Materials and Methods
MATERIALS AND METHODS

ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM FRUITS OF PIPER LONGUM

The fruits of *Piper longum* were purchased from Govindaraja Mudali and Sons, Parrys and authenticated by Dr.S.Brinda of Central Institute for Siddha Research, Chennai.

The fruits were shade dried and ground to a powder. The powder was stored in a sealed bottle at 4°C.

PREPARATION OF AQUEOUS EXTRACT

100 g of the powder was soaked in 250ml of sterile water for 24 hours after gentle warming. The supernatent was filtered centrifuged to remove particulate aggregates and stored in air tight bottle at 4°C (vol ≈ 100ml)

The colour of the extract was noted. Its pH and protein content was determined.
PROTEIN ESTIMATION  (Lowry O.H et al., 1951)

REAGENTS

1)  2g of sodium carbonate and 20 mg of Sodium potassium tartarate was dissolved in 100 ml of 0.1 N sodium hydroxide. The reagent was prepared fresh before use.

2)  50 mg copper sulphate was dissolved in 10 ml of distilled water.

3)  Folin’s ciocalteau reagent: Dissolved 100g of sodium tungstate and 25g of sodium molybdate in about 700 ml of distilled water in 2 litre round bottomed flask. Added 50 ml of 85% ortho phosphoric acid and 100 ml of concentrated hydrochloric acid. Refluxed for 10 hours. Added 150g lithium sulphate, 50ml of water and 15 ml of bromine. Boiled off excess bromine cooled and made upto 1 litre and filtered.

REAGENT A  -- Mixed 50 ml of (1) and 1 ml of (2).

REAGENT B  -- Mixed 5 ml of (3) and 9 ml of distilled water.
PROCEDURE

The standards were prepared in the range of 1.5mg % to 12 mg%. To 1ml of distilled water (Blank ), standards and test solution , 5ml of reagent. A was added. Left at room temperature for 10 minutes and added 0.5 ml of reagent B . Left at room temperature and read at 572 nm against distilled water.

CHEMICAL SEPARATION OF ALL BASIC COMPOUNDS FROM AQUEOUS EXTRACT

Acidification of the aqueous extract with sulphuric acid, followed by neutralization with sodium carbonate and addition of methanol yielded a white powder ( WP )which was a mixture of all basic compounds. The solubility of the WP in water and its pH was determined.

PREPARATION OF ACETONE EXTRACT AND ISOLATION OF COMPOUNDS

20g of the powder was soaked in 100 ml of acetone at 4°C in sealed bottles. In a separating funnel the extracts were partitioned with hexane and subsequently with chloroform. Preparative thin layer chromatography of the hexane fraction in silica gel with cyclohexane gave a yellow compound (Y) with $R_f = 0.9$. The purity of the compound was ascertained by redissolving the compound and
checking for a single band by TLC. UV light was used to view the developed chromatogram.

Similar chromatography of the chloroform fraction with a solvent system of hexane/acetone (65:35) gave a compound with $R_f$ 0.7 and the compound is hereafter designated as P. P is insoluble in petroleum ether. The purity of the compound was ascertained by redissolving the compound and checking for a single band by TLC. UV light was used to view the developed chromatogram.

The yields of the compounds after each solvent run were in the range of 10–20 mgs. The compounds Y and P were characterized by UV, IR and NMR spectra and compared with that of Piperine obtained from Sami Labs, Bangalore.

**IN VITRO STUDIES**

**(A) TESTING FOR ANTI BACTERIAL ACTIVITY**

The organisms used to study the anti bacterial activity are *E.coli* (gram negative), *S.aureus* (gram positive) and *M. smegmatis* (Mycobacteria). The cultures were obtained from Bacteriology Department of Tuberculosis Research Centre. *E.coli* and *S. aureus*
were sub cultured in nutrient agar and M. smegmatis was subcultured in Lowenstein Jensen medium.

The non pathogenic mycobacterium is comparatively safe to work since it grows rapidly in liquid culture in the laboratory than the pathogenic organism. (M. tuberculosis takes 15--21 days for optimal growth in stationary liquid culture.). Hence M. smegmatis was used to study the anti tubercular activity.

For comparison of anti bacterial activity standard drugs were used -- Ofloxacin against E.coli, Rifampicin against S.aureus , and Ethambutol against M. smegmatis. The anti bacterial and anti tubercular activity of WP was studied by measurement of turbidity in sauton's medium and that of the compounds Y and P was studied by disc diffusion method in solid media.

