MATERIAL AND METHODS
4.0 MATERIAL AND METHODS

4.1 PLANT MATERIAL

Before the actual experimental work authentication of plant material is one of the prime and important step. Since plant and plant derived products have become a major source of drugs globally, it is very essential to standardize the plant material before processing it for further scientific investigations[1]. World health Organization (WHO) has emphasized on the need of ensuring quality of herbal products by using modern techniques and suitable methods. At global level, different pharmacopoeias also provided monographs stating quality standards for herbs and their products. Indian Pharmacopoeia, British Pharmacopoeia, British Herbal Pharmacopoeia, United State Pharmacopoeia and Japanese Pharmacopoeia has specified the need of quality control of the herbals and their products. Roots of the plant *Piper longum linn* were procured from the commercial supplier from Pune region.

Identification and Authentification of material

Identification and authentification of the plant material was done on the basis of organoleptic characters, exomorphology and pharmacognostic study at Department of Pharmacognosy, MAEER’s Maharashtra Institute of Pharmacy, Pune 411038.

Chemicals and reagents

Petroleum ether (60-80) and ethyl alcohol of laboratory grade were purchased from Yash traders, Pune. All the chemicals and reagents for were purchased from Yash traders, Pune and were of good quality.

![Fig. 4.1 Roots of *Piper longum linn*](image-url)
4.2 STANDARDIZATION OF PLANT MATERIAL

Standardization of plant material was carried out as per the “Quality control of medicinal plant materials World Health Organization Geneva (1998)” guidelines.

Determination of Foreign Matter

Foreign organic matter is the material which is in the form of parts of organ or organs from which the drug is derived other than the part named in the definition and description or matter not coming from the source plant as moulds, insects, or other animal contamination.

500 g of each of original sample of dried roots of the plant *Piper longum linn* was spread as layer and inspected with unaided eye and also with 6X lens. Percent of foreign matter was determined from the weight of drug taken.

Determination of Ash values

The total ash determination is the important parameter to vegetable drugs or for preparations of such drugs. In the determination of total ash values the carbon must be removed at as low a temperature (Maximum $450^\circ$C) as possible to avoid loss of alkali chloride.

Total Ash

3 g of original samples of dried roots of plant *Piper longum linn* was taken in a silica dish and incinerated at a temperature $400^\circ$C until samples are free from carbon, which is then cooled and weight is taken.
Acid insoluble ash

Ash of roots of the plant *Piper longum linn* from above procedure was boiled with 25 ml of 2M HCl for 5 mins. Insoluble ash was collected on crucible dish and washed with hot water. The remaining residue was ignited, cooled and weight was taken. The percentage of acid-insoluble ash is calculated with reference to air-dried samples taken in above procedure.

Extractive values

The determination of water and ethanol soluble extractive is used as a means of evaluating drugs/constituents which are not easily estimated. Extraction of the drug is done by maceration or by continuous extraction process.

Ethanol soluble extractive

5 g of original sample of dried roots of the plant *Piper longum linn*, coarsely powdered, macerated with 100 ml of ethanol in a closed flask for 24 hrs. This closed flask containing sample was frequently shaked for first 6 hrs, then allowed to stand for 18 hrs, then filtered and evaporated to 25 ml. Filtrate of the sample was taken in flat bottomed dish, dried at 105°C and weight is taken. Percentage of ethanol soluble extractive is calculated with reference to weight of the air-dried sample taken.

Water soluble extractive

5 g of original sample of dried roots of the plant *Piper longum linn*, coarsely powdered, mixed with 50 ml of water at 80°C shaken, allowed to stand for 10 mins and cooled. Then 2 g of Kieselguhr powder was added and filtered. 5 ml filtrate was transferred to evaporating dish of 7.5 mm diameter. Solvent was evaporated then on water bath for 30 mins and finally dried for 2 hrs. Weight of residue was taken and percentage of water soluble extractive was calculated with reference to air-dried sample.

Loss on drying

Loss on drying is employed in British Pharmacopoeia and United State Pharmacopoeia. The loss in weight of samples tested is due to water. Although small amount of volatile material may also lost during such procedure.

Initially weight of empty glass bottle was noted, 125 g of sample was taken and bottle was covered and then weight of bottle and content was noted. Cover on bottle was removed and bottle was shaken for uniform distribution of content in bottle. Contents in bottle then dried directly in oven at 100°C up to constant weight of contents. After drying, bottle was removed, covered, cooled to room temperature,
finally weight of both bottle was taken and percentage loss in weight of sample is calculated with reference to the weight of samples taken previously.

4.3 EXTRACTION OF PLANT MATERIAL

Extraction of plant material in petroleum ether, alcohol and water were prepared as follows:

**Petroleum ether Extract**

Coarsely powdered root of *Piper longum linn* (500 g) was macerated for 48 hrs with occasional shaking with 2000 ml of petroleum ether (60-80°C) solvent, filtered through cloth and then filter paper to get clear solution. Macerate obtained were transferred to previously weighed Petri dish and evaporated to dryness at room temperature (35-40°C) to obtain dry extracts. The weight of Petri dish was taken. The yield of extract was obtained by subtracting the empty weight of Petri dish. The yield of the extract is reported as percentage yield.

**Alcoholic Extract**

Marc, the reminder of extract of petroleum ether extraction was dried in air. The dried marc was macerated by using the above procedure by using 90 % alcohol to obtain dried alcoholic extract.

**Water Extract**

Marc of alcoholic extract was dried in air. The dried marc was macerated as per the procedure of petroleum ether extract using distilled water (containing 0.25% Chloroform) as solvent. The extract was evaporated to dryness at 55°C.

**Storage of Extract**

Petroleum ether extract and alcohol extract were stored in tightly closed glass bottles at room temperature and water extract was stored in tightly closed glass bottle in refrigerator at 2-6°C.

4.4 PHYTOCHEMICAL ANALYSIS OF EXTRACTS

Phytochemical analysis of different extract was carried out as per the literature methods [2]. Test solution (T. S.) of petroleum ether extract, alcohol extract and water extract were prepared in petroleum ether, alcohol and water respectively in a concentration of 100 mg/ml each.
Test for Carbohydrates

Few drops (2-3) of α-napthol in alcohol were added to 2-3 ml of T. S. shaken for few mins and then 0.5 ml of conc. H₂SO₄ was added from the sides of the test tube. The formation of violet color ring at the junction of two solution indicate the presence of carbohydrates.

Test for reducing sugars

Fehling’s Test

Fehling A and B Solutions (1 ml each) were taken in test tube boiled for 1 min to this, 2-3 ml of T. S. was added and boiled for 10-15 mins in boiling water bath. The formation of yellow and then brick red precipitate indicate the presence of reducing sugar.

Benedicts test

Benedict reagent (1ml) and T. S. (1ml) was mixed in test tube and boiled in water bath for 5-10 mins. The change in color to yellow, green or red indicate the presence of reducing sugar.

Test for Monosaccharide

Barfoed’s Test

Barfoed’s reagent (1ml) and T. S. (1ml) was mixed in test tube and heated on boiling water bath for 1-2 min and then cooled. Appearance of red precipitate indicate the presence of Monosaccharide.

Test for Pentose sugar

Bial’s Test

To the boiling Bial’s reagent (2ml), 4 drops of T. S. was added. Appearance of green or purple color indicate presence of pentose sugar.

Test for Hexose sugar

Selwinoffs Test (For Fructose)

Selwinoffs reagent (3ml) and T.S. (1ml) was heated on water bath for 1-2 mins. Change in color to red indicate the presence of hexose sugar.

Cobalt-chloride Test

Cobalt-chloride solution (2ml) and T.S. (2ml) was mixed in test tube, heated on water bath for 2 mins, then cooled and NaOH Solution (2-5 drops) was added. Change in color of solution to greenish blue, purplish or upper layer greenish blue
and lower layer purplish indicate the presence of glucose, fructose or mixture of glucose and fructose respectively.

