EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 10
Issue: 1
221-236
Year: 2023

EVALUATION OF STANDARDIZATION PARAMETERS FOR BERBERINE IN AYURVEDIC FORMULATION

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Article Received on 07/11/2022 **Article Revised on 28/11/2022** **Article Accepted on 18/12/2022**

ABSTRACT
The aim of this work is Evaluation Of Standardization Parameters For Berberine In Ayurvedic Formulation. Berberine (Berberidaceae) is a very common substitute to " Daruharida" which is used in the Ayurvedic system of medicine. The crude drug was successively extracted by soxhlet assembly using methanol solvent Preliminary evaluation, microscopical evaluation, Phytochemical investigation and Determination of physical characteristic of powder. Phytochemical screening of Berberine showed the presence of Alkaloids, Glycoside, Carbohydratyes, Proteins, Flavonoids and Tannins in the crude drug.Quantitative estimation by UV-Spectroscopy and Quantitative estimation by High Performance Liquid Chromatographic Method for estimation of Berberine. The concentration of Berberine extract was determined using Zodiac-100 (150 mm × 4.6 mm,5µm Particle size) with Mobile phase of Trifluroacetic acid 0.5%: Acetonitrile 80:20 v/v,at flow rate 1.5ml/min and with UV detection at 336 nm gives good separation of Berberine at Rt 10.55 min. The berberine content was observed in methanol extract when compared to sample the proposed UV and HPLC method is rapid, sensitive, simple and accurate HPLC method has been developed for the Berberine.

KEYWORDS: Berberine, UV, HPLC, Daruharidra.

INTRODUCTION
The recognition of the herbal drugs day to day increasing and arriving new formulations in the market. A number of the pharmaceutical industry to introduce herbal products because of public demand and safety of the therapy. The rational use of herbal medicine is very limited adverse effects and traditionally practicing since long years ago to treat disease, but there is no documented evidence. In this regard, recent years documenting many of the herbal drugs with scientific support many documented evidence of Indian system of medicines such as Siddha and traditional medicines of Unaited and Ayurvedic

The caution of herbal medicines is very important and if using inappropriately to cause serious health issues even lethal. While the use of herbal medicines needs more documentary evidence and should be nontoxic with minimal or no side effects. Some of the herbal drugs having a more poisonous effect, then a beneficial effect. One of the global issues is poor quality due to contamination of heavy metals and poisonous substances; it needs proper guidelines to investigate the herbal medicines.

This is very helpful to treat multiple disease conditions without harming effects. Hundreds of polyherbal formulations are available in the market for the management of different disease conditions such as metabolic diseases and dermatological infections. Majority of formulations is more than eight drug combination; the main issues are arising from the standardization of drugsand quality. So need further investigations on quality control.

Advantages of Herbal Medicine
- They have large amount of use.
- They have better patient tolerance as well as acceptance.
- The medicinal plants have renewable source of cheaper medicines.
- Improvements in the quality, efficacy and safety of herbal medicines with the development of science and technology.
- They are not harmful.
- They are more effective than any synthetic drugs.
- They have longer duration of treatment.
- They do not provoke allergic reaction and do not have negative side effect.

BERBERINE STRUCTURE

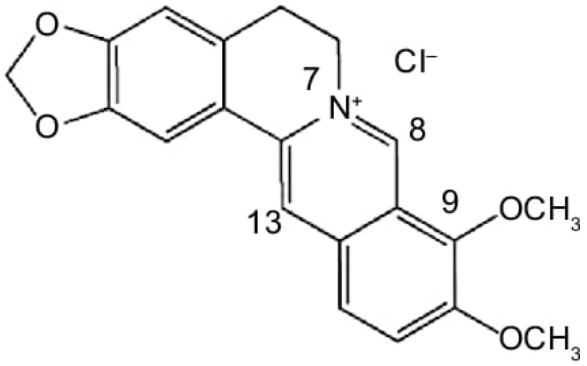


Figure no.1: Chemical structure of Berberine.

Table No 1: Drug Profile of Berberine.

Name of drug	Berberine
IUPAC Name	9,10-Dimethoxy-5,6-dihydro-2*H*-7λ^5-[1,3]dioxolo[4,5-*g*]isoquinolino[3,2-*a*]isoquinolin-7-ylium
Chemical formula	$C_{20}H_{18}NO_4^+$
Molar mass	336.366 g.mol^{-1}
Appearance	Yellow solid
Melting point	145℃
Biological Source	Berberine is a quaternary ammonium salt from the protoberberine group benzylisoquinoline alkaloids found in such plants as Berberis, such as Berberis vulgaris(Barberry), Berberis aristata(tree turmeric), Tinospori Cordifolia, Berberine is usually found in the roots, rhizomes, stems and bark. Family Berberidaceae.
Chemical constituents	Oxyberberine, Berbamine, Aromoline, Karachine, Palmatine, Oxyacanthine and Taxilamine.
Use	Bacterial infection, Inflammation, Diabetes, High cholesterol, High blood pressure, Obesity, Polycystic ovary syndrome.

MATERIALS AND METHODS

Plant materials

Plant material was collected from the local market of India .The plant was identified as Berberine Aristata family Berberidaceae from Government college of pharmacy, Amravati. The plant was carefully collected and air dried under shade. The air dried material were powered passed through 40 mesh sieve size and stored in an airtight container for further use.

Extraction of Berberine

The roots of Berberine were washed with water, chopped into small pieces and air dried at room temperature for 7 days and coarsely powdered of pulverized plant material was extracted by soxhlet extraction method with petroleum ether as solvent at 60-80℃ then extracted with 500 ml of methanol for 48 h. The obtained extract was filtered and evaporated of solvent and extract was concentrated.

Qualitative Evaluation

Organoleptic evaluation refer to evaluation of drug by colour, odour, taste etc, using organs of our body.

Appearance – Appearance takes place by visual examination.

Colour – Dried sample was the taken in a test tube, and colour was observed in the sunlight.

Odour – Freshly prepared sample was taken for evaluation of odour.

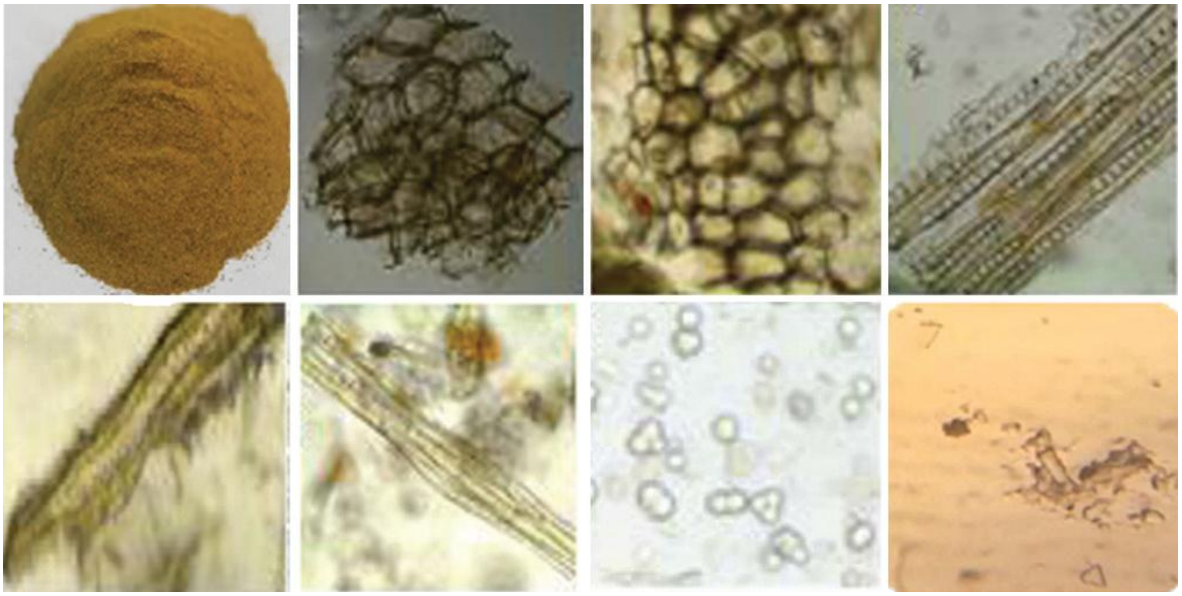
Taste – Freshly prepared sample was taken for evaluation of taste.