TESTING THE ACTIVITY OF WP BY TURBIDITY MEASUREMENT IN LIQUID SAUTON'S MEDIUM  
(Robert.F. Boyd., 1984)

The activity of a drug against given bacterial strain is determined by adding varying amounts of drug in closely graded steps to a series of tubes in liquid medium to which identical inoculae are added. The endpoint is the lowest concentration that prevents visible growth or a
concentration that produces definite reduction in turbidity after a period of incubation. (Minimum inhibitory concentration – MIC)

**COMPOSITION OF LIQUID SAUTON’S MEDIUM**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>30 ml</td>
</tr>
<tr>
<td>Potassium dihydrogen Ortho phosphate</td>
<td>0.5g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>2g</td>
</tr>
<tr>
<td>Ferric ammonium sulphate</td>
<td>0.05g</td>
</tr>
<tr>
<td>Sodium glutamate</td>
<td>4g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>950 ml</td>
</tr>
</tbody>
</table>

Adjusted to pH 7.0 with 40% Potassium hydroxide.

Made upto 1litre with distilled water and sterilized at 15 lbs for 10 minutes. Tween 80 was added to Sauton’s medium for measurement of turbidity in the case of *M. smegmatis*.

A fixed amount of inoculum was added to the medium containing fixed amount of phytochemical or drug. The controls included medium with only inoculum and medium with only drug or
phytochemical. After 24 hours or 48 hours incubation (in the case of
_M.smegmatis_) at 37° C, the turbidity was measured at 630nm.

**TESTING ACTIVITY OF COMPOUNDS Y AND P BY DISC DIFFUSION**
(Robert.F.Boyd,, 1984)  ·

For disc diffusion study, nutrient agar was used for _E.coli_ and
_S.aureas_ and modified solid sauton’s medium was used for
_M.smegmatis_.

**COMPOSITION OF SOLID SAUTON’S MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>-- 4 ml</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>-- 0.5g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>-- 0.05g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>-- 0.2g</td>
</tr>
<tr>
<td>Iron ammonium citrate</td>
<td>-- 0.005g</td>
</tr>
<tr>
<td>Tween 80</td>
<td>-- 0.2 ml</td>
</tr>
<tr>
<td>Asparagine</td>
<td>-- 4g</td>
</tr>
<tr>
<td>Bacto agar</td>
<td>-- 2g</td>
</tr>
</tbody>
</table>
Made up with distilled water to 100ml. Adjusted to pH 7.0 with 10N sodium hydroxid. Sterilized at 15 lbs for 10 minutes.

The compounds Y and P in different concentrations (10 μl) were loaded on to sterile discs which were placed on seeded medium. The plates were incubated at 37 °C for 24 or 48 hours and diameter of the zones of inhibition were measured and compared with that of the standard drug mentioned previously.

(B) BIO ENHANCER STUDIES:

The role of the extract as a bio enhancer was studied by disc diffusion method. The diameter of the zones of inhibition of the same concentrations of the drug alone and drug with Piper longum fruit extract was compared. For studying the bio enhancer activity with ofloxacin, E.coli was used. S.aureus was used to check the bio enhancer activity with rifampicin and M. smegmatis was used to check the bio enhancer activity with Ethambutol and Isoniazid.

STUDIES WITH EXPERIMENTAL ANIMALS

The animals used for the study were Swiss Albino mice. The animals were obtained from Madhavaram. The animals were housed
in well designed animal house in large spacious cages and were given food and water *ad libitum* during the course of the experiment.

**STUDIES WITH UNINFECTED ANIMALS**

The animals were divided into six groups of five animals each. (Males –2, Females –3). One group served as control. The other groups were treated as follows.

I group  
Normal control

II group  
The group was administered anti TB drugs. Ethambutol (25 mg/ kg body wt), Rifampicin (12mg/kg body wt), Isoniazid (10 mg / kg body wt), Pyrazinamide (35 mg / kg body wt).

III group  
AntiTB drugs with *Piper longum* Fruit extract(0.5g/ kg body wt)

IV group  
Anti TB drugs with Piperine (15mg / kg body wt)

V group  
*Piper longum* fruit extract only

VI group  
Piperine only
The drugs were administered in the form of a fine suspension with a feeding tube. The drugs were administered for 47 days and the animals were sacrificed on 48th day. The liver was dissected and washed in ice cold saline. A section of the liver was used for histopathological examination. A homogenate was prepared in 0.1M Tris buffer of pH 7.4 (20% solution). The liver enzymes, level of lipid peroxides and reduced glutathione was estimated in the homogenate as detailed below. Blood was collected and level of lipid peroxides was estimated in the serum sample.

PROTEIN ESTIMATION

The protein content of the homogenate was estimated by Biuret method.

BIURET REAGENT

(A) 4.5 g of sodium potassium trtarate was dissolved in 30ml of distilled water and added to 1.5 g of copper sulphate dissolved in 30ml of distilled water. The whole solution was made upto 100ml with distilled water.

(B) 0.5 g of potassium iodide was dissolved in 100ml of 2N sodium hydroxide.
20ml of (A) was mixed with 8ml of (B) and made up to 100ml and used.