**Test for Proteins**

**Biuret Test**

To the T.S. (3ml), 4% sodium hydroxide (2-5) drops and 1% copper sulphate was added. Change in color of solution to violet or pink indicate presence of proteins.

**Millions Test**

T.S. (3ml) and Millions reagent (5ml) was mixed in test tube. The appearance of white precipitate changing to brick red or dissolved and gave red color to solution on heating indicate the presence of proteins.

**Proteins containing Tyrosine and Tryptophan**

**Xanthoprotein test**

1 ml of Conc. H$_2$SO$_4$ was added in a test tube containing 3 ml of T.S., white precipitate turns to yellow on boiling and orange on addition of NH$_4$OH which indicate presence of tyrosine and tryptophan containing proteins.

**Proteins containing sulphur**

To a 5ml of T.S. 40 % NaOH (2ml) and 2 drops of Lead acetate solution was added and boiled on water bath for 5 mins. Change in color to brownish or black indicate the presence of sulphur containing proteins.

**Test for Amino Acids**

**Ninhydrin Test**

T.S. 3ml and 2 drops of 5% lead acetate solution was boiled on water bath for 10 mins. Change in color of solution to purple or blue indicate the presence of amino acids.

**Test for Tannins and Phenols**

Following reagents (3-5) drops were added to 2-3 ml of T.S. in test tube. Change in color to blue-black, appearance of white precipitate or decoloration of solution indicate the presence of tannins and phenols.

Reagents used for test:

- 5% Ferric chloride Blue-black
- Lead acetate – White acetate
- Potassium permanganate – decoloration.
Test for Glycosides

Cardiac glycosides

Legal Test
To the extract 1ml of pyridine and 1ml of sodium nitroprusside was added. Change in color to pink or red indicate the presence of cardiac glycosides.

Keller-Killani Test
To 2ml of T.S., 3-5 drops of glacial acetic acid, 1 drop of 5% FeCl₃ and Conc. H₂SO₄ were added. Formation of reddish brown color at the junction of two layers and bluish green in the upper layer indicate the presence of cardiac glycosides.

Anthraquinone glycosides

Borntragers Test
2ml of extract and dilute H₂SO₄ were boiled for few minutes mixture was filtered and to the filtrate 2ml of benzene or chloroform was added and the mixture was shaked well. To the organic layer ammonia solution was added which when turned to pink red indicate the presence of Anthraquinone glycosides.

Test for Saponins

Foam test
Plant extract (10-20mg) along with 1ml of water was vigorously shaken. Formation of persistent foam indicate the presence of saponins.

Test for Flavonoids

Shiroda test
To the dry extract (10-20mg), 5ml of ethanol (95%), 2-3 drops of HCl and 0.5 g of magnesium turnings were added. Color change to pink indicate presence of flavonoids.

Test for Alkaloids
To the 10-20 mg of extract 1-2 ml of dilute HCl was added. Mixture was shaken and filtered. The filtrate is subjected to following tests:

Mayers Test
To the 2-3 ml of filtrate 2-3 drops of Mayers reagent was added appearance of precipitate indicate the presence of alkaloids.
**Wagner Test**

To the 2-3 ml of filtrate 3-5 drops of Wagner's reagent was added. Appearance of reddish brown precipitate indicates the presence of alkaloids.

**Hager Test**

To the 2-3 ml of filtrate, 4-5 drops of Hager's reagent was added. Appearance of yellowish precipitate indicates the presence of alkaloids.

**Steroids**

**Salkowski reaction**

2 ml of Chloroform and 2 ml of H$_2$SO$_4$ were added to 2 ml of T.S., shaked well. Chloroform layer shows red color and acid layer when shows greenish yellow fluorescence, indicate the presence of steroids.

**Liebermann-Burchard reaction**

2 ml of T.S. was mixed with chloroform. To the solution 1-2 ml of acetic anhydride and 2 drops of Conc. H$_2$SO$_4$ was added from the sides of the test tube. Change in color first to red, then blue and finally to green indicate the presence of steroids.

### 4.5 METHOD OF ISOLATION

**Chemicals**

Petroleum ether, ethyl acetate, methanol, chloroform, benzene, toluene, Silica gel (chromatographic grade) for the separation was obtained from Rankem, India. All other chemicals used were of high purity.

Roots of the plant *Piper longum* have been selected for the isolation of different constituents from it. The plant material was collected from the local supplier and it has been authenticated from Department of Pharmacognosy, Maharashtra Institute of Pharmacy College, Pune.

Chromatographic methods have been used for separation, identification of compounds. These methods are widely employed in the pharmaceutical field for isolation of the active constituents from the plants. Many such methods are reported in the literature for isolation of active constituents from the plants.

The dried roots of the plant *Piper longum* were powdered and pass through appropriate mesh size so as to get uniform coarse powder.

**Selection of appropriate solvents**

In order to obtain correct information about selection of the solvent different solvents have been used. Thin layer chromatographic (TLC) techniques have been used to
find out the solvent which has given maximum number of spots on TLC. The list of such solvents is given Table 5.7

**Preparation of TLC plates**

The slurry was prepared by suspending silica gel G in distilled water (1:2). Measured amount of slurry was put on the dry and clean glass plate which was kept on a level surface. The plate was then tripped back and forth to spread the slurry uniformly over the surface. The plates were dried in air for 30 mins and then in oven at 110 °C for another 60 mins for the activation of adsorbent layer [3].

A spot of sample was applied on the starting line which was parallel and about 10 mm above the lower edge, with the help of glass capillary. Sample spots were allowed to dry at room temperature.

In TLC chamber, sufficient quantity (5-6ml) of mobile phase was poured to achieve saturation. Chamber was closed and allowed to stand for 15-20 mins. The plate was placed as nearly vertical as possible in to the chamber ensuring that the points of application were above the surface of the mobile phase. Chamber was closed and mobile phase was allowed to ascend to specified distance. Plate was removed from position, mobile front was marked and mobile phase was allowed to evaporate at room temperature.

Plate was observed in day light, under the UV light and then in iodine chamber. After each observation the central points of spots appeared on chromatograph were marked with needle.

Retention factor was calculated by following formula:

\[ R_f = \frac{X}{Y} \]

Where, \( X \) = Distance between point of application and central point of spot of material being examined.

\( Y \) = Distance between point of application and the mobile phase front.

Thin Layer Chromatography (TLC) in different solvents was carried out in two phases:

**Thin Layer Chromatography (TLC) at room temperature**

1g of dried powder of the plant *Piper longum linn* was taken in a test tube and 10 ml of solvent was added in the tube with frequent shaking the whole mixture was stirred for about 30 mins and then filtered. Filtrate was used to carry out TLC.

Solvent system used: Pet ether and Ethyl acetate (9:1)

The \( R_f \) values of the different spots was recorded in Table 5.7
Thin Layer Chromatography (TLC) in hot condition

1g of dried powder of the plant *Piper longum linn* was taken in a test tube and 10 ml of solvent was added in the tube with frequent shaking, the whole mixture was heated for about 60 mins and then filtered. Filtrate was used to carry out TLC.

Solvent system used: Pet ether and Ethyl acetate (8:2)

The Rf values of the different spots was recorded in Table 5.7

The dried roots were powdered and then thin layer chromatographic analysis was carried out in different solvents such as water, benzene, acetone, ether, ethyl acetate, ethanol, hexane and chloroform. Among the different solvent extract petroleum ether extract, alcohol extract showed maximum number of spots. Since petroleum ether has shown presence of maximum spots on TLC plate it was selected for isolation. TLC plates were observed under Ultra-violet light chamber and iodine chamber. The plates were observed by spraying the anisaldehyde-sulfuric acid reagent, prepared by mixing anisaldehyde (0.5 ml) with glacial acetic acid (10 ml), methanol (85 ml) and concentrated sulfuric acid (5 ml). This solution was sprayed on the plate, which was then heated at 110°C for 19 mins.

**Apparatus**

Borosilicate glass column was obtained from Yash Traders, Pune.

(Height 60 cm and Diameter 3 cm).

