Chapter II

Material and Methods

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- Administration of Mandur bhasma
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In present project it is proposed to study the effects of Mandur bhasma on rifampicin induced hepatic injury in male albino rats.

The animals and chemicals used throughout the project are described in this chapter along with the details of methods used to induce hepatic toxicity by rifampicin in albino rats. The histological methods and bioassay methods used for different parameters are given in detail.

Animals :

Male albino rats which were originally derived from Haffkine Institute, Mumbai, were maintained and bred in departmental animal house (Registered for "Research and Breeder" No.233/CPCSEA) under standard laboratory conditions. The rats were fed standard pellet diet (rat feed) prepared by Amrit feeds (Navmaharashtra Chakan Oil Mills, Sangli, Maharashtra, India) and water *ad libitum.* during breeding, maintenance and experimental work.

Chemicals :

Tris and bovine serum albumin malondialdehyde, paraformaldehyde, guanidine hydrochloride were purchased from Sigma chemical Company, St. Louis USA. All other chemicals were of highest purity obtained from BDH and E. Merk, India.

Pathological diagnostic kits were purchased from Nirmal laboratory, Chopada (Jalagaon), India.

Mandur Bhasma Preparation :

Mandur bhasma is the ore of the iron in the form of ferric oxide obtained from mines. It was purchased from the local suppliers of Ayurvedic medicines. It was grinded to form very fine dust. It was soaked in the juice of fruits of *Garcinia indica* for twenty-four hours and washed thoroughly with water. 1Kg powder of Mandur was triturated with the five liters of juice of leaves of *Aloe vera* and dried. The bhasma was prepared by giving Gaja puta.

The quality test for Mandur bhasma was conducted and described in the Ayurvedic and Sidha tests.

The quality test

1 gm.....Mandur bhasma.

200 mg.....Ghee

200 mg.....Jaggury.

200 mg .....*Abrus precatorious* seeds.

200 mg.....Honey.

200 mg..... Borax.

Mandur bhasma (1g) was triturated with the above mixture and roasted at very high temperature. On cooling no particles of iron were formed. This test showed that with above treatment, Mandur bhasma was not altered. Therefore the quality of the Mandur bhasma was good and no untraced metal was present in the bhasma.

Rifampicin :

Rifampicin and other derivatives of Rifamycin were considered as relatively non-toxic antibiotics having a broad antibacterial spectrum and are active against Mycobacterium species. It is available at medical shops in injectable form, in suspension and powder in encapsulated form. Rifampicin in free powder form was obtained directly from manufacturer- Li-Taka Pharmaceuticals, Pune. We thank respective authorities of the company for supply of free sample.

Rifampicin is widely used as anti-tubercular drug throughout the world. However the drug is also known for the induction of hepatotoxicity.

Dose of rifampicin :

To study the histological and biochemical alterations occurring in liver, kidney and blood during experimental work healthy male albino rats were given desired dose of Rifampicin (50 mg/kg body wt/day) for 30 days. A paste of drug in water was prepared and was fed by using feeding tube. 24 hours after last dose they were sacrificed and they were tested for desired parameters.

Mandur bhasma :

Mandur bhasma is an Ayurvedic drug which is already tested as hepatoprotective and hepatocurative drug. It was also proposed to use to test its influence on Rifampicin induced hepatotoxicity.

Dose of mandur bhasma :

Mandur bhasma was given as per body weight (10 mg/kg/day) for 30 days. A colloidal solution in water was prepared and was given by using feeding tube.

Experimental protocol :

As mentioned earlier the experiments were conducted in four sets- The rats weighing 130 to 140 g were used for the present experiment. The animals were grouped into 4 groups [each containing 6 animals] and

the rifampicin, rifampicin + mandur bhasma and mandur bhasma oral treatments were given daily between 8-00 to 9-00 a.m. Following were the Groups of the animals.

Group I - Normal Rats

Normal male albino rats were provided with the normal diet and water *ad libitum* until experimental set was completed.