The concentration of bovine serum albumin was 60 mg/ml. To 50 µl of standard or sample, 2ml of 0.9% sodium chloride was added and 2ml of Biuret reagent was added. A blank was prepared with 50µl of 0.9% sodium chloride and treated similarly. The absorbance was read at 540nm. The concentration of protein in the sample was estimated by comparison with the standard calibration graph.

**LIPID PEROXIDES ASSAY** (Okhawa et al., 1979)

**REAGENTS**

1. **Standard** -- 5µl of di ethyl acetal and 3µl of 6N Hydro chloric acid to 10 ml. The working standard was prepared by a 1 in 100 dilution of the stock standard with 6N Hydrochloric acid.

2. **Thio barbituric acid** -- 0.6%

3. **Trichloroacetic acid** -- 10%

To 0.5 ml of the homogenate or serum sample 3ml of tri chloro acetic acid and 2ml of thio barbituric acid was added and kept in
a boiling water bath for 10 minutes. The samples were then cooled and the absorbance was recorded at 535 nm. The concentration of the working standard was 25 n moles of malondialdehyde/ml. A series of standards were pipetted out and made upto 3ml and treated as mentioned above. The concentration of the sample was determined by comparison with the standard calibration graph. The amount of lipid peroxide was expressed as n moles of malondialdehyde/g tissue or /g protein in the homogenate and µM of malondialdehyde /l of serum.

ESTIMATION OF REDUCED GLUTATHIONE
(Moron et al., 1979)

REAGENTS

(1) Standard -- 20 mg of reduced glutathione was dissolved in 100ml of distilled water.

(2) Phosphate buffer -- 0.2 M ,pH 8.0

(3) Dithio nitro benzoic acid -- 0.6 mM in 0.2 M phosphate buffer.

(4) Trichloroacetic acid -- 5%

To 0.5ml of the homogenate 2ml of 5% tri chloro acetic acid was added, mixed well and centrifuged. To 0.5 ml of the supernatent 2ml of 0.2M phosphate buffer of pH 8.0 and 0.5 ml of 5,5’di thio bis 2
nitrobenzoic acid was added. The absorbance was read at 412 nm. A series of standards were pipetted out and treated similarly. The concentration of reduced glutathione in the sample was calculated by comparison with standard calibration graph.

ASSAY OF LIVER ENZYMES

ASSAY OF ALKALINE PHOSPHATASE
(King and Armstrong, 1980)

REAGENTS

(1) 0.1M carbonate–bicarbonate buffer buffer pH 10

(2) Buffered substrate – 218 mg of disodium phenyl phosphate was dissolved in 100 ml of buffer, slightly warmed and cooled.

(3) Folin’s phenol reagent

(4) 15% sodium carbonate in water

(5) Standard phenol-- 100mg of phenol was dissolved in 100 ml of 0.1 N Hydrochloric acid. Working standard with a concentration of 25μg/ml was prepared.

To 2 ml of buffered substrate, 0.1 ml of liver homogenate was added and incubated at 37 °C for ten minutes. The reaction was
arrested with 1ml of Folin's phenol reagent. In the case of control 0.1 ml of liver homogenate was added after the reaction was arrested. After centrifugation, to 0.2 ml of supernatant added 2 ml distilled water and 1ml of 15% sodium carbonate. The tubes were incubated at 37°C for ten minutes and the colour developed was read at 650nm. 2 to 10 μg of phenol was used as standard. From the standard graph, the activity of alkaline phosphatase was calculated and expressed as μ moles of phenol liberated / sec/ g protein.

ASSAY OF ASPARTATE TRANSAMINASE
(Mohur and Cook., 1957)

REAGENTS

(1) Phosphate buffer-- 0.1M, pH 7.5

(2) Substrate – 1.33 g of DL aspartic acid and 15 mg of 2--oxo glutaric acid were dissolved in 20.5 ml of 1N sodium hydroxide and made upto 50 ml with phosphate buffer.

(3) Sodium hydroxide – 0.4N in distilled water.

(4) 2,4-- Dinitro phenyl hydrazine-- 0.02% in 1N Hydrochloric acid.

(5) Standard pyruvate solution – 11mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer.
To 1 ml of the substrate 0.2 ml of homogenate was added and incubated at 37° C for an hour. 1 ml of 2,4 di nitro phenyl hydrazine was added to arrest the reaction and incubated at room temperature for twenty minutes. To the control tube, homogenate was added after the reaction was arrested. 10 ml of 0.4N sodium hydroxide was added to both the test tubes. After five minutes the absorbance was read at 540 nm. The amount of pyruvate formed was obtained from the standard calibration graph and the activity was expressed as μ moles of pyruvate produced / sec/ g protein.