**Chemicals**

Petroleum ether, ethanol, ethyl acetate (Emerk) purchased from Yash Traders, Pune. Column grade silica (#100-200) was obtained from S.D. Fine Chem. Limited, Mumbai and TLC grade silica (S. D. Fine Chem) was obtained from Yash Traders, Pune.

**Column Chromatography**

Slurry of column grade silica (#100-200) was prepared and column was packed with slurry. Sample was then loaded on packed column and after stabilization, column was eluted with mobile phase. Different fractions of the column were collected after elution. Fractions were analyzed by using thin layer chromatography.

**Activation of silica**

Column grade silica (#100-200) was kept in oven and dried at 110°C for 3hrs to prevent it from moisture content.

Weighed quantity of activated silica was added in beaker containing mobile phase and slurry was prepared.

**Preparation of mobile Phase**

Solvents like petroleum ether and ethyl acetate were first distilled and used in a ratio 8:2.
Packing of Column

Clean and dry borosilicate glass column (Height 60 cm and Diameter 3 cm) was aligned in vertical position by using a clamp of supported stand. Cotton soaked with mobile solvent was tamped down slowly with glass rod. The bottom of column was packed with cotton soaked in mobile phase. Column was filled with 1/3rd portion of mobile phase. Column was then slowly and evenly filled with the prepared slurry to 5/6th volume. Stopcock was operated to remove excess of mobile phase. The side of chromatographic column was gently tapped so that the silica gel gets compactly settled in the column. The stopcock was used to remove excess of mobile phase. The levels of the mobile phase were maintained constant just above the silica level.

100 g of dried root powder of the plant *Piper longum linn* was dissolved in 500 ml of the petroleum ether with intermittent shaking and extracted at room temperature for about 48 hrs. This extract was then filtered and the filtrate was then evaporated under reduced pressure in rotary evaporator to obtain a viscous mass (8 gm) by mixing silica gel (2 gm). The dried mass was then poured in column slowly and the mass was completely settled down. Then the cotton having a diameter of column size was placed on the top surface of mass so that it does not get disturb by the addition of mobile phase during elution process.

Elution of Column

Column was eluted by gravity at a flow rate of 1ml/min. The fractions (20ml) were collected in amber color bottles and the collected volumes were evaporated at room temperature to 1/4th volume. The concentrates of fractions were subjected for TLC analysis. Fractions showing similar spots and Rf values were combined together. The fraction which was not eluted properly was eluted by increasing polarity by ethyl acetate and stored as ethyl acetate fraction.

4.6 CHARACTERIZATION OF THE ISOLATED CONSTITUENTS

General experimental procedure

Melting points were determined in a open capillary (Gallenhamp melting point apparatus) and were uncorrected. Infra red (IR) spectra were recorded on Shimadzu 8400 FT Infrared spectrometer. \(^1\)H Nuclear Magnetic Resonance (NMR) spectrum was recorded on Varian-Mercury (300 MHz) instrument with chemical shift data reported in ppm. Gas chromatograph-Mass spectrums (GCMS) were recorded on GC-MS Shimadzu (QP 5050) instrument. [4]

The isolated compounds were then purified and their general properties were noted. The melting points were taken and readings uncorrected.

The purified constituents were then subjected for spectral studies for structural determination and identification.
The IR, NMR, $^1$NMR and GCMS spectra's have been reported (Fig. 5.1 to 5.13). Total four different constituents were isolated. The first constituent was found to exhibit structural similarity with that of piperine. The other constituents isolated were in minor quantity.

**4.7 ACUTE TOXICITY STUDIES**

*Preparation of dosage forms*

Dosage forms of individual extract were prepared as per the following method:

**Petroleum ether extract**

Emulsion of petroleum ether extract of *Piper longum linn* was prepared by triturating with Tween 80 (2.5%) in glass mortar with gradual addition of water for injection to make volume.

**Alcoholic extract**

Emulsion of alcoholic extract of *Piper longum linn* was prepared by triturating extract with Tween 80 (2.5%) and CMC (0.5%) in glass mortar with gradual addition of water for injection to make volume.

**Aqueous extract**

Solution of aqueous extract of *Piper longum linn* was prepared in water for injection.

**Storage**

All the dosage form of extracts and drug solutions were prepared freshly on the day of experiment and stored in tight amber colored vials.

**Method**

Prepared solvent extracts of plant were administered in a doses of 30, 100, 300, 1000, 2000 and 5000 mg/kg or vehicle (5ml/kg) intra peritoneally. The rats were observed for mortality or any sign of toxicity for 24 hrs. Study was carried out as per OECD (Guideline 425) as follows:

Only one rat received a dose at a particular time. First animal received a dose of 30 mg/kg i. p. or p. o. Animal was observed for 3 hrs after injection for any toxicity signs, survival or death. If the first animal died or appeared morbid, the second animal received lower dose (10 mg/kg). The dose progression or reduction factor was 3.2 times of the previous dose. If no mortality was observed in the first animal then the second animal received a higher dose (100 mg/kg). Dosing of the next animal was continued depending on the outcome of the previously dosed animal for a fixed time interval (3hrs). The test was stopped when one of the stopping criteria was meet as following:

- 5 reversals occur in any 6 consecutive animal tested.
- 3 consecutive animals died at one dose level.
Survival of animals was observed for outcome for a period of 24 hrs.

4.8 SCREENING FOR CARDIAC ACTIVITY

**Plant materials**
Aqueous, pet ether and alcoholic extracts prepared as per procedure described in section 4.7

**Method**
Frogs (Rana Tigrina), weighing 150-200 gms were pithed [5-6], the heart was exposed and isolated from body after cannulation of the inferior vena cava using symes canula. The canula was connected to the reservoir containing frog’s Ringer solution. The solution was continuously bubbled with air, at room temperature. The composition of frog Ringer in mM was Na⁺ 110.7, Cl⁻ 114.2, K⁺ 1.2, Ca²⁺ 1.10, HCO₃⁻ 2.8, H₂PO₄⁻ 0.1 and glucose 11.1 (pH 7.6-8.0). The basal cardiac contraction was recorded on a smoked kymograph drum after the administration of frog Ringer’s solution and gum acacia (5%). The administration of gum acacia was done to see that it did not contribute to the effects of pet ether extract, alcoholic extracts and the isolated fractions. The drugs and extracts were administered through the canula. The average basal heart rate were 70 beats/min and 18 mm respectively. The effects obtained with the drugs and extracts were transposed to the respective percentage of the basal values. Graded dose-response was recorded for each extract and the fraction. The dose which causes the maximum effect was chosen as the experimental dose. The frog heart was washed with the Ringer solution after every administration of extracts and drugs till it was brought back to the normal state.

The frog heart was perfused with adrenaline at 2.5 x 10⁻⁵M and propranolol at 3 x 10⁻⁵M concentration in frog Ringer solution for 60 seconds followed by the administration of extracts, fraction and readings were noted.

4.9 ANTIOXIDANT ACTIVITY

**Plant materials**
Pet ether extract and fraction 1 were prepared as per procedure described in section 4.7

**Chemicals**
1,1-Diphenyl-2-picryl-hydrazyl (DPPH), was purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals used were of analytical grade and were obtained from Merck and Sigma (Sigma-Aldrich GmbH, Sternheim, Germany).
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Radical scavenging activity

DPPH free radical scavenging activity:

The DPPH free radical scavenging activity of extracts was measured according to DPPH radical scavenging method [7-8]. Briefly, 0.1 mM solution of DPPH in ethanol was prepared and 1 ml of this solution was added to 3 ml of extract solution (1,10,50 mg/ml concentration) in water. The mixture was shaken vigorously and incubated at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer (8500 II, Bio-Crom GmbH, Zurich, Switzerland) against a blank. The free radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

\[ \text{DPPH scavenging effects (\%)} = 100 - \left[ \frac{(A_0 - A_1)}{A_0} \right] \times 100 \]

Where \( A_0 \) was the absorbance of the control reaction and \( A_1 \) was the absorbance in the presence of the sample of plant extract and fraction 1.