Group II - Rifampicin treated Rats

To these animals daily dose of 50 mg /kg body wt/day of Rifampicin was given for 30 days.

Group III - Rifampicin + mandur bhasma treated Rats.

These rats were fed with 50 mg rifampicin/kg body wt/day and 10 mg mandur bhasma /kg body wt/day for 30 days.

Group IV Mandur bhasma treated Rats

This group of rats was maintained as Control for Mandur bhasma by giving 10 mg mandur bhasma/kg body wt/day for 30 days.

Immediately after the oral dose, food was supplied to the animals. They were deprived of food for 12 hrs prior to killing. The

rats were killed after 24 hr of the last dose given by giving deep ether anesthesia.

To test the effects of mandur bhasma on rifampicin induced toxicity on liver and kidney the following parameters were used.

A. Serological parameters

Collection of serum :

On the completion of the experimental schedule giving deep ether anesthesia killed animals. The blood samples were aspirated from the left ventricle with the syringes and were allowed to clot at room temperature. On clotting the serum samples were obtained by centrifuging the clots using tabletop centrifuge. The colorless samples were stored at 10^0 C until use.

Liver Function Tests :

Diagnosis of liver function in human is performed using serum levels of AST/Aspartate aminotransferase/GOT and ALT/alanine aminotransferase/GPT and alkaline phosphatase (ALP) and bilirubin (conjugated and nonconjugated). Therefore in the present study liver function tests were studied in albino rats.

AST, ALT and bilirubin were estimated using commercial diagnostic kits available from Nirmal laboratory, Chopada,

Jalagaon). The assay methods of AST and ALT were as described by Reitman and Frankel [1957] and that of bilirubin was as described by Jendrassik and Grof (1938). Alkaline phosphatase was assayed according to the method described by Bergmeyer [1965].

Assay of alkaline phosphatase:

Chemicals-

1. Subhstrate buffer -

Glycine	375 mg
NaOH	166 mg
$MgCl_2$	10 mg
p-nitrophenyl phosphate	165 mg

Dissolve above chemicals in distilled water and make final volume 100 ml. The pH of the solution will be 9.2

2. NaOH (0.02 N)- Dissolve 80 mg NaOH in 100 ml distilled water.

Assay of serum alkaline phosphatese

Take 1.0-ml substrates buffer and 0.1-ml suitably diluted serum and incubate at 37^0 C for 30 minutes. Stop the reaction by

the addition of 10 ml 0.02 N NaOH. Do not add the serum to control tube during incubation. Add serum to control tube stopping the enzyme activity by NaOH. Take reading at 405 nm. Calculate the activity by converting OD to the amount of p-nitrophenol released.

Unit of enzyme activity was defined as the amount of p-nitrophenyl phosphate required to release 1μ M of p-nitrophenol per minute under standard assay conditions.

Kidney function tests

Diagnosis of kidney function in humans is performed using serum levels of serum urea and serum creatinine.

Serum urea and serum creatinine were also estimated using commercial kits available from Nirmal laboratory, Chopada, Dist. Jalgaon. The assay methods of urea and creatinine were as described by Henry (1991) and Heinegard and Tiderstorm (1973).

A. Histology:

Normal histological appearance of any organ reflects normal physiology of the animal. Histological alterations are used for the diagnosis of pathological condition. During toxicological and pathological studies the variations in the histology are exploited for the evaluation of physiological state of the animal. Therefore during present work also the histopathological alterations were studied.

Preparation of tissue for histology:

Always pieces were selected from central region of central (main lobe) of liver for histological studies. Kidneys were removed and were stripped off peritoneal wall and fat. The middle region pieces were used for studies ensuring that circular piece/half circular piece was selected. Always new razor blade was used to cut tissue into pieces.

Fixation and microtechnique:

Livers and kidneys were dissected out and cut with the precautions described above into pieces of 0.5 x 0.5 x 0.5 cm, fixed in Bouin's fixative, 10% buffered paraformaldehyde. The fixatives were prepared as follows

Bouin's fixative:

It was prepared as described by Thompson (1966).