ASSAY OF ALANINE TRANSAMINASE  
(Mohur and Cook., 1957)

REAGENTS

(1) Phosphate buffer-- 0.1M, pH 7.5

(2) Substrate – 1.78 g of DL--alanine and 30 mg of α ketoglutaric acid were dissolved in 20 ml of buffer. 0.5 ml of 1N sodium hydroxide was added and made upto 100 ml with phosphate buffer.

(3) Sodium hydroxide – 0.4N.

(4) 2,4-- Dinitro phenyl hydrazine-- 0.02% in 1N Hydrochloric acid.
(5) Standard pyruvate solution – 11mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer.

To 1ml of the substrate, 0.2 ml of homogenate was added and incubated at 37° C for an hour. 1ml of 2,4 di nitro phenyl hydrazine was added to arrest the reaction and incubated at room temperature for twenty minutes. To the control tube, homogenate was added after the reaction was arrested. 10 ml of 0.4N sodium hydroxide was added to both the test tubes. After five minutes the absorbance was read at 540 nm. The amount of pyruvate formed was obtained from the standard calibration graph and the activity was expressed as µ moles of pyruvate produced / sec/ g protein.

The values obtained were compared using students ‘t’ test.

HISTOPATHOLOGICAL STUDIES

Autopsy bits were preserved in 10% formalin solution for minimum one hour. Dehydration of tissue was done by three changes of acetone. Cleaning of tissue from acetone was effected by three changes of xylene in a total duration of three hours. Incubation of the processed tissue in melted paraffin wax was done by two changes for three to four hours at 58--60 ° C. After incubation, the tissue was fixed
in paraffin wax by cooling and slice of 1--3 μ was prepared using a microtome. The paraffin section was taken on a glass slide and cleaned by immersion in xylene. The section was stained with hematoxylin and eosin and observed under microscope.

**STUDIES WITH INFECTED ANIMALS**

The animals were divided into five groups of five animals each (Males--3, Females--2)The first group served as normal control. The other four groups were infected with H37Rv by a tail vein injection of the suspension after counting using Arnold .R.Horwell Thoma counter. After the infection was established (two weeks), the groups were treated as follows for fifteen days.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
</tr>
<tr>
<td>II</td>
<td>Infected, No treatment</td>
</tr>
<tr>
<td>III</td>
<td>Infected and administered anti TB drugs. Ethambutol(25 mg/ kg body wt), Rifampicin (12mg/kg body wt), Isoniazid (10 mg / kg body wt), Pyrazinamide (35 mg / kg body wt).</td>
</tr>
</tbody>
</table>
IV group -- Anti TB drugs with *Piper longum* fruit extract (0.5g/kg body wt)

V group -- *Piper longum* fruit extract alone

After fifteen days, the animals were sacrificed. The liver was dissected and washed with ice cold saline. A homogenate was prepared in 0.1M Tris buffer of pH 7.4 (20% solution). The liver enzymes, level of lipid peroxides and reduced glutathione were estimated in the homogenate as mentioned earlier. The values obtained were compared using students ‘t’ test. Histopathological studies were carried out as mentioned earlier.

**STUDIES WITH HEALTHY HUMAN VOLUNTEERS**

The volunteers were selected in the age group of 25--50 years, the body weight ranging from 50--75 kg. They did not suffer from any hepatic or renal dysfunction.

**BIOAVAILABILITY STUDY DURING ADMINISTRATION OF ANTI TUBERCULOSIS DRUGS WITH FRUITS OF *PIPER LONGUM***

To study the pharmacokinetic parameters, the drug concentrations were monitored at regular time intervals after the administration of the dose. The dosages of anti TB drugs used was Rifampicin 450 mg, Isoniazid 600 mg, Pyrazinamide 1500 mg and
Ethambutol 1200mg. The concentrations of the drugs were measured at time points of 0 hr, 1 hr, 2 hr, 3 hr and 6 hr. Drug concentrations were measured in the urine sample collected between 0–8 hours. The concentrations of different drugs were measured using techniques like HPLC, Spectrofluorimetry, Spectrophotometry etc. After a wash out period of ten days the same concentration of anti TB drugs was administered with 0.5 g of fruits of Piper longum and the investigation was repeated as above.

ESTIMATION OF ISONIAZID (INH) AND ACETYL INH IN URINE
(Rao K.V.N et al., 1971)

REAGENTS

1) Standard INH and Acetyl INH – 1 mg/ml

2) 0.1 N Sodium hydroxide

3) Ammonium sulphate

4) Chloroform butanol mixture (7:3)

5) 0.1 N sulphuric acid

For INH the standards used were 10 μg/ml and 20 μg/ml and for acetyl INH the standard concentrations used were 30 μg/ml and 60 μg/ml. To 2.5 ml of the standards 2.5 ml of distilled water was added.
5ml of water served as blank. 5 ml of urine sample was taken for the test. To all the flasks 2ml of 0.1N sodium hydroxide was added. One spoonful of ammonium sulphate powder was added to all flasks. 30 ml of chloroform butanol mixture in the ratio 7:3 was added to each flask and the flasks were shaken for 30 minutes. Two to three spoonfuls of ammonium sulphate was added to absorb water. Filtered through cotton plugged funnels into test tubes and measured 20 ml into the respective marked flasks containing 6 ml of 0.1 N sulphuric acid. The flasks were again shaken for fifteen minutes. Suitable dilutions of the acid extract was prepared with 0.1 N sulphuric acid. The reagents for colour development were added as follows.