4.10 ENZYMATIC ACTIVITY OF PIPER LONGUM LINN AND ITS CONSTITUENTS

Since myocardial ischemia is one of the serious complications in the cardiac studies, it was decided to use rat as a animal model for studying the cardiac activity of the extract and isolated constituents [9]. Myocardial ischemia is induced by the administration of isoproterenol and then the extracts and constituent are administered to study their effects on the heart.

The diagnostic marker enzymes of myocardial infarction are creatine kinase (CK), lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate transaminase (AST). Rats administered with isoproterenol exert a significant decrease in activities of enzymes such as CK, LDH, AST and ALT in the heart with subsequent increase in their activities in serum when compared to normal.

A significant increase in the levels of lipid peroxides in serum and heart on isoproterenol administration indicates enhanced lipid peroxidation by free radicals. Due to increased lipid peroxidation, glutathione levels might be significantly lowered in blood and heart of rats. All such physiological changes need to be verified and the exact effect of this changes after the administration of pet ether extract and isolated plant constituent has to be verified. Hence all above-mentioned parameter were estimated and the values are expressed in Table 5.12 and 5.13.

Method

Chemicals

Petroleum ether, alcohol was purchased from Yash traders, Pune.

Equipment

Auto analyzer BIOTRON BTR 830 was used for enzymatic estimations.
Drugs
Isoproterenol injection, water for injection was obtained from the local medical supplier.

Preparation of dosage forms
Dosage forms of extract and fraction 1 were prepared as per the following method.
Emulsion of petroleum ether extract of *Piper longum linn* was prepared by triturating with Tween 80 (2.5%) in glass mortar with gradual addition of water for injection to make volume. Similarly fraction 1 dosage form were prepared.

Storage
All the dosage form of extracts and drug solutions were prepared freshly on the day of experiment and stored in tight amber colored vials.

Route of administration
Extracts were administered by i.p./oral route. Isoproterenol was administered by subcutaneous route.

Volume of Injection
The volume of injection was calculated based upon the body weight of animal. Volume of injection was kept constant with respect to their body weight.

Animals
Adult male albino rats of Wistar strain weighing 120-150 g were used for the study. They were fed with commercial pelleted rat chow and given water *ad libitum* and should be maintained in a clean polypropylene cages at 25°C. The rats were divided into five groups, each consisting of 6 animals.

Approval of protocol
All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of National Institute of Virology, Pune, constituted under committee for Purpose of Control and supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Ethical guidelines were strictly followed during all the experiments.

Procedure
Group I served as control with normal diet and saline, Group II rats were administered with isoproterenol (20mg/100g subcutaneously twice at an interval of 24 hrs). Group III rats were administered with 100mg/kg of pet ether extract for a period of 7 days orally. Group IV rats were pretreated with pet ether extract for 7 days and were given isoproterenol (20mg/100g subcutaneously twice at an interval of 24 hrs) at the end of treatment period. Group V rats were pretreated with fraction
1 for 7 days and were given isoproterenol (20mg/100g subcutaneously twice at an interval of 24 hrs) at the end of treatment period.

After the experimental period, the rats were scarified by cervical decapitation. Blood was collected and the serum separated was used for the assay of marker enzymes Creatine Kinase (CK), Lactate Dehydrogenase (LDH), Aspartate Transaminase (AST) and Alanine Transaminase (ALT) in control and experimental animals.

The heart was dissected out, immediately washed in ice-cold saline and a homogenate was prepared in 0.1 M Tris-HCl buffer (pH 7.4). Homogenate was centrifuged and supernatant was used for the assay of marker enzymes, glutathione and lipid peroxides in serum and heart homogenate.

4.11 HISTOPATHOLOGICAL STUDIES

Chemicals and Drugs
Stains like haematoxylin and eosin was purchased from Yash traders, Pune.

Equipment
Microtome and microscope.

Animals
Adult male albino rats of Wistar strain weighing 120-150 gm. were used for the study. They were fed with commercial pelleted rat chow and given water ad libitum and maintained in a clean polypropylene cages at 25°C. The rats were divided into five groups, each consisting of 6 animals.

Approval of protocol
All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of National Institute of Virology, Pune, constituted under committee for Purpose of Control and supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Ethical guidelines were strictly followed during all the experiments.

After sacrificing the rats, systematic post mortem examination was conducted. Pieces of hearts of rats were fixed in 10% buffered formal saline as well as 70% alcohol for histopathological studies [10]. After fixation samples were dehydrated with increasing grades of alcohol and blocks were prepared. These blocks were cut at 5μ thickness with the help of microtome. The slides were stained with haematoxylin and eosin and observed under microscope for the effects of necrosis in animals.
4.12 ANTIBACTERIAL STUDIES

Plant material
The dried roots of the *Piper longum linn* were obtained from local market of Pune, India.

Chemicals
n-Hexane, ethyl acetate, methanol, chloroform, benzene, toluene and dimethyl formamide (DMF) were purchased from Merck India Ltd., Mumbai. Nutrient agar was purchased from Hi-media, Mumbai. Streptomycin (Nicholas, India) was purchased from the local market of Pune. All other chemicals used were of high purity.

Bacterial strains
*Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Klebsiella pneumoniae, Salmonella typhi, Serratia marcescens, Shigella dysenteriae and Staphylococcus aureus* were obtained from the Department of Microbiology, University of Pune, Pune. All the strains were maintained on nutrient agar medium.

Method
Antibacterial activity of the extract and fraction 1 was determined by the well diffusion method [11]. The microbial cultures were grown at 37°C for 24 hrs and then approximately diluted by sterile saline (0.9 % w/v) solution to obtain a cell suspension of $10^5$ CFU/ml. Diluted inoculum (0.2 ml, $10^5$ CFU/ml) of test microorganisms were spread on nutrient agar plates. Wells of 6 mm diameter were punched into the agar medium and filled with 20 μl each of extracts at a concentration of 10 mg/ml. The plates were incubated for 18-24 hrs at 37°C. The antibacterial activity was evaluated by measuring the zone of inhibition. The antibiotic streptomycin (100 μg/ml) was used in the test system as positive control. The average of the zone of inhibition was obtained from three replicates.

Minimum Inhibitory Concentration Assay (MIC)
MIC values were determined only with those microorganisms that showed promising inhibitory zones [12]. Pet ether extract and piperine had showed the maximum inhibitory zones against two organisms, hence has been selected for MIC assay. The microorganisms selected for the study were *Pseudomonas aeruginosa* and *Bacillus cereus*. The dilutions of extract and fraction 10, 5, 2.5 and 1 mg/ml were prepared in DMF to obtain an effective concentration of 2, 1, 0.5 and 0.25 mg/well. The assay was carried out and zone of inhibition was measured. The MIC values were determined as the lowest concentration of the extract and fraction 1 (piperine), which completely inhibited the growth of bacteria.
### 4.13 INSTRUMENTS AND MATERIAL FOR FORMULATION STUDIES

Table 4.1 Instruments for formulation study

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Make</th>
<th>Model No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical Balance</td>
<td>AND, Shimadzu</td>
<td>GR 200</td>
</tr>
<tr>
<td>Balance</td>
<td>AND</td>
<td>GF 3000</td>
</tr>
<tr>
<td>Double cone blender</td>
<td>Rimek Kalweka</td>
<td>HD – 410</td>
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<td>Compression machine</td>
<td>CADMACH</td>
<td>CMD 4-16/MT</td>
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<tr>
<td></td>
<td>Rimek (8 Station)</td>
<td>Minipress - II</td>
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<tr>
<td>Vernier</td>
<td>Mitutgo Digimatic Calliper</td>
<td>-</td>
</tr>
<tr>
<td>Freeze Dryer</td>
<td>Audiotronics</td>
<td>-</td>
</tr>
<tr>
<td>Hardness tester</td>
<td>Monsanto Hardness Tester</td>
<td>-</td>
</tr>
<tr>
<td>Incubator Shaker</td>
<td>Samarth</td>
<td>--</td>
</tr>
<tr>
<td>Friability tester</td>
<td>Roche Friability tester</td>
<td>--</td>
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<tr>
<td></td>
<td>Lab Hosp., Kumar</td>
<td>--</td>
</tr>
<tr>
<td>Dissolution test apparatus</td>
<td>Electro Lab</td>
<td>TDT-08L</td>
</tr>
<tr>
<td></td>
<td>Lab India Disso 2000</td>
<td>-</td>
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<tr>
<td>UV Spectrophotometer</td>
<td>Perkin Elmer</td>
<td>EZ 201</td>
</tr>
<tr>
<td></td>
<td>Shimadzu</td>
<td>UV-1700 PharmaSpec</td>
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<tr>
<td>pH meter</td>
<td>Equip-tronics</td>
<td>EQ 621</td>
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<tr>
<td>Ultrasonicator</td>
<td>Ultrasonics</td>
<td>5.5L 15OH</td>
</tr>
<tr>
<td>Stability Chamber</td>
<td>Thermolab</td>
<td>-</td>
</tr>
</tbody>
</table>
DRUG AND EXCIPIENTS [13-15]