Table No 02: Organoleptic properties of Berberine drug.

Sr.No	Sample	Apperence	Colour	Taste	Odour
1	Berberine	Powder	Yellow-brown	Bitter	Woody

Microscopical Evaluation

The transverse section of Berberis root were prepared using sharp razor then section were treated with few amount of chloral hydrate. Best section was selected and mounted glycerin temporally and observed under light microscope.



Powder microscopy – (a) Powdered root (b) Cork cells (c) Parenchymatous cells containing starch grains (d) Tracheids and vessels along with medullary rays (e) Lignified and pitted tracheid (f) Fibers (g) Prismatic crystals of calcium oxalate (h) Starch grains.

Figure No 02: Observation of Powder Microscopy of Berberine.

Phytochemical Investigation

a) Detection of Alkaloids

(Hager´s Test) Small amount of drug was taken in test tube, Hager´s reagent was added, shaken well and filtered. Yellowish colour was formed showing the presence of alkaloids.

b) Detection of Cardiac Glycosides

(Baljet´s test) Treat was test solution with picric acid shake well, Yellowish brown coloured was formed showing the presence of glycosides.

c) Detection of Carbohydrates

(Osazone formation test) Heat was drug with solution of phenylhydrazine hydrochlorides, sodium acetate and acetic acid. Yellow crystal was formed showing under microscope the presence of carbohydrates.

d) Detection of Proteins

(xanthoproteic test) A Small quantity of test solution was taken, 1ml of concentrated nitric acid was added and boil, Yellow precipitate was formed. After cooling it, 40% sodium hydroxide solution was added orange coloured was formed.

e) Detection of Flavonoid

(Alkaline reagent test) A small quantity of test solution was taken, few drpos of sodium hydroxide solution was added, intense yellow coloured was formed which turns to colorless on addition of few drops of dilute acid indicate presence of flavonoids.

f) Detection of Tannins

(Ferric chloride test) Treat was extract with ferric chloride solution, blue coloured was appeared if hydrolysable tannins are present and green coloured was appears if condensed tannins were present.

Table No 03: Observations and Result of Phytochemical Investigation.

Sr No.	Test	Observation	Result
1	Alkaloids	Yellowish coloured was observed	Present
2	Glycosides	Yellowish brown colured was observed	Present
3	Carbohydrates	Yellow coloured was observed	Present
4	Proteins	Yellow coloured was observed	Present
5	Flavonoids	Yellow coloured was observed	Present
6	Tannins	Blue coloured was observed	Present

Determination of Physical Characteristics of Powder

Physical characteristics like Bulk density, Tap density, Angle of repose and Carr´s index were determined for different formulation.

a) Bulk Density and Tap Density

The term bulk density refers to a measure used to describe a packing of particles or granules. The equation for determining bulk density (D),Db.=M/Vb.

Where, M = Mass of the particles and

V =Total mass of the packing.

The volume of packing can be determined in an apparatus consisting of a graduated cylinder mounted on a mechanical tapping device (Jolting Volumeter) that has specially cut rotating can 100 gm. of weighed formulation powder was taken and carefully added to the cylinder with the aid of funnel. Typically the initial volume was noted and the sample was then tapped until no further reduction in volume was noted. The initial volume gave the Bulk density value and after tapping the volume reduced, giving the value of tapped density.

Table No 04: Observation and Result Bulk density of Berebrine.

Sr.No	Sample	Mass of power (gm)	Bulk volume of powder (ml)	Bulk density (g/cc)
1	Berberine	20	36	0.555

Table No 05: Observation and Result Tapped density of Berebrine.

Sr.No	Sample	Mass of powder (gm)	Tapped volume of powder	Tapped density
1	Berberine	20	27	0.740

b) Angle of repose

The internal angle between the surface of the pile of powder and the horizontal surface is known as the angle of repose. The powder is passed through funnel fixed to a burette at a height of 4 cm. A graph paper is placed below the funnel on the table. The height and the radius of the pile were measured. Angle of repose of the powder was calculated using the formula.

Angle of repose = tan-1 (h/r)

Where, H=height of the pile

r =radius of the pile

Table No 06: Observation and Result Angle of repose of Berebrine.

Sr.No	Sample	Height of the pile (cm)	Radius of circle (cm)	Angle of repose = $\tan^1$ (h/r)	Flow
1	Berberine	2.8	4.7	30.75	Excellent

c) Carr´s Index

It is the propensity of the the powder to be compressed. Based on the apparent bulk density and tapped density the percentage compressibity of the powder can be determined using the following**.**

% compressibility = [(tapped density – bulk density)] / tapped density] ×100

Table No 07: Observation and Result Carr´s Index of Berberine.

Sr.No	Sample	Tapped density	Bulk density (g/cc)	Carr´s Index
1	Berberine	0.740	0.555	25.0

Physical Evaluation

Physical Standards are determined for drug. These are rarely constant for crude drug, but may help in evaluation, specifically with reference to moisture content, specific gravity, density optical rotation, refractive index, melting point and solubility in different solvents.

a) Solubility

The presence of adulterant in a drug was indicate by solubility studies-

The solubility of Berberine is soluble in organic solvents such as Ethanol, Methanol and Dimethyl formamide.

b) Melting Point

The determination of a melting point to identify a sample, purity and determine thermal stability of the sample.

The method involves placing the sample in capillary tube and running the heat sample until it reaches melting point, The melting point of Berberine 143-145℃.

c) Moisture Content (Loss on drying)

The drying oven method is a thermogravimetric method (Loss on drying) in which the sample was dried for a defined period of time at constant temperature. The moisture content was determined by weighing the sample before and after drying and determining the difference.

10 g of the sample (without preliminary drying) was weighed and placed in tarred evaporating dish. It was dried at 105℃ for 5 hours, and at 1 hour interval until difference two successive weighing corresponding to not more than 0.25%.

Loss of drying = a/b × 100

Where, a = Weight of crucible

b = Weight of crucible after incineration

Table No 08: Observation and Result of Loss on Drying.

Sr. No	Sample	Weight.of crucible with sample	Weight. Of crucible after incineration	Percentage (%)	Mean ± SD
1	Berberine	5	4.2	0.84%	0.8 ± 0.04
2		5	4.0	0.8%	
3		5	3.8	0.76%	

d) Determination of Total ash

About 2 to 3g of sample was accurately weighed in a tarred silica dish at a temperature not exceeding 450℃ until it was free from carbon. Then it was cooled and weighed. The percentage of total ash was calculated with reference to the air dried.