COLOUR DEVELOPMENT FOR ACETYL INH

REAGENTS

1) 0.1 N sodium hydroxide

2) 0.5 M phosphate buffer ( pH 6.0 )

3) 20 % potassium cyanide

4) 12.5% chloramine T

5) Acetone
To 0.5 ml of the acid extract, 0.4 ml of 0.1N sodium hydroxide, 0.5 ml of 0.5 M potassium phosphate buffer (pH 6), 0.5 ml of 20% potassium cyanide and 2.0 ml of 12.5% chloramine T were added. Waited for precipitate to form after addition of chloramine T and after two minutes added 2.5 ml of acetone. Pink colour formed was read at 550 nm. The colour was stable for thirty minutes. The absorbance of the test was compared with that of standards.

**COLOUR DEVELOPMENT FOR INH**

**REAGENTS**

1) 2% vanillin in 25% ethanol

To 3 ml of the acid extract 0.3 ml of 2% vanillin in 25% ethanol was added and the yellow colour developed was read at 380 nm. The absorbance of the test was compared with that of standards.

**ESTIMATION OF INH IN PLASMA**
(William.A.Olson et al., 1977)

**REAGENTS**

1) Standard Isoniazid (1 mg/ml)

2) Trichloro acetic acid-- 10%

3) Salicylaldehyde – 100 µl of salicylaldehyde is added to 3 ml of ethanol. To this added 13.67g of sodium acetate
trihydrate and 2.1 ml of 10N sodium hydroxide. Made up to 250 ml.

4) Sodium bisulphate – To 26.2 g of sodium acetate trihydrate and 1.38 g of sodium meta bisulphite, 8.3 ml of 10 N sodium hydroxide was added and made up to 500 ml with water.

The technique employed for estimation of INH in plasma is spectro fluorimetry. All the reagents were prepared in milli Q water. Standards were prepared in the range of 3.12 – 100 µg/ml. To 25 µl of the standard 225 µl of pooled plasma was added. For the blank 25 µl of water was taken and 225 µl of pooled plasma was added. 250 µl of the plasma sample was taken for the test. 1.25 ml of 10% trichloro acetic acid was added to all the tubes, vortexed and centrifuged for 10 minutes. 0.8 ml of the supernatent was transferred to another tube and 0.8 ml of milli Q water and 1 ml of salicylaldehyde was added to all the tubes. Checked whether the pH was between 3.9--4.1. Otherwise adjusted with 1N sodium hydroxide or 1N hydrochloric acid. Left for fifteen minutes. Then added 2 ml of sodium bisulphate and 200 µl of ascorbic acid. Checked whether the pH was between 5.65--5.75. Otherwise adjusted with 1N sodium hydroxide or 1N hydrochloric acid. Kept at 50º C water bath for ten minutes. Added 3 ml of iso butyl alcohol to all the tubes and vortexed for one minute. Kept in ice immediately for ten minutes. Top organic layer removed or
fluorescence measured as such at an emission wavelength of 462nm, the excitation wavelength being 375 nm.

ESTIMATION OF RIFAMPICIN IN URINE BY HPLC
(Hemanth kumar et al., 2004)

REAGENTS

Stock standard: Rifampicin (RMP) and Desacetyl rifampicin (DRMP) (1000 µg/ml)

2 Internal standard: Rifapentine (RPN) (12.5 mg / 10 ml)

3 Buffer: 1 ml of 1.5 M citric acid added to 13 ml of 1.5 M sodium hydrogen phosphate

4 Chloroform:

5 Potassium di hydrogen phosphate 0.05 M (Mobile phase) Chloroform

The stock standard was diluted to 100 µg/ml with urine. Standards in the range of 2.5 – 80 µg/ml were prepared by diluting with urine. To 2 ml of blank, standards, or test 40 µl of rifapentine, 1 ml of buffer and 2 ml of chloroform were added. Vortexed for one minute and centrifuged at 2500 rpm for ten minutes. 0.5 ml of the
chloroform layer transferred to small tubes and evaporated. Reconstituted in 500 µl of mobile phase and injected into a C 18 Merck column of 4 diameter at a flow rate of 1.2 ml/ min. A UV detector at 254 nm was used. The retention time was compared. (DRMP-- 1.9 min, RMP-- 2.9 min, RPN -- 5.8 min) By comparing the peak heights of the sample with that of standards, the concentration of the drug was determined.