Propranolol Hydrochloride

**Chemical Name:** (RS)-1-isopropylamino-3-(1-naphthyloxy)propan-2-ol hydrochloride.

**Structure:**

![Chemical structure of Propranolol Hydrochloride](image)

**Molecular Formula:** C\textsubscript{16}H\textsubscript{21}NO\textsubscript{2}·HCl

**Molecular weight:** 295.81

**Colour:** White or almost White powder.

**Odour:** Odourless.

**Taste:** Bitter to taste.

**Solubility:** 1 gm in 20 ml water or 20 ml alcohol, slightly soluble in chloroform, practically insoluble in ether.

**pKa:** 9.45

**Melting Point:** 163-166°C

**pH:** between 5.0 and 6.0, determined in a 1.0% w/v solution.

**Pharmacology:** Propranolol is a nonselective, beta-adrenergic receptor-blocking agent possessing no other autonomic nervous system activity. It specifically competes with beta-adrenergic receptor-stimulating agents for available receptor sites. When access to beta-receptor sites is blocked by Propranolol, the chronotropic, inotropic, and vasodilator responses to beta-adrenergic stimulation are decreased proportionately.

At dosages greater than required for beta blockade, Propranolol also exerts a quinidine-like or anesthetic-like membrane action, which affects the cardiac action potential. The significance of the membrane action in the treatment of arrhythmias is uncertain.

**Mechanism of Action:** The mechanism of the antihypertensive effect of Propranolol has not been established. Among the factors that may be involved in contributing to the antihypertensive action include: decreased cardiac output, inhibition of renin production, and...
release by the kidneys, and diminution of tonic sympathetic nerve outflow from vasomotor centers in the brain.

Although total peripheral resistance may increase initially, it readjusts to or below the pretreatment level with chronic use of Propranolol. Effects of Propranolol on plasma volume appear to be minor and somewhat variable.

**Pharmacokinetics and Drug Metabolism:**

**Absorption:** Propranolol is highly lipophilic and almost completely absorbed after oral administration. However, it undergoes high first pass metabolism by the liver and on average, only about 25% of Propranolol reaches the systemic circulation. Peak blood levels following dosing with Propranolol hydrochloride extended-release occur at about 6 hrs.

**Distribution:** Approximately 90% of circulating Propranolol is bound to plasma proteins (albumin and alpha-1-acid glycoprotein). The binding is enantiomer-selective. The S(-)-enantiomer is preferentially bound to alpha-1-glycoprotein and the R(+)-enantiomer preferentially bound to albumin. The volume of distribution of Propranolol is approximately 4 liters/kg.

Propranolol crosses the blood-brain barrier and the placenta, and is distributed into breast milk.

**β-CYCLODEXTRIN**

**Synonym:** beta-cycloamylose, cycloheptaamylose, beta-cycloheptaamylose, cycloheptaglucan, cycloheptaglucosan, cyclomaltoheptaose, beta-dextrin, βCD, BCD, β Schardinger dextrin.

**Definition:** A non-reducing cyclic saccharide consisting of seven alpha-1,4-linked D-glucopyranosyl units manufactured by the action of cyclodextrin transglycolase on hydrolysed starch followed by purification of the β-cyclodextrin; purification is by preparation of a β-cyclodextrin/solvent inclusion compound followed by steam-stripping of the solvent before final purification.

**Chemical name:** Cycloheptaamylose

**Structural Representation:**
Fig. 4.4 Chemical structure of β-Cyclodextrin

**Chemical formula:** C_{42}H_{70}O_{35}

**Molecular Weight:** 1135

**Description:** Virtually odourless, slightly sweet tasting white or almost white crystalline solid

**Solubility:** Sparingly soluble in water; freely soluble in hot water; slightly soluble in ethanol

**Functional Category:** Solubilizing agent, stabilizing agent, encapsulation agent for food additives, flavouring and vitamins

**Applications:**

- Cyclodextrins are 'bucketlike' or 'conelike' toroid molecules, with a rigid structure and a central cavity, the size of which varies according to the cyclodextrin type. The internal surface of the cavity is hydrophobic and the outside of the torus is hydrophilic; this is due to the arrangement of hydroxyl groups within the molecule. This arrangement permits the cyclodextrin to accommodate a guest molecule within the cavity, forming an inclusion complex.

- Cyclodextrins may be used to form inclusion complexes with a variety of drug molecules, resulting primarily in improvements to dissolution and bioavailability owing to enhanced solubility and improved chemical and physical stability. Cyclodextrin inclusion complexes have also been used to mask the unpleasant taste of active materials and to convert a liquid substance into a solid material.

- β-Cyclodextrin is the most commonly used cyclodextrin, although it is the least soluble. It is the least expensive cyclodextrin; is commercially available from a number of sources; and is able to form inclusion complexes with a
number of molecules of pharmaceutical interest. However, β-cyclodextrin is nephrotoxic and should not be used in parenteral formulations.

- β-Cyclodextrin is considered to be nontoxic when administered orally, and is primarily used in tablet and capsule formulations. β-cyclodextrin derivatives tend to be nontoxic when used either orally or parenterally, and the derivatives 2-hydroxypropyl-β-cyclodextrin and 3-hydroxy propyl-β-cyclodextrin are becoming increasingly important in pharmaceutical formulations.

- In oral tablet formulations, β-cyclodextrin may be used in both wet-granulation and direct compression processes. The physical properties of β-cyclodextrin vary depending on the manufacturer. However, β-cyclodextrin tends to possess poor flow properties and requires a lubricant, such as 0.1% w/w magnesium stearate, when it is directly compressed.

- In parenteral formulations, cyclodextrins have been used to produce stable and soluble preparations of drugs that would otherwise have been formulated using a non-aqueous solvent.

- In eye drop formulations, cyclodextrins form water-soluble complexes with lipophilic drugs such as corticosteroids. They have been shown to increase the water solubility of the drug; to enhance drug absorption into the eye; to improve aqueous stability and to reduce local irritation.

- Cyclodextrins have also been used in the formulation of solutions, suppositories, and cosmetics.

HYDROXYPROPYL-BETA-CYCLODEXTRIN

Hydroxypropyl beta-cyclodextrin (HPBCD) is produced from beta-cyclodextrin (BCD) by addition of propylene oxide to some of the hydroxyl groups of BCD. This modification results in greater solubility of HPBCD and its complexes compared to BCD. Therefore, HPBCD is used in applications where the solubility of BCD is not sufficiently high and in applications where faster dissolution rates are needed. Other benefits obtained by complexation with the basic BCD, such as protection against chemical degradation and volatilisation are retained by the HPBCD. HPBCD, in dry form or in solution, is applied in a wide variety of compounds in diverse applications such as pharmaceutical, personal care/cosmetics, diagnostics, chemical processing and many more.

**Chemical Structure:**

Hydroxypropyl beta-cyclodextrin (HPBCD) is partially substituted poly (hydroxypropyl) ether of beta cyclodextrin (BCD).