Total ash value = 100 (z – x) /y%

Where, Z = weight of ash

Y = Weight of drug taken

Table No 09: Observation and Result of Total Ash content.

Sr. No	Sample	Wt.of crucibile with sample(g)	Wt.of crucible after incineration	Wt.of ash(Z)	Percentage of ash	Mean±SD
1	Berberine	34	33.86	0.14	7	5.6±1.25
2		34	33.89	0.11	5.5	
3		34	33.91	0.09	4.5	

e) Determination of acid insoluble ash

Total total ash obtained was boiled for 5 minutes with 25 ml of dilute hydrochloric acid, the insoluble matter obtained was collected on an ash less filter paper, washed with hot water and ignited to constant weight. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

Acid insoluble ash = 100 × a / y %

Where, a =Weight of insoluble after ignition

Y = Weight of drug taken.

Table No 10: Observation and Result of Acid Insoluble Ash.

Sr.No	Sample	Wt of total ash in 25ml dil hcl	Wt.of insoluble matter	Wt.of insoluble matter after ignition	Percentage of acid insoluble ash	Mean±SD
1	Berberine	2	0.14	0.08	2	1.83±0.35
2		2	0.11	0.05	2.5	
3		2	0.09	0.02	1	

f) Water Soluble Ash

The ash obtained in the determination of total ash was boiled for 5 minute with 25 ml of water. The insoluble matter was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into a tarred silica crucible and ignited for 15 minute at a temperature not exceeding 450℃. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight was considered as the water soluble ash was calculated with reference to the air dried drug.

Water insoluble ash = 100 × a/y %

Where, a = weight of insoluble after ingnition

Y = weight of drug taken

Table No 11: Observation and Result of Water Soluble Ash.

Sr.No	Name of Sample	Wt.of total ash in 25 ml water	Wt.of insoluble matter	Wt.of insoluble matter after ignition	Percentage of water insoluble ash	Mean±SD
1	Berberine	2	0.16	0.12	6	4.66±1.25
2		2	0.13	0.09	4.5	
3		2	0.11	0.07	3.3	

g) Determination of Water Soluble extractive

5 g of test sample was weighed and macerated with 100 ml of chloroform water in a closed flask for twenty-four hours, Shaking frequently during six hours and allowing standing for eighteen hours. It was filtered rapidly, taking precaution against the loss of solvent 25 ml of the filtered was taken and evaporated to dryness in a tarred flat bottomed shallow dish at 105℃, to constant weight and weighed the percentage of warer soluble extractive was calculated with reference to the air dried sample.

Water soluble extractive value of sample = 80x %

Where, x = Weight of residue

Table No 12: Observation and Result of Water Soluble Extractive.

Sr No.	Sample	X value	80x%	Mean ± SD
	Berberine			12.26 ± 2.44
1		0.12	9.6	
2		0.18	14.4	
3		0.16	12.8	

h) Determination of Alcohol Soluble Extractive

Procedure for water soluble extractive was followed for the determination of alcohol soluble extractive but 90 % ethanol was used instead of chloroform water.

Alcohol soluble extractive value of sample = 80x %

Where, x = Weight of residue

Table No 13: Observation and Result of Alcohol Soluble Extractive.

Sr No.	Sample	X- value	80x%	Mean±SD
1	Berberine	0.13	10.4	16.55±5.32
2		0.24	19.2	
3		0.25	20	

QUANTITATIVE ESTIMATION BY UV – SPECTROSCOPY

Development of UV-Spectrophotometric Method For Berberine

a) Selection of Solvent

Berberine is soluble in ethanol and methanol. The spectra of Berberine show the feasibility of using ethanol for spectrophotometric analysis for this drug.

b) Preparation of Standard Stock Solution: (150µg/ml)

An accurately weighed quantity of berberine 1.5 mg of drug was transferred into 10 ml of volumetric flask. About 7 ml of ethanol was added to the volumetric flask and sonicated to dissolve the drug. The solution was cooled to the room temperature and volume made up to the mark.

c) Study of Spectra, Selection of scanning range and detecting wavelength.

Standard solution scanned in 1cm cell in a range of 200-400 nm and spectra were recorded.From the spectra obtain Figure no 03.

The wavelength selected for estimation of Berberine were at 336 nm.

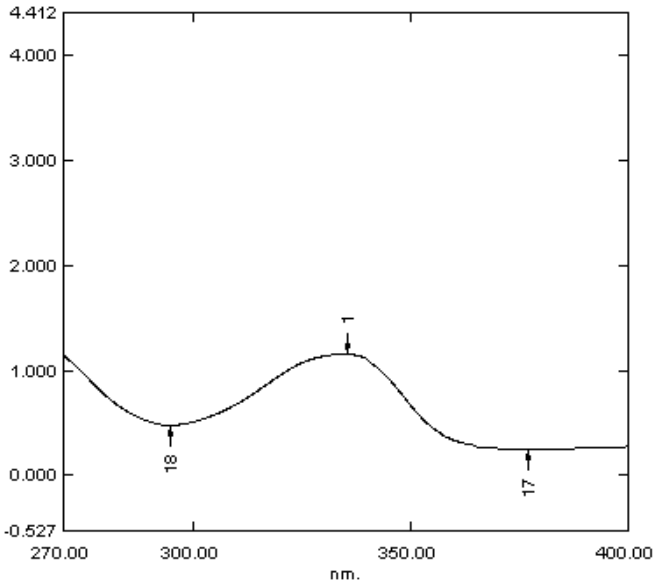


Figure No 03: Determination of λmax of Berberine.

d) Study of Beer - Lambert´s Law

Calibration curve for the Berberine (5-30µg/ml) appropriate volume of aliquots from standard Berberine stock solution was transferred to different volumetric flasks. The volume was adjusted to the mark with the diluent to obtain the concentration of 5, 10,15,20,25 and 30 µg/ml. The curve of each solution against the diluent was recorded at 336 nm was measured and the plot of absorbance v/s concentration was plotted. The absorptivity of drug at wavelength 336 nm was found by straight-line equation. It shows straight line meaning the calibration curve obeys Beers law.

Table No 14: Calibration Data.

Sr No.	Concentration	Absorbance
1	5	0.473
2	10	0.897
3	15	1.306
4	20	1.721
5	25	2.028
6	30	2.462
Slope		0.0809
R^2		0.9976

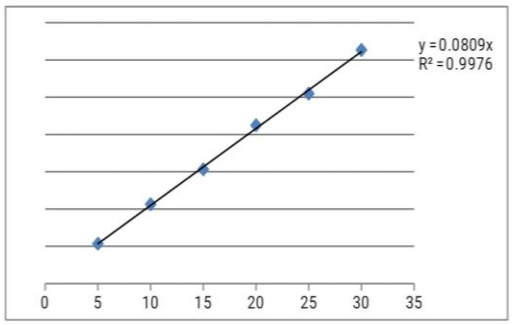


Figure No 04: Plot of Beer - Lambert´s Law of Berberine.

Application of proposed method to standard laboratory sample.

a) Preparation of Standard Stock Solution: (150 µg/ml)

An accurately weighed quantity of berberine 1.5 mg of drug were transferred into 10 ml of volumetric flask. About 7 ml of ethanol was added to the volumetric flask and sonicated to dissolve the drug. The solution was cooled to the room temperature and volume made up to the mark.

b) Study of Spectra, Selection of scanning range and detecting wavelength.

Standard solution scanned in 1cm cell in a range of 200-400 nm and spectra were recorded.From the spectra obtain Figure no 05.