ESTIMATION OF RIFAMPICIN IN PLASMA BY HPLC (Hemanth kumar et al., 2004)

REAGENTS

1) Stock standard Rifampicin and DRMP – 1 mg / ml (100 µl of ascorbic acid added to stock)

2) Internal standard Rifapentine – 12 5 mg / 10 ml of acetonitrile

3) Mobile phase – 0.05 M Potassium dihydrogen phosphate buffer : acetonitrile 55 : 45

4) Ascorbic acid-- 500 mg in 10 ml
Standards were prepared in the range of 0.25 μg/ml to 20 μg/ml with pooled plasma. To 200 μl of blank, standard or sample, added 300 μl of acetonitrile and 20 μl of internal standard. Vortexed for one minute. Micro centrifuged at 10,000 rpm for five minutes. Transferred 300 μl of supernatent to another tube. Freezed at -45 °C for half an hour. Vacuum evaporated for approximately one hour. Reconstituted in 200 μl of mobile phase. Injected 20 μl into a C 18 Merck column of 4 diameter at a flow rate of 1.2 ml/ min. A UV detector at 254 nm was used. The retention time was compared. (DRMP-- 1.9 min, RMP-- 2.9 min, RPN -- 5.8 min) By comparing the peak heights of the sample with that of standards, the concentration of the drug was determined.

ESTIMATION OF PYRAZINAMIDE IN PLASMA
Prema Gurumurthy et al., 1980)

REAGENTS

1) Stock standard – Pyrazinamide (1 mg / ml )

2) Trichloro acetic acid (TCA)--10%

3) Sodium nitroprusside – 0.2%

4) Sodium hydroxide – 2 N

To 3.5 ml of plasma added 1.8 ml of TCA and the contents were stirred and centrifuged. 3 ml of protein free supernatent was
applied to a column (75 x 6 mm) of Dowex 1--x8 (chloride form, 200--400 mesh) and the column was washed with small amounts of water till 10 ml of the eluate was collected. To 3 ml of this eluate, added 0.5 ml of freshly prepared solution of 0.2% sodium nitroprusside and 0.5 ml of 2N sodium hydroxide. The tubes were left at room temperature for fifteen minutes and the optical densities were recorded at 495 nm. By comparing the absorbance with that of standards, the concentration of the drug was determined.

**ESTIMATION OF PYRAZINAMIDE IN URINE**
(Prema Gurumurthy et al., 1980)

**REAGENTS**

1) Stock standard – Pyrazinamide and pyrazinoic acid (1 mg / ml)

2) Trichloro acetic acid (TCA)

3) Sodium nitroprusside – 0.2%

4) Sodium hydroxide – 2 N

Standards in concentration of 50 and 100 µg/ml was prepared in pooled urine. Loaded 3 ml of urine into a column of Dowex (details mentioned previously). Pyrazinamide was eluted from the column by washing it with small amounts of water till 15 ml of the eluate was collected. For pyrazinoic acid elution the column was first washed with 15 ml of water followed by small amounts of 0.5 M sodium
chloride till 15 ml of the eluate was collected. To 3 ml of respective eluates added 0.5 ml of sodium nitroprusside and 0.5 ml of sodium hydroxide. After fifteen minutes colour developed was read at 495 nm. By comparing the absorbance with that of standards, the concentration of the drug was determined.

From each series of plasma concentrations at different hours, Maximum concentration (C_max) and the time to attain C_max (t_max) were determined by direct visual inspection of data. Area under the time concentration curve (AUC_0→t) was computed using the linear trapezoidal rule. AUC_0→α was obtained from the sum of AUC_0→t and concentration at t hours divided by the elimination constant. Elimination constant was calculated by log linear decline of concentration.

The values obtained were compared using students 't' test.

STUDY OF HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS AFTER ADMINISTRATION OF FRUITS OF *Piper longum*

Hematological parameters and the levels of metabolites, electrolytes, enzymes and hormones were estimated in healthy volunteers before the start of the study. 0.5g of powdered fruits of *Piper longum* was administered for 40 days and above mentioned
estimations were carried out once again to check the effect of administration of *Piper longum* fruits on various parameters.

**HEMATOLOGICAL PARAMETERS**

**TOTAL ERYTHROCYTE (RBC) COUNT**  (Huxtable.R.J., 1990)

**REAGENTS**

RBC diluting fluid -- (Hayem’s fluid) -- 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200 ml of distilled water.

With the help of a RBC pipette, blood was drawn up to 0.5 mark and diluted up to mark 101 with diluting fluid. The dilution was 1:200. It was then filled in a neubauer counting chamber and counted.