The empirical formula is: \( (C_{42}H_{70-n}O_{35}) (C_3H_7O)_n \)
It contains not less than 10.0% and not more than 45.0% hydroxypropoxy groups. The structure is shown below where R represents either hydrogen or a hydroxypropoxyl group.

**Structural Representation:**

![Chemical structure of HP-β-Cyclodextrin](image)

where $R = \text{CH}_2\text{CHOHCH}_3$ or H

Fig. 4.5 Chemical structure of HP-β-Cyclodextrin

The basic closed circular structure of BCD is maintained in HPBCD. The glycosidic oxygen forming the bond between the adjacent glucose monomers and the hydrogen atoms lining the cavity of the cyclodextrin impart an electron density and hydrophobic character to the cavity. Organic compounds interact with the walls of the cavity to form inclusion complexes. The hydroxyl groups and the hydroxypropyl groups are on the exterior of the molecule and interact with water to provide the increased aqueous solubility of the HPBCD and the complexes made with the HPBCD.

**Degree of substitution:**

The hydroxypropyl groups are randomly substituted onto the hydroxyl groups of the cyclodextrin and the amount of substitution is reported as average Degree of Substitution (DS) or number of hydroxypropyl groups per cyclodextrin and is the preferred manner of describing the substitution. An alternative measurement is Molar Substitution (MS) or the average number of substitutions per anhydro glucose unit in the ring of the cyclodextrin. Molar substitution is used with polymers whose molecular weight is not determined or contains a mixture of polymers of different degrees of polymerisation. Substitution occurs randomly with respect to which hydroxyl groups and which glucose units are modified. There is a distribution around the average degree of substitution of the number of hydroxypropyl groups per cyclodextrin, with some molecules having more than the average and some less than...
the average degree of substitution. The result is a mixture of many molecular species with respect to the number and location of substitutions around the ring of the cyclodextrin. The reaction to produce HPBCD is highly controllable and repeatable, so the product is consistent from batch to batch. Substitution can have an effect on the binding of guests to the HPBCD. At low degrees of substitution, binding is very similar to that of the unmodified beta-cyclodextrin. Increasing substitution can lead to weaker binding of the guest due to steric hindrance. The effect is dependent upon the particular guest. It is even possible to obtain increased binding as a result of an increase in surface area to which the guest can bind. With most guests, these differences in binding at different degrees of substitution are small, if detectable at all.

**Applications:**

- Improving the solubility of drugs. Insoluble drugs including Chinese herbal medicines might be turned into injections by the derivatives of cyclodextrins.

- Improving the bioavailability of drugs. For example, effectively control release oral dosing of injection of 5-fluorocarbamidopyrimidine might be prepared with an improved bioavailability, which might bring in a decreasing of the dosage but an increasing of the curative effect.

- Adjusting or controlling the releasing of the drugs. For example, lasting effective dosings of testosterone and progesterone made with an improved stability.

- Decreasing the toxicities of the drugs. For example, to decrease the haemocytolysis of the drugs. The stimulating to eyes or nasal cavities of the related drugs cold also be decreased.

- Improving the stabilities of the drugs. For example, to prevent the volatilization, sublimation, oxidation decomposition etc.

- Concealing or rectifying the badness odor of testing.

- Improving the synthesis effects as well as qualities of the drugs by chiral selective catalysis of cyclodextrin derivatives. It is very important for a chiral structure in drugs.

- Constructing stational chiral phases for the separation of isomers of drugs.

- Exploring molecular probes for examining differentiation of gene etc.

- Looking for new drugs or new coordination materials by more proper modifications of cyclodextrins.
Table 4.2 Physicochemical properties of HP-βCD

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Item</th>
<th>Standard Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Appearance</td>
<td>White powder</td>
</tr>
<tr>
<td>2</td>
<td>Content</td>
<td>&gt;= 99%</td>
</tr>
<tr>
<td>3</td>
<td>Water Solubility</td>
<td>&gt;= 80%</td>
</tr>
<tr>
<td>4</td>
<td>Degree of Substitution</td>
<td>4-6</td>
</tr>
<tr>
<td>5</td>
<td>Moisture</td>
<td>&lt;= 0.3%</td>
</tr>
<tr>
<td>6</td>
<td>pH of 1% Solution</td>
<td>6.8-7.2</td>
</tr>
<tr>
<td>7</td>
<td>Heavy Metal (as Pb)</td>
<td>&lt;= 0.001%</td>
</tr>
<tr>
<td>8</td>
<td>Ash</td>
<td>&lt;= 0.2%</td>
</tr>
<tr>
<td>9</td>
<td>Arsenic</td>
<td>&lt;= 0.0008%</td>
</tr>
<tr>
<td>10</td>
<td>Sodium Chloride</td>
<td>&lt;= 0.2%</td>
</tr>
<tr>
<td>11</td>
<td>Propylene Glycol content</td>
<td>&lt;= 0.2%</td>
</tr>
</tbody>
</table>

**SODIUM ALGINATE**

*Non Proprietary Names:* BP, USP Sodium alginate, PhEur: Natrii alginas

*Grade:* Keltone Hvcr (Viscosity) 400

*Synonym:* Algin, alginic acid, sodium salt, sodium polymannuronate

*Empirical Formula & Molecular Formula:* Sodium alginate mainly consists of sodium salt of alginic acid, which is mixture of polyuronic acids composed of residues of D- mannuronic acid and L- guluronic acid.

*Description:* Sodium salt of alginic acid. Free-flowing white to off-white powder.

*Properties:* Insoluble at pH below 4, hydrates readily in water, forms gels in the presence of divalent cations, gives wide range of rheological profiles, forms clear, glossy, uniform films.

*Applications:*
- Suppresses drug release in hydrophilic matrix solid dosage forms for sustained release.
> Prevents dose dumping of basic drugs in acidic gastric fluid.
> Forms films suitable for aesthetic or enteric tablet coatings.
> Encapusulates proteins, live cells, and enzymes.
> Forms exudate absorbent fibers suitable for wound care.
> Delivers lubricity to coatings for medical devices.
> Creates gelatinous raft to prevent gastric reflux in liquid antacid formulations.
> Provides thickening and stabilization for liquid dosage forms (syrups).
> Imparts emulsion stabilization and acts as suspending agents in semi-solid, liquid and topical formulations.

**DIRECTLY COMPRESSIBLE LACTOSE (DCL)**

**Non Proprietary Names:** Lactose monohydrate, USP-NF, Ph.Eur., JP.

**Description:** It is a free-flowing, white, spray-dried powder consisting of spherical particles. Each sphere is composed of minute lactose alpha-monohydrate crystals bonded with amorphous lactose.

**Definition:** DCL grades have excellent flow properties because the particles are spherical and have a narrow size distribution. The particles consist of fine crystals of \(\alpha\)-lactose monohydrate and amorphous lactose to enhance compatibility. The particle structure affords excellent blend and tablet content uniformity. Coarse sieved fraction of \(\alpha\)-lactose monohydrate (100 mesh) is used in direct compression due to its flowability. It contains about 5% w/w water. Compared to other filler-binders, \(\alpha\)-lactose monohydrate exhibits relatively poor binding properties. It consolidates mainly by fragmentation. It has higher brittleness compared to spray-dried lactose and anhydrous \(\alpha\)-lactose. \(\alpha\)-lactose monohydrate (100 mesh) is often combined with microcrystalline cellulose. This combination results in a stronger synergistic effect on disintegration time, whereas the crushing strength increases as the percentage of microcrystalline cellulose in the blend is increased. The strength of tablets compressed from \(\alpha\)-lactose monohydrate increases with a decrease in particle size of the excipient.

This lactose powder is specifically engineered for pharmaceutical direct compression tableting and is particularly well-suited in high dose formulations of poorly compressible actives.

**Application:**
> Excellent flow characteristics.
> Improved tablet weight uniformity.
> Exceptional carrying capacity.
Chapter 4 Material And Methods

- Rapid tablet disintegration.
- Excellent compressibility.
- Low hygroscopicity.
- Low reactivity with active ingredients.
- Excellent physical, chemical and microbiological stability.
- Solubility aids in drug dissolution.
- Low dose formulations.

