatogram of sample 3 (Kozhikode) HPLC chromatogram ofsample 4(Alappuza)

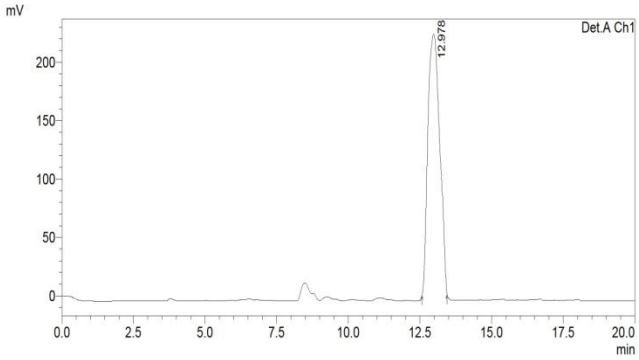


Fig. 11: chromatogram of Kozhikode sample.

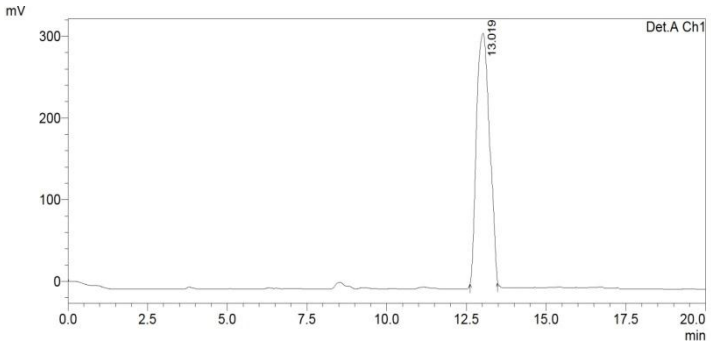


Fig. 12: chromatogram of Alappuza sample.

PeakTable

Detector A Ch1 343nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	12.978	4062447	169831	100.000	100.000
Total		4062447	169831	100.000	100.000

PeakTable

Detector A Ch1 343nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	13.019	5541868	232734	100.000	100.000
Total		5541868	232734	100.000	100.000

HPLC chromatogram of sample 5(Palakkad) HPLC chromatogram of sample 6(Thiruvananthapuram)

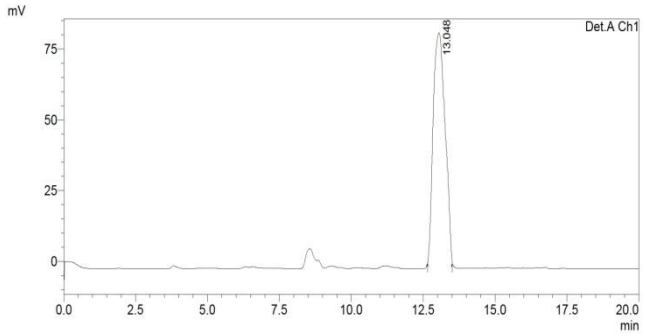


Fig. 13: chromatogram of palakkad sample.

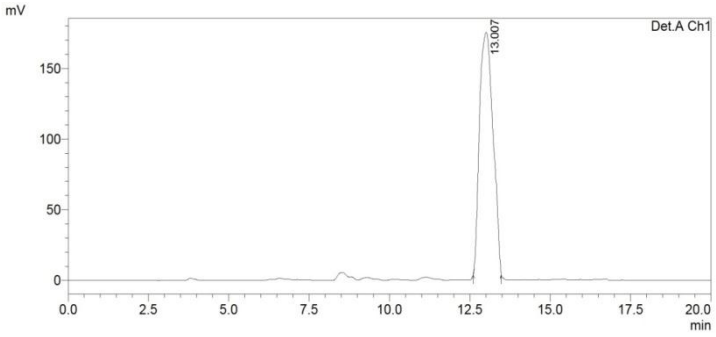


Fig. 14: chromatogram of Thiruvananthapuram sample.

PeakTable

Detector A Ch1 343nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	13.048	1456660	61595	100.000	100.000
Total		1456660	61595	100.000	100.000

PeakTable

Detector A Ch1 343nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	13.007	3108795	128748	100.000	100.000
Total		3108795	128748	100.000	100.000

Calibration curve

Standard solutions of 100 – 6400 ng/mL of piperine were analyzed to check the linearity of response.

Table No. 12: linearity and range of standard piperine.

S.No	Concentration(ng/mL)	Peak area
1	100	218299
2	200	566010
3	400	1132080
4	800	2264250
5	1600	4528575
6	3200	9057207
7	6400	17224405

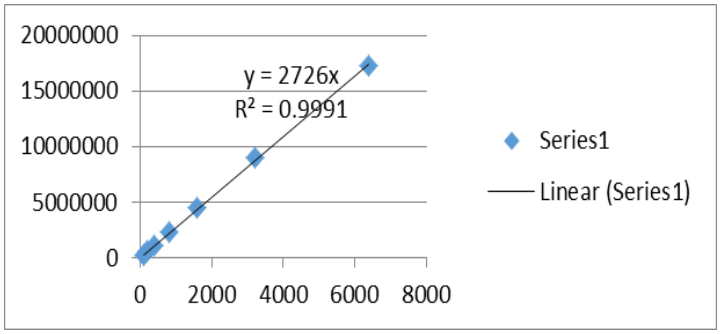


Fig. 15: calibration curve of standard piperine.

ASSAY

Standard and sample solutions were injected in HPLC system. The amount of piperine present per gram or per ml of piper nigrum extract was calculated by comparison of the areas measured for the sample with the calibration curve constructed from peak area obtained from standard solution of piperine The percent content of piperine was found to be1.66%, 2.66%, 4.48%, 6.12%, 1.61% and 3.43% in the piper nigrum samples collected from Wayanad, Idukki, Kozhikode, Alappuza, Palakkad, and Thiruvananthapuram respectively.