**TOTAL LEUCOCYTE (WBC) COUNT**  (Raghuramulu *et al.*, 1983)

**REAGENTS**

1) WBC diluting fluid  
   a) Acetic acid --  1.5 ml  
   b) Crystal violet --  1ml  
   c) Distilled water --  98.5 ml

With the help of A WBC pipette, 0.02 ml of blood was drawn up to 0.5 mark and diluted up to mark 11 with the diluting fluid. The dilution
was 1:20. It was then filled in a neubauer counting chamber and counted.

**TOTAL THROMBOCYTE (PLATELET) COUNT** (Brecher., 1964)

**REAGENTS**

1) Diluting fluid

3.8g of Sodium citrate, 0.2 ml of formalin and 0.1g of Brilliant cresol blue were dissolved in 100 ml of distilled water and filtered before use.

With the help of a RBC pipette, blood was drawn up to 0.5 mark and diluted up to mark 101 with diluting fluid. The dilution was 1:200. It was then filled in a neubauer counting chamber and counted.

**ESTIMATION OF HEMOGLOBIN**

Hemoglobin was estimated colorimetrically by cyanmethaemoglobin method and the absorbance was read at 540nm. (Drabkin and Austin., 1932)

**REAGENTS**

1) Drabkin’s reagent -- Dissolved 200 mg potassium ferricyanide, 50 mg potassium cyanide and 1g sodium carbonate
in water and made up to 1 litre. The reagent had a pale yellow colour and pH of 9.6.

2) Cyanomethaemoglobin standard – 16g/dl.

0.02 ml of blood was diluted with 5 ml of the reagent. The diluted blood was mixed well and allowed to stand for ten minutes to ensure the completion of the reaction. The absorbance was read at 540 nm and compared with the standard.

BIOCHEMICAL PARAMETERS
ESTIMATION OF GLUCOSE

Glucose was estimated colorimetrically by o-toluidine colour reaction. (Sasaki et al., 1972)

REAGENTS

(1) Trichloro acetic acid – 10% in distilled water.

(2) Glucose Standard – 10mg in 100 ml.

(3) Ortho toluidine boric acid reagent.

2.4 g of boric acid and 2.5 g of thiourea were dissolved in 100 ml of solution containing distilled water, glacial acetic acid and freshly distilled ortho toluidine in the ratio of 10:75:15.

0.1 ml of blood was mixed with 3 ml of 10% tri chloro acetic acid to precipitate the proteins. 1 ml of the supernatant was mixed with 4 ml of ortho toluidine reagent. For blank 1 ml of distilled water was
used. The colour developed was read at 640 nm and compared with that of standards.

ESTIMATION OF UREA
Urea was estimated colorimetrically using Diacetyl monoxime (Varley., 1988)

REAGENTS

(1) Diacetyl monoxime and thio semi carbazide reagent – The reagent contained 36 mM Diacetyl monoxime and 61.7 mM thiosemi carbazide in 2% acetic acid. (DAM – TSC)

(2) Acid Ferric reagent – This acid contained 3.6 Sulphuric acid. 0.12 Ferric chloride and 38.6 ml ortho phosphoric acid.

(3) Urea standard – 10 mg in 100 ml of distilled water.

To 2ml of blood 2.8 ml of 10% trichloro acetic acid was added to precipitate the proteins. To 2ml of the supernatent 1ml of DAM – TSC reagent and 1.5 ml of acid ferric reagent were added and kept in a boiling water bath for 15 minutes. The colour was read at 520 nm and compared with that of the standards.
ESTIMATION OF CREATININE

Creatinine was estimated colorimetrically by developing a red orange coloured complex with alkaline picrate and reading the absorbance at 520nm (Slot., 1965)

REAGENTS

1) Creatinine (standard) -- 100mg of creatinine was dissolved in 100 ml of 0.1N hydrochloric acid to prepare the stock standard. 1ml of the stock was diluted to 100 ml to prepare the working standard.

2) Picric acid -- 1.2 g of picric acid was dissolved in 1 litre of distilled water.

3) Sodium hydroxide -- 30g was dissolved in 1 litre. Equal volumes of 1 and 2 were mixed just before use.

To 0.1 ml of blood, 3.9 ml of 10% TCA was added to precipitate the proteins. To 3 ml of the deprotenized supernatent 2 ml of alkaline picrate was added and the colour was read at 520 nm after 30 minutes.
ESTIMATION OF URIC ACID

The method is based on the reaction of phosphotungstic acid reagent with uric acid to give a blue colour. (Caraway. W.I., 1963)

REAGENTS

(1) Uric acid standard -- 100mg of uric was dissolved in 150 ml of water containing 60 mg of lithium carbonate by heating at 60°C. The solution was cooled to room temperature and added 2ml of formaldehyde and diluted to 500 ml after slightly acidifying with sulphuric acid. 1 ml of the stock was diluted to 10 ml to prepare the working standard.