MAGNESIUM STEARATE

Nonproprietary Names: BP, USPNF, JP: Magnesium stearate, PhEur: Magnesii stearas

Synonyms: Magnesium octadecanoate; octadecanoic acid, magnesium salt; stearic acid, magnesium salt.

Chemical Name: Octadecanoic acid magnesium salt

Empirical Formula and Molecular Weight: $C_{36}H_{70}MgO_{4}, 591.34$

Description: Magnesium stearate is a very fine, light white, precipitated or milled, impalpable powder of low bulk density, having a faint odor of stearic acid and a characteristic taste. The powder is greasy to the touch and readily adheres to the skin.

Solubility: practically insoluble in ethanol, ethanol (95%), ether and water; slightly soluble in warm benzene and warm ethanol (95%).

Definition: The USPNF 23 describes magnesium stearate as a compound of magnesium with a mixture of solid organic acids that consists chiefly of variable proportions of magnesium stearate and magnesium palmitate ($C_{32}H_{62}MgO_{4}$). The PhEur 2005 describes magnesium stearate as a mixture of magnesium salts of different fatty acids consisting mainly of stearic acid and palmitic acid and in minor proportions other fatty acids.

Structural Formula: $[CH_3(CH_2)_{16}COO]_2Mg$

Functional Category: Tablet and capsule lubricant.

Density (bulk): 0.159 g/cm$^3$

Density (tapped): 0.286 g/cm$^3$

Density (true): 1.092 g/cm$^3$

Flash point: 250°C

Applications:

Cardiac Activity and Formulation Evaluation of Isolated Constituents of Piper longum linn
Magnesium stearate is widely used in cosmetics, foods, and pharmaceutical formulations. It is primarily used as a lubricant in capsule and tablet manufacture at concentrations between 0.25% and 5.0% w/w.

4.14 COMPLEXATION STUDY [16-24]

Phase solubility studies
Phase solubility studies were carried out in distilled water according to the literature reported method. Excess amount of piperine was added to 10 ml of aqueous solutions of containing various concentrations of βCD and HP-βCD (2.5 mM to 15 mM). Then the suspensions were sealed and shaken at (25 ±2) °C for 7 days. After equilibrium attainment, the samples were filtered through 0.45 μm membrane filter and properly diluted. The concentration of Piperine was determined spectrophotometrically at 340.5 nm. The presence of βCD and HP-βCD did not interfere with the spectrophotometry assay of piperine. Each experiment was repeated thrice. The stability constant was calculated from the phase solubility diagram, with the assumption of 1:1 stoichiometry, according to the following equation:

\[
K_s = \frac{\text{Slope}}{S_0(1 - \text{Slope})}
\]

Where,

\(K_s\) = Apparent stability constant

\(S_0\) = Solubility of Piperine in absence of cyclodextrin

Slope = Corresponding slope of the phase solubility diagram.

Preparation of inclusion complexes of piperine:
The inclusion complexes of piperine and cyclodextrin (CD) were prepared using four methods:

1. Physical binary mixture (PM)
Piperine and βCD, Piperine and HP-βCD physical mixture were prepared by simply blending the previously sieved (63-169 μm sieve) βCD; Piperine and HP-βCD: piperine for 30 mins with 1:1 molar ratio uniformly in a glass mortar.

2. Kneaded binary system (KN)
Piperine and βCD, Piperine and HP-βCD were triturated in a glass mortar with a small volume of water-methanol (1:1) solution. The thick slurry was kneaded for 45 min and then dried at 45°C until dry.
3. **Lyophilized binary system (LPh) / Freeze Drying (FD)**

βCD, and HP-βCD were added to distilled water. Piperine was then added to this solution under stirring, according to the stoichiometry 1:1. The solution stirring was maintained for 06-08 hrs. Furthermore the resultant solution was frozen at -40°C and then frozen solution was lyophilized in a freeze dryer.

4. **Solvent Evaporation (SE) / Coevaporation method**

The aqueous solution of βCD and HP-βCD were added to alcoholic solution of piperine. The resulting mixture was stirred for 06hrs and evaporated at a temperature of 37°C until dry on temperature controlled stirrer. Residue was collected and dried at room temperature.

The obtained inclusion complexes were evaluated for thermal analysis (DSC), X-ray Diffraction study (XRD), Infrared spectrum (FTIR), percent yield, percent drug entrapment, aqueous solubility study, dissolution profile and stability studies.

**Thermal Analysis**

Differential scanning calorimetry (DSC) measurements of the pure material and binary systems were carried out using a Perkin-Elmer DSC7 differential scanning calorimeter. The accurately weighed sample was placed in an aluminum pan, and an empty aluminum pan was used as reference. The scanning rate was 10°C/ min and the nitrogen flow was 20 ml/ min.

**X-ray Diffraction study**

The powder X-ray diffraction was recorded using XD-98 diffractometer, operated at a voltage of 40 kV and a current of 36 mA. The samples were analyzed in the 2θ angle range of (3-40)° and the process parameters were set as : scan step size of 0.02°, scan step time of 1.54 s.

**Infrared spectrum**

Infrared spectrum of the inclusion complex was obtained using a Shimadzu FTIR-8900 spectrometer according to potassium bromide disk method. The IR spectra of pure βCD, HP-βCD as well as inclusion complexes of 1:1 molar ratio were obtained by the same procedure for comparison. The scans were executed at a resolution of 8 cm⁻¹, from 4000 to 400 cm⁻¹.
Material And Methods

Evaluation of Complexes

Method Development

Solvent used - Methanol, Distilled Water

Preparation of standard stock solution

A stock solution of Piperine (1000 µg/ml) was prepared by dissolving 10 mg of Piperine in 5 ml of methanol and the volume of the solution was made up to 10 ml with methanol. 1 ml of stock was taken in 100 ml volumetric flask and volume was made up with distilled water. (10 µg/ml)

Selection of analytical wavelength

Aliquots of stock solution of drug were transferred to volumetric flasks and volume was made up with distilled water in order to get working standard solutions of Piperine in concentration range of 1-10 µg/ml. These solutions were scanned from 200-400 nm from which the analytical wavelength was selected. Absorbance of the solutions was measured against reagent blank.

Selection of analytical concentration range

The aliquots of stock solution of drug were transferred to volumetric flasks and volume was made up with distilled water. The absorbance of solutions was measured at 340.5 nm against reagent blank and plotted against concentration. The range in which Piperine obeyed Beer’s law was determined.

Calibration curve

10 mg of the drug was dissolved in 10 ml methanol. This solution (1000 µg/ml) was used as the stock solution for the preparation of further dilutions. Dilutions were made using distilled water to get concentration in range of 1-10 µg/ml. The calibration curve was obtained by recording the absorbance on a UV spectrophotometer at $\lambda_{\text{max}}$ 340.5 nm.

Percent Drug Entrapment

3 mg of complex were dissolved in 10 ml Acetonitrile. From which then 0.1 ml of sample were transferred to volumetric flasks and volume was made up 10 ml with Acetonitrile. The absorbance of this sample were determined spectrophotometrically at $\lambda_{\text{max}}$ 340.5 nm.

Aqueous Solubility Study

Excess of the complexes were dissolved in 10 ml of distilled water and shake in incubator shaker for 48 hrs at 37°C. From this sample 0.5 ml were transferred to
volumetric flasks and volume was made up 10 ml with distilled water. The absorbance were determined spectrophotometrically at $\lambda_{\text{max}}$ 340.5 nm.

**Dissolution profile of the inclusion complexes**

The dissolution profiles of the inclusion complexes previously obtained were collected using a dissolution test apparatus of Lab India Disso 2000. The study was performed by using USP Type- I basket. The dissolution media consisted of 900 ml of distilled water. The media were previously filtered, degassed and maintained at 37 ± 0.5°C according to USP XXVIII. The stirred speed was set at 100± 2 rpm and the temperature was maintained at 37 ± 0.5°C. Aliquots from samples containing 10 mg of piperine or its equivalent in different prepared complexes were withdrawn each 10 min for a period for 120 min and analyzed by UV-VIS spectroscopy (UV-1700 Pharmaspec; Shimadzu) at 340.5 nm. Three replicates have been made for each experiment. Percentage drug release was then calculated using PCD Disso v3 software.