The wavelength selected for estimation of Berberine were at 336 nm

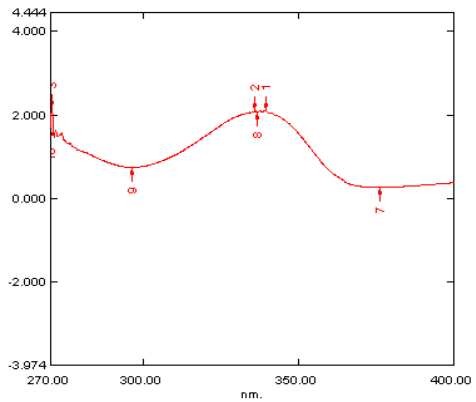


Figure No 05: Determination of λ max of standard solution of Berberine.

Application of proposed method to prepared sample

a) Preparation of Standard Stock Solution: (150µg/ml)

An accurately weighed quantity of berberine 1.5 mg of drug were transferred into 10 ml of volumetric flask. About 7 ml of ethanol was added to the volumetric flask and sonicated to dissolve the drug. The solution was cooled to the room temperature and volume made up to the mark.

b) Study of Spectra, Selection of scanning range and detecting wavelength

Standard solution scanned in 1cm cell in a range of 200-400 nm and spectra were recorded.From the spectra obtain Figure no 06.

The wavelength selected for estimation of Berberine were at 336 nm.

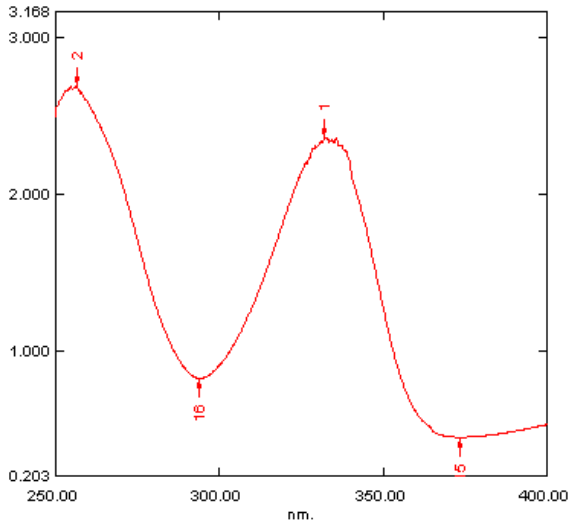


Figure No 06: Determination of λmax of prepared sample of Berberine.

QUANTITATIVE ESTIMATION BY HPLC

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

1. Method development of HPLC

a) Preparation of standard stock solution and selection of detecting wavelength standard stock solution

An accurately weighed quantity of berberine 1.5 mg of drug was transferred into 10 ml of volumetric flask, dissolved in HPLC grade and volume was made up to mark with same solvent.(Conc.1.5 mg/ml).The aliquot portion of standard stock solution was diluted appropriately with same solvent to obtain concentration of 60.50µg/ml. This solution was scanned in 1 cm cell using double beam UV-visible spectrophotometer over range of 200-400 nm and wavelength selected for estimation of drug was 336 nm as shown in Figure 07.

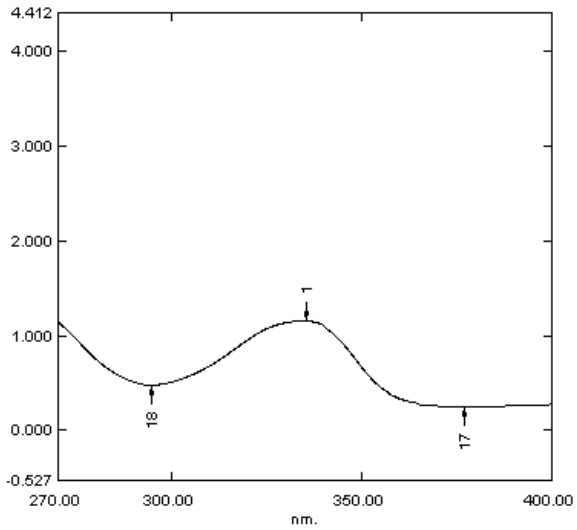


Figure No 07: UV absorption spectrum of Berberine.

b) Selection of mobile phase

The drug of Berberine was injected into HPLC system and run in different solevent systems. Each mobail phase was allowed to equilibrium with stationary phase until steady baseline was obtained .Different mobile phases like Methanol and Trifluroacetic acid and Acetonitrile in various proportions were tried. Different individual solvents as well as combinations of solvents were tried to get a stable peak. Each mobile phase was filtered through 0.45µm nylon membrane filter and sonicated on ultra sonic bath. After several trials Trifluroacetic Acid (0.5 %): Acetonitrile (80: 20 v/v) was found to be most satisfactory since it gave sharp, peak with symmetry within limits and significant reproducible retention time.

c) Selection of chromatographic mode and Selection of stationary phase

The analysis of the drug was carried out on Shimadzu SCL 10Ave inbult with binary pump (LC-10ATvp) System, UV Detector: HPLC/GC Column Zodic-100 (150mm×4.6mm, Particle size: 5 micron), UV Absorbance detector and running UV Probe Software.

d) Selection of flow rate

Different mobile phase flow rates (1ml/min) were investigated. The optimum flow rate for which the column plate number (N) was Maximum, with the best resolution obtained and with a short run time was selected.

The following chromatographic conditions were established by trial and error and were kept constant throughout the experimentation.

Table No 15: Chromatographic parameter.

Sr.no	Parameter	Specification
1	HPLC	Shimadzu SCL-10Ave;Binary pump equipped (LC 10ATvp)
2	Software	LC Solution
3	Column	Zodiac-100 (150mm x 4.6 mm, 5 µm partical size)
4	Stationary phase	Zodiac-100 (C18)
5	Mobile phase	TrifluroaceticAcid(0.5%):Acetonitrile(80:20v/v)
6	Detection wavelength	336 nm wavelength
7	Flow rate	1 ml/min
8	Temperature	28℃
9	Run time	15 min
10	Filter paper	0.45µ

2. Study of Berberine on chromatographic condition used in method development of HPLC for the following mobile phase were tried For Method development of HPLC

Table No 16: Selection of Mobile Phase Used.

Sr.no	Mobile phase
1	Trifluroacetic acid(0.5%):Acetonitrile (80:20v/v)
2	Acetonitrile(60): Water(40):Methanol(0.1)
3	Acetonitrile(90):Water(10):Trifluroacetic acid (0.1)
4	Acetonitrile(80): Water(20):Trifluroacetic acid(0.1)

3. Analysis was done by following parameter

- Solubility
- UV spectra and Determination of λmax
- HPLC chromatogram

a) Preparation of standard stock solution:

An accurately weighed quantity of Berberine about 1g was transferred into 100 ml Amber coloured volumetric flask, dissolve with 0.5% Trifluroacetic acid and acetonitrile(80:20v/v) using a 20 ml. Volumeteric flask and completing the final volume by adjusting with either 5% Trifluroacetic acid and Acetonitrile, based on their solubility in particular solvents. Furthermore, freshly prepared sample solution was sonicated for 10-20 minutes and filtered through 0.20µ nylon filters. Required serial dilution was made for evaluating the validation studies.

Preparation of Working Standard Stock Solution:
Working stock solution of Berberine (60.50µg ml-1) was prepared by serial dilution of 4ml of its stock solution in a 100 ml volumetric flask by completing volume with the mobile phase.