(2) Tungstic acid -- Added 50 ml of 10% sodium tungstate to 50 ml of 0.6N sulphuric acid. A drop of Phosphoric acid was added and made upto 800ml with water.

(3) Phospho tungstic acid – 50 gm of sodium tungstate was dissolved in 400 ml of water. Added 40ml of 85% phosphoric acid and refluxed gently for 2 hours. Cooled and made upto 500 ml. Diluted 1 in 5 hours before use.

(4) 10% sodium carbonate 5.4 ml of tungstic acid was added to 0.6 ml of serum. The contents was mixed and
centrifuged. To 3ml of supernatent in the test tubes, 0.6 ml of sodium carbonate and 0.6 ml of phospho tunstic reagent were added, mixed and kept in a 25°C water bath for 10minutes. The blue colour developed was read at 700 nm and compared with that of standards.

ESTIMATION OF BILIRUBIN

Bilirubin was estimated by coupling with diazotised sulphanilic acid and reading the absorbance of the red dye formed at 530nm. (Malloy and Evelyn., 1937)

REAGENTS

(1)Bilirubin standard – 5mg of bilirubin was dissolved in 50ml of chloroform.

(2)Diazoblank – 10 g of sulphanilic acid was dissolved in 500 ml of distilled water. 200 ml of concentrated hydrochloric acid was added. Warmed to dissolve and made up to 1 litre with distilled water.

(3)Sodium nitrite – 20 g of sodium nitrite was dissolved in 100 ml of distilled water. 1 ml of the stock was diluted to 20 ml.
(4) Diaz reagent – To 10 ml of diazo blank, 0.3 ml of sodium nitrite was added before use.

To 0.2 ml of serum, 6.2 ml of distilled water and 0.7 ml of diazo reagent was added and the colour developed was read at 540 nm and compared with standards. For blank 6.4 ml of distilled water was taken.

ESTIMATION OF CHOLESTEROL

Cholesterol was estimated by colour development with ferric chloride and sulphuric acid and the pink colour developed was read at 560nm (Harold Varley, 1988).

REAGENTS

1) Cholesterol standard -- Stock was prepared by dissolving 100mg of cholesterol in 100 ml of acetic acid after adding a drop of chloroform. 4 ml of the stock was diluted to 100 ml with ferric chloride reagent.

2) Ferric chloride reagent -- 500 mg of ferric chloride was weighed dissolved and made up to 100 ml with aldehyde free acetic acid. From the above stock 10 ml was made up to 100 ml.
3) Concentrated sulphuric acid --To 0.1 ml of serum, 4.9 ml of ferric chloride reagent was added, followed by 3 ml of concentrated sulphuric acid and the colour developed was read at 590 nm.

ESTIMATION OF PROTEIN

Total protein was estimated by Biuret method using alkaline copper sulphate and the absorbance was read at 546 nm according to the procedure mentioned earlier.

ELECTROLYTES

(1) Chloride was estimated colorimetrically by forming a complex of chloride ions with mercury thio cyanate and reading the absorbance at 460nm. (Krupp., 1980). To 20μl of standard, sample or deionized water serving as blank 2ml of the reagent was added and after 5 minutes the absorbance was read at 460nm.

(2) Bicarbonate was estimated by titration against sodium hydroxide using bromothymol blue as indicator.

(3) Sodium and potassium were estimated by flame photometry.
(1) Alanine transaminase was assayed by employing a coupled assay technique. The pyruvate formed as end product reacts with reduced form of Nicotinamide adenine dinucleotide (NADH) in the presence of lactate dehydrogenase to form lactate. The rate of decrease in absorbance at 340 nm due to decrease in concentration of NADH is directly proportional to ALT activity. The assay was carried out using kits. (Liqui)

(2) In the case of Aspartate transaminase the oxaloacetate formed reacts with NADH in the presence of malate dehydrogenase to form malate. The rate of decrease in absorbance at 340 nm due to decrease in concentration of NADH is directly proportional to AST activity. The assay was carried out using kits. (Liqui)

(3) Alkaline phosphatase was assayed using the substrate para nitrophenyl phosphate and monitoring the amount of paranitrophenol formed by reading the absorbance at 405 nm (Bessy et al., 1946). The assay was carried out using kits. (Ecoline)
HORMONES

Free T4 and T3 are estimated by competitive enzyme immuno assay involving immobilized antibody, enzyme antigen conjugate (Enzyme Horse radish peroxidase) and serum containing the native free antigen (Teitz., 1976). The substrates used were tetra methyl benzidine and hydrogen peroxide. The absorbance was measured at 450 nm. The enzyme activity in the antibody bound fraction is inversely proportional to the native free antigen concentration which is determined from the calibration graph. The estimation was carried out using kit. (Monobind)

The values obtained were compared using students ‘t’ test.