Table No. 13: Peak table of HPLC chromatogram.

S.No.	Samples	Ret.time	Area	Height	Area %	Height %
1	Standard piperine	12.653	9057207	392468	100.00	100.00
2	Wayanad	13.013	1506876	20961	100.00	100.00
3	Idukki	13.037	2411256	101293	100.00	100.00
4	Kozhikode	12.978	4062447	169831	100.00	100.00
5	Alappuzha	13.019	5541868	232734	100.00	100.00
6	Palakkad	13.048	1456660	61595	100.00	100.00
7	Thiruvananthapuram	13.007	3108795	128748	100.00	100.00

The percent content of piperine were calculated present in piper nigrum (black pepper) collected from different geographical source. The result shows, the pecent content differs from location to location and the percent content lays between 1.61% and 6.12%. The sample collected from Alappuzha gives high percent content (6.12%) and sample collected from Palakkad gives low percent content (1.61%). The percent content of piperine were found to be First prepapre the calibration curve using standard solutions of the analyte in a particular mobile phase. Then take extracted or purified sample's HPLC chromatogram for its peak at the retention time. Then find out the concentration of the analyte in the extracted sample using linear equation. The formula of % purity can be applied.

$$\text{Amount} = \frac{\text{Peak Area of sample}}{\text{Peak Area of standard}} \times \frac{\text{Concentration of standard}}{\text{Concentration of sample}} \times \text{Average weight}$$

Assay Data

Table No. 14: percent content of piperine.

S.No.	Sample	Mean concentration	% content
1	Wayanad (n=6)	0.0166±0.00004 mg/ml	1.66%
2	Idukki (n=6)	0.0266±0.00002 mg/ml	2.66%
3	Kozhikode (n=6)	0.0448±0.00011 mg/ml	4.48%
4	Alappuzha (n=6)	0.0619±0.0o007 mg/ml	6.12%
5	Palakkad (n=6)	0.0161±0.00013 mg/ml	1.61%
6	Thiruvananthapuram (n=6)	0.0343±0.00009 mg/ml	3.43%

CONCLUSION

In summary, simple, environment friendly, cost-effective, and convenient method for the Quantitative analysis of piperine was achieved with specific reagents and conditions.

This research work describes the isolation,identification and Quantitative analysis of piperine from *Piper nigrum linn.* of various regions of kerala by using RP-HPLC method along with spectroscopic methods,TLC and phytochemical screening.

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the methods were complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of piperine in piper nigrum linn.(black pepper).

A very few analytical methods appeared in the literature for the determination of piperine, simple analytical methods were planned to develop with sensitivity, accuracy, precision and low cost. This methode also helpful in herbel industry.

The result obtained from quantitative estimation of piperine in black pepper shows the percentage content of piperin deffer from location to location. This comparative study helpful for the determination of piperin in pepper and the result shows the amount of piperine present in the pepper collected from Alappuza containe more piperine than other samples from kerala.And the piperin content of Palakkad shows low piperine content.

ISOLATION

Piperine was successfully isolated with high purity. Possible improvements could be made to the experiment to improve the percentage recovery of the piperine, such as increasing the time the black pepper is refluxed.

IDENTIFICATION

Phytochemical analysis include Simple and economical chemical tests were conducted and it give positive results. TLC method used for the calculation of R_f value of piperin. The values are matching with standard values.

SPECTROSCOPIC METHODS

Development and validation of spectrophotometric method for the estimation of piperine in piper nigrum could be used as a valuable analytical tool in routine analysis of piperine in polyherbal formulations containing piperine and other crude drugs. The UV-VIS spectrophotometric method demonstrated were simple, sensitive, accurate and precise. In addition to the positive requirement for analytical method, the striking advantage is that they are economical also.the method was validated in terms of sensitivity, accuracy and precision. Hence this method can be used for the routine determination of crude drug and pharmaceutical dosage formulations.

IR data indicates the presence of piperine.All signals were annotated accordingly, and matched the predicted spectra for piperine, indicating that the desired product has been isolated and contain piperine.The peak obtained after the FT-IR spectra of piper nigrum compared with the standard IR absorption value of pure piperine. The values are matching with standard values.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This research work describes simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of piperine from *piper nigrum linn.* The developed method was found to be more sensitive, accurate and precise compared to the spectrophotometric methods. This method can be used for the routine determination of piperine in polyherbal formulations containing piperine.

The main objective of this study was to increase the solubility of Carvedilol using hydrophillic polymers β-cyclodextrin and Plasdone K-30. Further the incorporation of the prepared solid dispersion was done in the formulation of press-coated pulsatile drug delivery system for the treatment of hypertension. The results of saturation solubility, drug content and*in-vitro* dissolution study of solid dispersions of Carvedilol indicated that the solubility of Carvedilol was increased with increasing the concentration of hydrophillic polymers.

The batch SD6 with drug to Plasdone K-30 ratio of 1:3 showed the greater increase in solubility than the pure drug. The FTIR spectroscopy study showed that there was no interaction between Carvedilol, β-cyclodextrin and Plasdone K-30. The DSC study of the pure drug showed melting endotherm at 118^0C corresponding to the melting point of the drug indicating that the drug is crystalline. Whereas the DSC study of solid dispersion indicated that the drug is in amorphous state. Thus it can be concluded that spray drying is effective in increasing the solubility of Carvedilol. Further the results of SEM study showed that the spray dried solid dispersion particles of batch SD6 are spherical in shape with greater porosity. This indicates that the solubility was increased which is further confirmed from the *in-vitro* dissolution studies.

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