Chromatographic Condition
Chromatographic separation was achieved on a Zodiac – 100 C18,5µm; 150×4.6 mm ID,5µ particle size applying an gradient elution 0-3 min(20% B),3-10 min (80 % B),10-17 min(80 % B) based on 0.5 % Trifluroacetic acid (A) and Acetonitrile (B) (80:20v/v) as a mobile phase. The ultraviolet detector was operated at 336 nm. The buffer solution was filtered through 0.2µm nylon membrane filter and degassed for 10 – 20 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 1 ml.min^{-1}. The column temperature was adjusted to 28℃ and the injection volume was 20 µl.

Trials of HPLC Method
Trial 1:
Chromatographic Parameter
Table No 17: Chromatographic parameter trial no.1.

Sample name	Berberine
Mobile Phase	0.5% Trifluroacetic Acid,80:20v/v Acetonitrile
Flow rate	1 ml/min
Wavelength	336 nm wavelength
Run time	10.56 min
Sample Volume	20µl

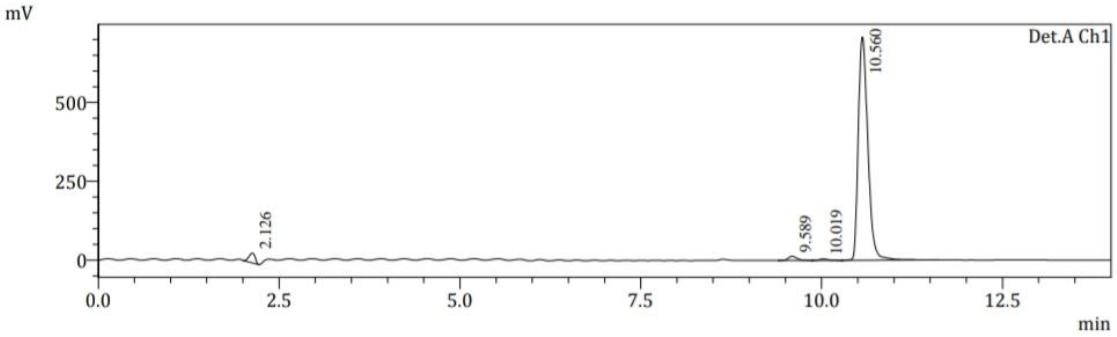


Fig No 08: Chromatogram of Berberine using 0.5% Trifluroacetic acid,80.20v/v Acetonitrile.

Trials 2
Chromatographic Parameter
Table No 18: Chromatographic Parameters Trial 2.

Sample name	Berberine
Mobile phase	Acetonitrile(60):Water(40):Methanol(0.1)
Flow rate	1ml/min
Wavelength	336nm
Run time	10.38min
Sample volume	20µl

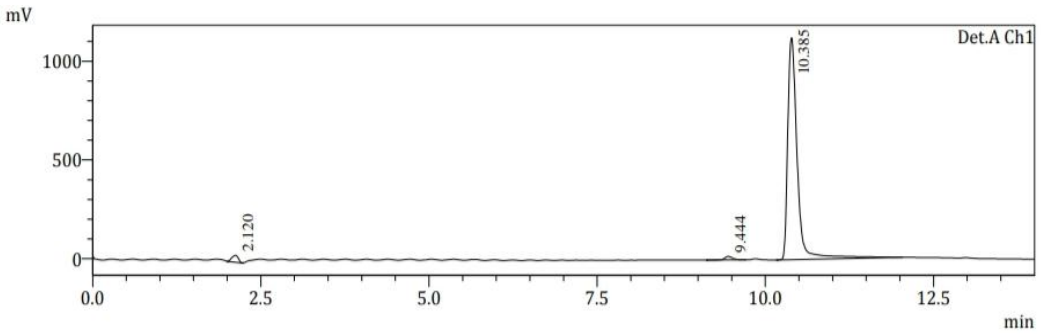


Fig No 09: Chromatogram of Berberine using Acetonitrile: Water: Methanol (60:40:0.1%v/v).

Trials 3

Chromatographic parameter

Table No 19: Chromatographic Parameters Trial 3.

Sample name	Berberine
Mobile phase	Acetonitrile(90):Water(10):Methanol(0.1)
Flow rate	1.5 ml/min
Wavelength	336nm
Run time	7.02
Sample volume	20 µL

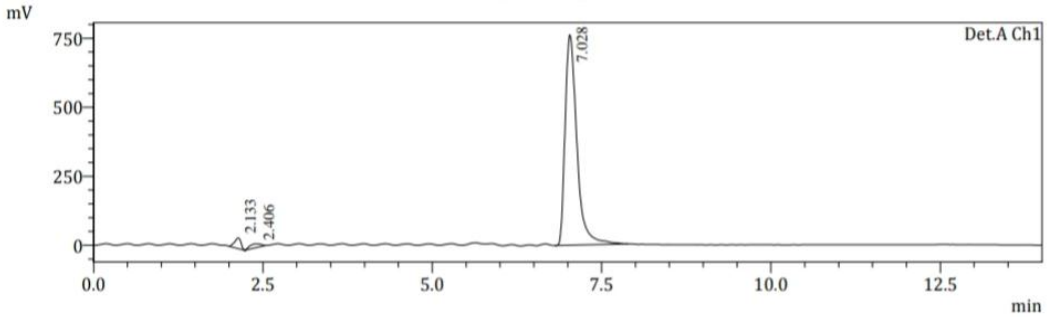


Fig No 10: Chromatogram of Cholecalciferol using Acetonitrile: Water: Methanol (90:10:0.1% v/v).

- **Finalized Chromatography Parameters**

Table No 20: Chromatographic Parameters.

Sample name	Berberine
Mobile phase	Acetonitrile(80):Water (20):Trifluroacetic acid(0.1)
Flow rate	1.5 ml/min
Wavelength	336nm
Run time	10.55min
Sample volume	20 µL

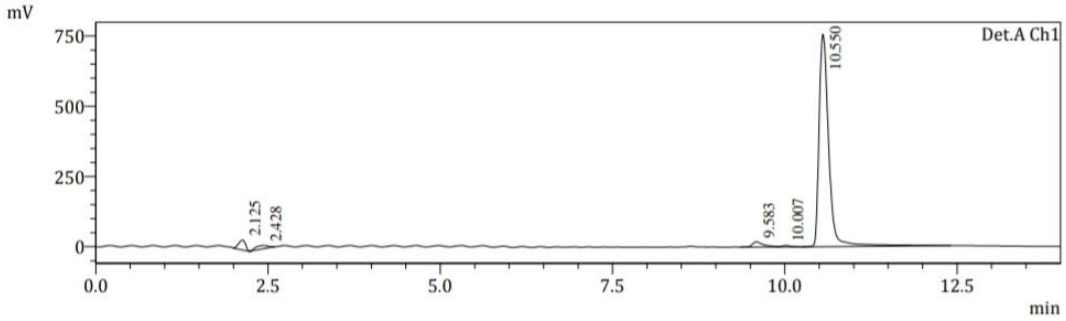


Fig No 11: Chromatogram of Berberine using Acetonitrile: Water: Trifluroacetic acid (80:20:0.1% v/v).

Analysis Specification

Table No. 21: Analysis Specification of Berberine.