**Stability studies for Complexes**

Stability studies were carried out to see the effects of environment on drug stability. The prepared complexes F1 to F8 were selected and stored at the following conditions for a period as prescribed by ICH guidelines for accelerated studies.

1. 25 ± 1 °C and RH 60 % ± 5%
2. 30 ± 1 °C and RH 65 % ± 5%
3. 40 ± 1 °C and RH 75 % ± 5%

The sample were withdrawn after a period of 7, 14 days, 1, 2, 3 months and analyzed for UV spectra, % drug entrapment and IR studies.

**4.15 FORMULATION OF TABLET DOSAGE FORM [25-30]**

Complexes F1 (Freeze dried βCD: Piperine) and F2 (Freeze dried HP- βCD: Piperine) and piperine have been subjected for formulation of their dosage form in tablet. To check whether addition of beta blocking agent will interfere the release of piperine, combined tablets (Piperine and Propranolol) were also prepared and evaluated. The tablets were prepared by direct compression technique.

*Cardiac Activity and Formulation Evaluation of Isolated Constituents of *Piper longum linn*


**Screening of excipients**

Table 4.3 Screening of excipients

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Use</th>
</tr>
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<tbody>
<tr>
<td>Directly compressible lactose</td>
<td>Diluent</td>
</tr>
<tr>
<td>Sodium alginate HVCR</td>
<td>Polymer</td>
</tr>
<tr>
<td>Magnesium Stearate</td>
<td>Anti-adherant, lubricant</td>
</tr>
</tbody>
</table>

**Formula 1 FT1:**

- βCD: Piperine (F1) (equivalent to 10 mg Piperine) 50 mg
- Directly compressible lactose 108 mg
- Sodium alginate HVCR 40 mg
- Magnesium Stearate 02 mg

200 mg

**Formula 2 FT2:**

- HP- βCD: Piperine(F2) (equivalent to 10 mg Piperine) 58 mg
- Directly compressible lactose 100 mg
- Sodium alginate HVCR 40 mg
- Magnesium Stearate 02 mg

200 mg

**Formula 3 FT3:**

- Piperine 10 mg
- Directly compressible lactose 148 mg
- Sodium alginate HVCR 40 mg
- Magnesium Stearate 02 mg

200 mg

*Cardiac Activity and Formulation Evaluation of Isolated Constituents of *Piper longum* linn*
Chapter 4

Material And Methods

**Formula 4 FT4:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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</thead>
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<td>βCD: Piperine (F1) (equivalent to 10 mg Piperine)</td>
<td>50 mg</td>
</tr>
<tr>
<td>Propranolol</td>
<td>10 mg</td>
</tr>
<tr>
<td>Directly compressible lactose</td>
<td>98 mg</td>
</tr>
<tr>
<td>Sodium alginate HVCR</td>
<td>40 mg</td>
</tr>
<tr>
<td>Magnesium Stearate</td>
<td>02 mg</td>
</tr>
<tr>
<td></td>
<td>200 mg</td>
</tr>
</tbody>
</table>

**Formula 5 FT5:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP- βCD: Piperine(F2) (equivalent to 10 mg Piperine)</td>
<td>58 mg</td>
</tr>
<tr>
<td>Propranolol</td>
<td>10 mg</td>
</tr>
<tr>
<td>Directly compressible lactose</td>
<td>90 mg</td>
</tr>
<tr>
<td>Sodium alginate HVCR</td>
<td>40 mg</td>
</tr>
<tr>
<td>Magnesium Stearate</td>
<td>02 mg</td>
</tr>
<tr>
<td></td>
<td>200 mg</td>
</tr>
</tbody>
</table>

**Formula 6 FT6:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperine</td>
<td>10 mg</td>
</tr>
<tr>
<td>Propranolol</td>
<td>10 mg</td>
</tr>
<tr>
<td>Directly compressible lactose</td>
<td>138 mg</td>
</tr>
<tr>
<td>Sodium alginate HVCR</td>
<td>40 mg</td>
</tr>
<tr>
<td>Magnesium Stearate</td>
<td>02 mg</td>
</tr>
<tr>
<td></td>
<td>200 mg</td>
</tr>
</tbody>
</table>

**Manufacturing Procedure**

I) *Weighing:* The calculated quantities of ingredients as per the respective formulae were weighed on an electronic balance.

II) *Sifting:* All the weighed ingredients were sifted separately using sieve no 40

III) *Dry Mixing:* Sifted ingredients (except magnesium stearate) were mixed in a double cone blender for 20 minutes at 10 rpm.
IV) **Lubrication:** Sifted Magnesium stearate was then added to mixture and mixed in a double cone blender for 5 minutes at 10 rpm.

V) **Compression:** Lubricated blend was kept in a double polybag for compression. It was then compressed using a 8 station compression machine. Tablets were packaged in a plastic bag which was stored in a desiccator.

**Selection of appropriate punch size And shape of the tablets**
Round shape of 8mm flat-face bevel edge (FFBE) Plane on both side.

**Evaluation of Tablets** [18]
Tablets from all the formulation were subjected to following quality control test:

**General Appearance**
The general appearance of a tablet, its visual identity and overall “elegance” is essential for consumer acceptance. Include in are tablet’s size, shape, presence or absence of an odor, taste, surface texture, physical flaws and consistency and legibility of any identifying marking.

**Tablet thickness**
Tablet thickness is an important characteristic in reproducing appearance and also in counting by using filling equipment. Some filling equipment utilizes the uniform thickness of the tablets as a counting mechanism. Twenty tablets were taken and their thickness is recorded using micrometer. The size of the tablet can be dimensionally described, monitored and controlled.

**Uniformity of weight**
USP procedure for uniformity of weight was followed, twenty tablets were taken and their weight was determined individually and collectively on a digital weighing balance. The average weight of one tablet was determined from the collective weight. The weight variation test would be a satisfactory method of determining the drug content uniformity.

**Tablet hardness**
Hardness of tablet is defined as the force applied across the diameter of the tablet in the order to break the tablet. Under condition of storage, the resistance of the tablet
to chipping, abrasion or breakage transformation and handling before usage depends on its hardness. Hardness of tablet was determined using Monsanto hardness tester.

**Friability**

Friability is the measure of tablet strength. Friability of tablets was determined by using Roche friabilator. Twenty tablets were weighed properly and placed in Roche Friabilator. After 100 rotations, the tablets were weighed and percentage loss in tablet weight was determined. The extent of friability was calculated using following formula:

\[
\% \text{ Friability} = \frac{\text{loss in weight}}{\text{Initial weight}} \times 100
\]

**Dissolution studies for Tablet**

The in Vitro drug release was studied by conducting dissolution test for tablets. Dissolution was carried out using USP XIII dissolution apparatus type II-Paddle type. Tablets accurately weighed 200 mg each were subjected to dissolution test using paddle stirrer rotated at 75 rpm ± 2rpm in 900 ml of 0.1 N HCl maintained at 37± 2°C for first two hours and then in 900 ml of 6.8 pH phosphate buffer for next 22 hours. Samples of 5ml were withdrawn from 1, 2, 4, 6, 8, 10,12,16,20 and 24hrs. The volume so withdrawn was replaced with fresh medium. Samples withdrawn were filtered through Whatman filter paper. The filtrate was assayed on UV-VIS spectrophotometer at about 340.5 nm. Three replicates have been made for each experiment. The reading was then processed for dissolution data using PCD Disso v3 software.
4.16 REFERENCES


Chapter 4

Material And Methods


24. ICH: Harmonised Tripartiate Guidelines Stability testing of new drug substances and products, Q1A (R2), www.ich.org


Cardiac Activity and Formulation Evaluation of Isolated Constituents of Piper longum linn