Analytes Selected	Berberine
Analyte concentration	62.50ppm
Marketed Formulation	Healthy-Hey Nutritional(Berberine 95% in 300mg capsules);Healthy Hey Foods LLP.
HPLC/GC Column Specification	Zodiac-100;150×4.6mm.ID.5µ Partical size
Eluents/Solvents Composition	0.5% TrifluroaceticAcid,Acetonitrile(80:20v/v)
Elution Type	Gradient Elution;0-3min(20%B) 3-10min(60%B),10-17min(60%B)
Wavelength Selected	336nm
Flow Rate	1ml/min
Temperature	28℃
Instrument Name and Inbuilt Specification	Shimadzu SCL-10AVP;Quaternary pump equipped with VWD.

b) Application of proposed method to standard laboratory mixture:

Preparation of standard stock solution:

An accurately weighed quantity of Berberine about 1g was transferred into 100 ml Amber coloured volumetric flask, dissolve with 0.5% Trifluroacetic acid and acetonitrile(80:20v/v) using a 20 ml. Volumeteric flask and completing the final volume by adjusting with either 5% Trifluroacetic acid and Acetonitrile, based on their solubility in particular solvents. Furthermore, freshly prepared sample solution was sonicated for 10-20 minutes and filtered through 0.20µ nylon filters.

Preparation of Working Standard Stock Solution:

Working stock solution of Berberine (60.50µg ml-1) was prepared by serial dilution of 4ml of its stock solution in a 100 ml volumetric flask by completing volume with the mobile phase.

BERBERINE EXTRACTED

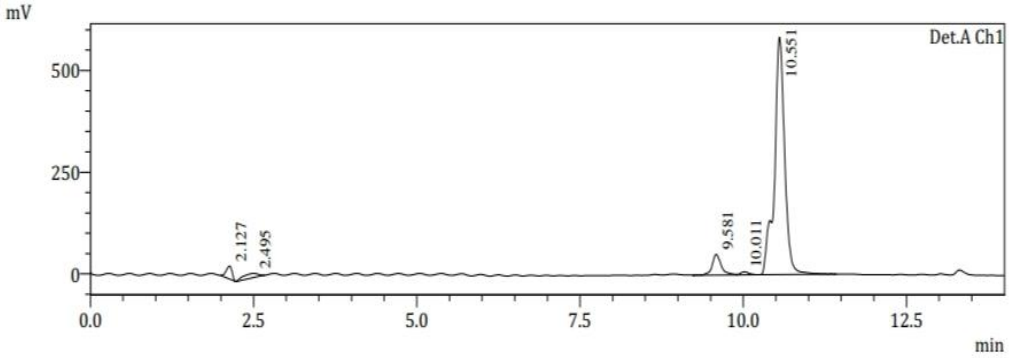


Figure No 12 : Chromatograph of Berberine Extracted (10.55 min).

Table No 22 : Observation and Result of Berberine Extracted.

Peak #	Ret. Time	Area	Theoretical plate #	Area %	Tailing Factor	k´
1	2.127	192991	2289.156	2.636	0.884	0.000
2	2.495	178400	622.795	2.437	0.785	0.173
3	9.581	488915	25282.341	6.679	1.253	3.505
4	10.011	66815	27430.684	0.913	0.000	3.708
5	10.551	6393254	26998.428	87.335	0.945	3.962
Total		**7320375**		**100.000**		

c) Application of proposed method to prepare sample:-

Preparation of standard stock solution

An accurately weighed quantity of Berberine about 1g was transferred into 100 ml Amber coloured volumetric flask, dissolve with 0.5% Trifluroacetic acid and acetonitrile(80:20v/v) using a 20 ml. Volumeteric flask and completing the final volume by adjusting with either 5% Trifluroacetic acid and Acetonitrile, based on their solubility in particular solvents. Furthermore, freshly prepared sample solution was sonicated for 10-20 minutes and filtered through 0.20µ nylon filters.

Preparation of Working Standard Stock Solution:

Working stock solution of Berberine (60.50µg ml-1) was prepared by serial dilution of 4ml of its stock solution in a 100 ml volumetric flask by completing volume with the mobile phase.

BERBERINE MARKETED FORMULATION

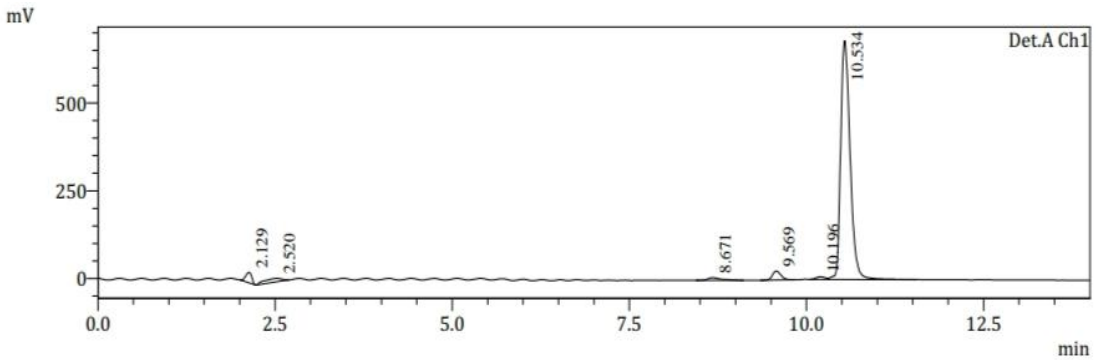


Figure No 13:- Chromatograph of Berberine marketed formulation (10.53 min).

Table No 23: Observation and Result of Berberine marketed formulation.

Peak #	Ret. Time	Area	Theoretical Plate	Area %	Tailing Factor	k´
1	2.129	172569	2537.513	2.439	0.915	0.000
2	2.520	180878	564.843	2.556	0.754	0.184
3	8.671	105147	11598.943	1.486	1.550	3.074
4	9.569	214702	27783.843	3.034	1.217	3.496
5	10.196	69307	23716.892	0.980	0.000	3.790
6	10.534	6333039	28810.635	89.505	1.412	3.949
Total		**7075641**		**100.000**		

Table No 24: Observation and Result of marketed formulation analysis.

Sr No.	Sample	Peak area	% Label claim
1	**Standard**	6393254	
2	**Sample**	6333039	99.05%
3		6292029	98.41%
4		6301019	98.55%
5		6281009	98.24%
6		6401007	100.12%
		Mean	**98.93**
		S.D	**0.883**
		R.S.D	**0.893**

Validation of Analytical Method

The method was validated as per ICH Q2 (R1) guidelines.

a) Smple preparation for drug recovery studies

Exactly 100 mg of Berberine were weighed separately, Powdered and mixed in a morter. An accurately weight 10 mg amount of Berberine was transferred to 25 ml volumetric flask. It was then mixed with 10 ml of equal volume of 0.5 % Trifluroacetic acid and Acetonitrile and sonicated for 20 minutes. Furthermore the solution was filtered through 0.20µ filter and then analysed with HPLC technique.

Repeatability data of Berberine

Table No. 25: Repetability data of Berberine.

S. No.	Drug Name **Berberin**
	Peak Area; **Conc. 62.50 ppm**
1	8466486
2	8434210
3	8484056
4	8498075
5	8488880
6	8389002
Mean	**8460118.16**
STD. DEV.	**41497.65**
RSD (%)	**0.49**

b) Sample preparation for Linearity and construction of Calibration Curve

Accurately measured aliquotes of stock solution equivalent to 62.50µg of Berberine, respectively were tranfered separately into series of 10 ml Volumeteric flask. The final volume was adjusted with same mobile phase, and then 20µL were injected into HPLC. A calibration curve (Linearity graph) was plotted by calculating peak area against concentration.

In these parameter range and linearity of Berberine concentration range was (R^2=0.9951), so that these parameter properly performed. Also concentration properly plot calibration graph on HPLC.

Table No 26: Liniearity of Berberine.

Name of Drug:-Berberine			
Sr No.	**Conc(µg m)**	**Area**	**Average (Mean)**
1	62.5	8126982	8126982
2	31.25	4019914	4019914
3	15.62	2688398	2688398
4	7.81	1674118	1674118
5	3.91	963454	963454
6	1.95	749463	749463
Regression Equation		**Y=119302x+590571**	
Correlation Coefficient(R^2)		**0.9951**	
Std.error of intercept		**123235.2399**	
Std.Dev. of intercept		**275562.3736**	
LOQ		**18.45**	
LOD		**13.62**	

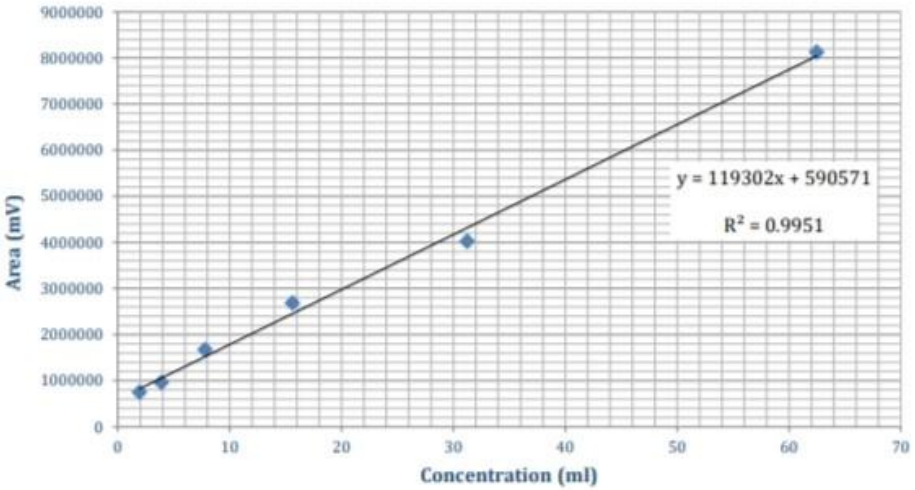


Figure No 14: Standard Calibration Curve Of Berberine.

c) Precision studies of the proposed method

Nine similar concentration of the Berberine was analyzed within the same day (Intraday precision). Also the mentioned concentration were analzed on three successive days using the same procedure; to determine the intermediate precision.

Intraday precision

The intraday study was performed by applying the proposed method on same sample of Berberine on same same day at interval.

Interday precision

The interday study was performed by applying the proposed method on same sample of Berberine on different day.

Precision studies were performed by method for Intraday and interday precision. The proposed method was determined by analyzed the solution of Berberine of three replicates of three same concentration,62.50ppm on the same and different days.

INTRADAY

The samples were analyzed on different times on same day by proposed method. The %RSD was calculated and found less than 2% shown in table.

Table No 27: Observation and Result Precision data of Intraday Berberine

Drug Name:-Berberine				
Sr No.	**Conc. (µg mL)**	**Area**	**Mean±SD**	**% RSD**
1	62.50 ppm	8484056		
	62.50 ppm	8498075	7122.164488	0.08
	62.50 ppm	8488880		
2	62.50 ppm	8389002		
	62.50 ppm	8359172	15843.84464	0.19
	62.50 ppm	8383345		
3	62.50 ppm	7467226		
	62.50 ppm	7212702	129628.9918	1.76
	62.50 ppm	7437226		
Average of RSD(%) 0.08-1.76				

INTERDAY

The samples were analyzed on different time on different day by proposed method. The %RSD was calculated and found less than 2% shown in table.

Table No 28: Observation and Result Precision data of Interday Berberine.

Drug Name:-Berberine				
Sr No.	**Conc(µg mL)**	**Area**	**Mean±SD**	**%RSD**
1	62.50 ppm	8434210		
	62.50 ppm	8389002	47545.85488	0.56
	62.50 ppm	8484056		
2	62.50 ppm	8112702		
	62.50 ppm	8298075	94973.02882	1.16
	62.50 ppm	8241267		
3	62.50 ppm	8988880		
	62.50 ppm	9234252	181544.9237	1.98
	62.50 ppm	9343342		
Average of RSD(%) 0.56-1.98				

d) Robustness for the chromatographic method:

The flow rate of mobile phase was changed by ± 2 decimal from 1 ml/min to 1.2 ml/min to 0.8 ml/min to evaluate the effect of the flow rate; similarly the variation of organic modifier used as acetonitrile was changed by ± 2 % from 25 % to 23 % and 27 % to monitor the peak area and retention time. Finally the effect of wavelength was monitored by making deliberate variation from 254 ± 2nm to 252 nm and 256 nm were selected and then differences in system sutability parameter such as retention time, peak tailing, capacity factor, resolution and theoretical plates were tested and evaluted.

Table No 29: Observation and Result of Roubustness for Berberine.

Drug Name: Berberine				
Variables	**t_R (min)**	**K´**	**T_f**	**N**
Flow rate (±0.2mL.min^{-1})	9.79	4.51	1.4	31657
Flow rate (±0.2mL.min^{-1})	11.59	3.17	1.42	26751
CH_3 OH (±2%)	10.14	3.76	1.41	27946
CH_3 OH (-2%)	10.88	4.13	1.41	33506
Wavelength (+2nm)	10.47	3.94	1.41	29941
Wavelength (-2nm)	10.5	3.94	1.41	31038
Mean ± SD	**10.56 ± 0.62**	**3.91 ± 0.44**	**1.41 ± 0.01**	**NA**

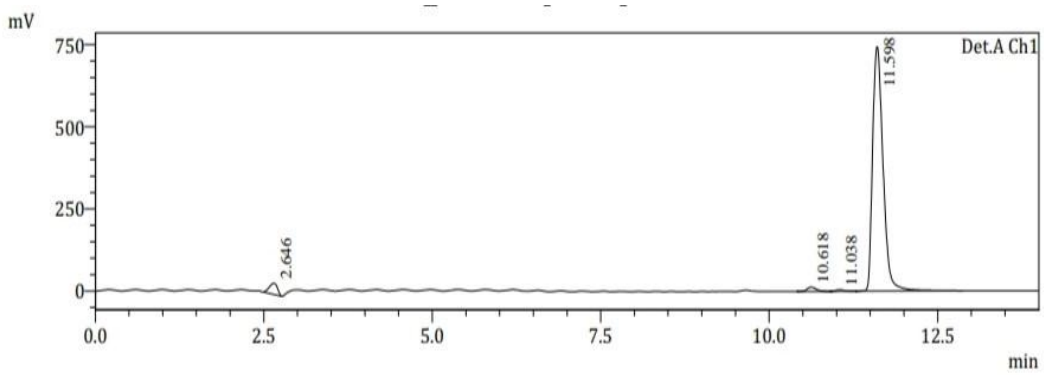


Figure No 15: Chromatograph of Robustness for Berberine (11.59min) at flow rate 0.8mL.min.

Detector A CH1 336nm

Table No 30: Observation and Result of Robustness for Berberine.

Peak #	Ret.Time	Area	Therotical Plate #	Area %	Tailing Factor	k´
1	2.646	281205	2054.395	3.309	0.877	0.000
2	10.618	125998	28188.945	1.483	1.301	3.012
3	11.038	40557	33371.747	0.477	1.425	3.171
4	11.598	8050556	26751.727	94.731	1.385	3.383
Total		**8498317**		**100.000**		

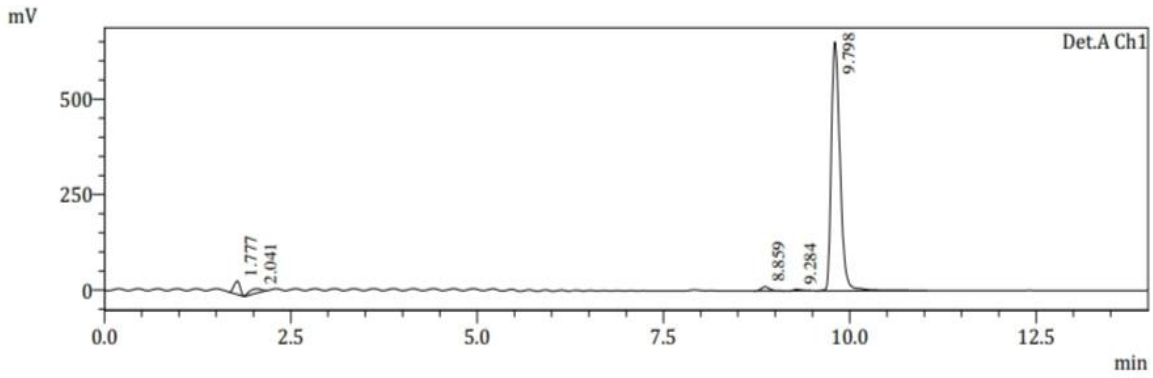


Figure No 16: Chromatograph of Berberine (9.79min) at flow rate 1.2 mL.min^{-1}.

Detector A CH1 336nm

Table No 31: Observation and Result of Berberine for Robustness.

Peak #	Ret. Time	Area	Therotical Plate #	Area %	Tailing Factor	k´
1	1.777	197934	1820.501	3.448	0.945	0.000
2	2.041	131013	882.300	2.283	0.879	0.148
3	8.859	77870	28228.036	1.357	1.343	3.984
4	9.284	23272	37712.868	0.405	1.405	4.223
5	9.798	5309769	31657.930	92.507	1.402	4.512
Total		**5739858**		**100.000**		

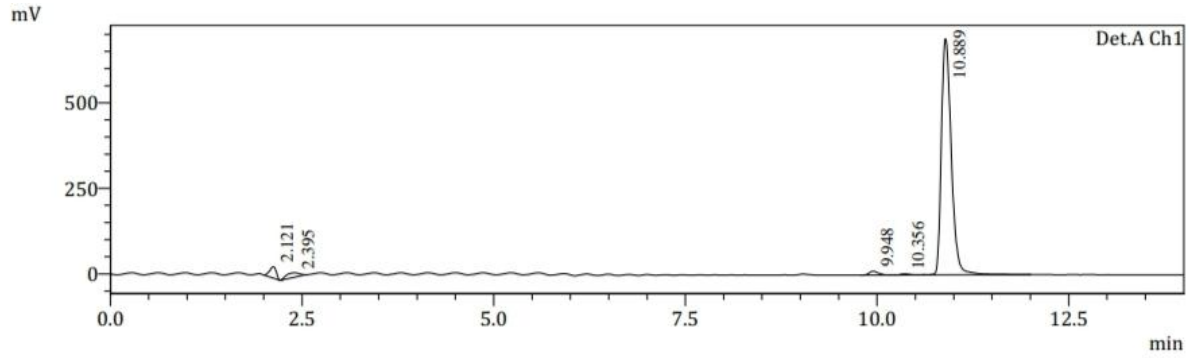


Figure No 16: Chromatograph of Berberine (10.88 min).

Detecor A CH1 336nm

Table No 32: Observation and Result of Robustness for Berberine.

Peak #	Ret. Time	Area	Therotical Plate #	Area %	Tailing Factor	k´
1	2.121	194179	2406.708	2.890	0.890	0.000
2	2.395	165035	1001.502	2.456	0.918	0.129
3	9.948	92778	30837.282	1.381	1.340	3.690
4	10.356	28345	34418.905	0.422	1.383	3.883
5	10.889	6238537	33506.152	92.851	1.410	4.134
Total		**6718874**		**100.000**		

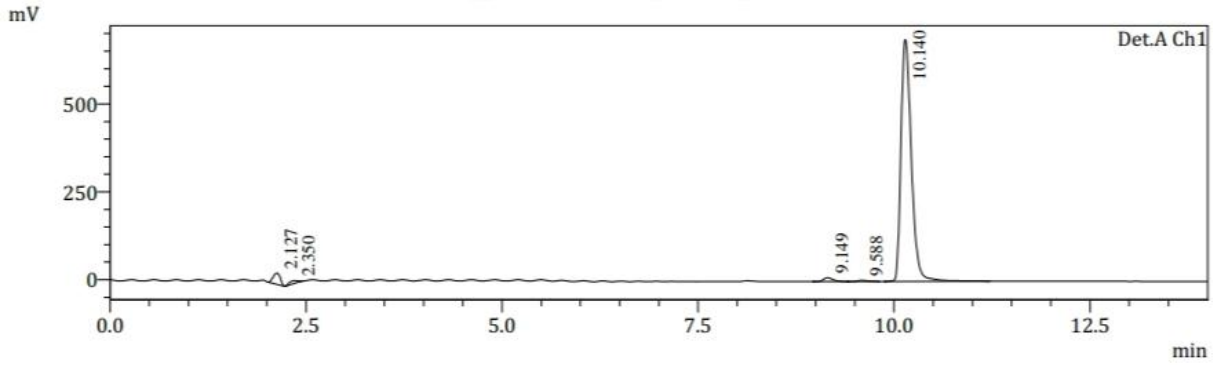


Figure No 17: Chromatograph of Robustness for Berberine (10.14 min).

Detector A CH1 336 nm

Table No 33: Observation and Result of Robustness for Berberine.

Peak #	Ret.Time	Area	Therotical Plate #	Area %	Tailing Factor	k´
1	2.127	198843	2238.614	2.968	0.932	0.000
2	2.350	63485	2170.710	0.947	0.902	0.105
3	9.149	90637	26080.076	1.353	1.326	3.302
4	9.588	26178	30605.232	0.391	1.471	3.509
5	10.140	6321321	27946.413	94.342	1.415	3.768
Total		**6700463**		**100.000**		

RESULT AND DISCUSSION

The present work berberine was yellow in colour, bitter in test and woody odour, berberine was analyzed for various physicochemical parameters, and method was conducted to identifying and quantifying the berberine from drug plant. Berberine peaks from solution of extract like methanol, ethanol were identified by comparing their Rt value with these obtained by chromatography of the standard under the same condition.

The peaks of Rt value 10.55 min,was observed in the chromatogram obtained from fractions like methanol, trifluroacetoic acid and acetonitrile the chromatogram of standards and test sample are shown in above figure.The more amount of berberine was present in methanol extract when compared to other fraction.

CONCLUSION

The HPLC and UV technique is quite, simple, accurate, precise, reproducible and sensitive .This method can be used for routing analysis of berberine in crude drug and prepared formulation and also it is used for standardization and quality control of herbal products.

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