A Bouin's Fluid ----Stock

75%------Saturated picric acid.

25%------formaldehyde.

B Working Bouin's Fluid.

95%--------solution A

5%----------Glacial acetic acid

Adjust the pH to 1.5-1.7.

1) Buffered 10% formalin :

a) Phosphate buffer

Dibasic anhydrous sodium phosphate (Na2HPO4) 6.5 g

Monobasic acid potassium phosphate (KH_2PO_4) 4.0 g

B] Paraformaldehyde

Dissolve 80-g paraformaldehyde in distilled water by heating at 60^0 C till complete dissolution. A

Mix equal volumes of phosphate buffer and paraformaldehyde solution. pH of this fixative should be 7.00

The tissues were processed for wax sectioning as described by Pears (1968) and Thompson (1966). The sections were cut of 0.5 µ a stained with hematoxylin and eosin and with Mallory's triple stain.

Preparation of chemicals :

1. Harris' hematoxylin

Hematoxylin (national aniline No. 564)	5.0 g
Ethyl alcohol (absolute)	50.0 ml
Aluminum ammonium sulfate	100.0 g
Mercuric oxide	2.5 g
Distilled water	1000.0 ml

Dissolve aluminum ammonium sulfate in 900 ml boiling water. Similarly dissolve 5g hemaoxylin in small quantity of absolute alcohol. Mix above two solutions and boil rapidly. Remove from the heat and add mercuric oxide very slowly. Shake well and plunge in a cold water. This is ready for use as soon as it cools and this stain can be stored indefinitely.

2. Eosin

Eosin Y (National aniline No. 516)	2.0 g
Absolute ethyl alcohol (AA)	200.0 ml

Dissolve eosin in alcohol and filter the stain.

3. Mallory's aniline blue

i. 1 % alcoholic iodine

Iodine	1.0 g
AA	100.0 ml

ii. 5 % sodium thiosulfate

Sodium thiosulfate	5.0 g
Dstilled water (Dist. W)	100.0 ml

iii. 0.5 % aqueous acid fuschin (national aniline No. 402)

Acid fuschin	0.50 mg
Distilled water	100.0 ml

iv. aniline blue – orange G

Aniline blue	0.5 g
(water soluble national aniline No.426)	
Orange G national aniline No. 696)	2.0 g
Phosphoric acid	1.0 g
Distilled water	100.0 ml

Mix all the above chemicals and use.

Staining procedures :

A] Heamoxylin and eosin

1) Dewax the sections for 30' in xylene
2) Hydrate through descending alcohol grades
3) Wash with distilled water
4) Treat the sections with Harris' hematoxylin for 8' at room temperature
5) Wash with distilled water repeatedly
6) Dehydrate through ascending grades of alcohol
7) Bring slides in absolute alcohol
8) Stain with eosin for 1'
9) Wash with absolute alcohol

10) Clear in xylene

11) Mount in DPX

B) Mallory's aniline blue stain

a) dewax the slides in xylene for 30'

b) bring the slides to absolute alcohol

c) treat the section with 1% alcoholic iodine for 5'

d) wash with water

e) Treat the slides with 5%acid fuschin for 5'

f) Treat the sections with aniline blue – orange G for 50 to 60'

g) Wash with 95 % alcohol

h) Wash with absolute alcohol

i) Clear in xylene

j) Mount the sections in DPX

B. Biochemical assays :

Preparations of tissue homogenates:

The pieces of liver from central region of central lobe, full transverse sections of kidney were used ensuring the inclusion of medulla and cortex in natural proportion and Known quantities of liver, kidney were homogenized in Teflon homogenizer with 4 strokes at 1300 rpm. The homogenates were diluted with distilled water to a known volume.

Lipid peroxidation assay : (Buege and Aust, 1978).

Chemicals :

TCA-TBA-HCL reagent :

15% TCA -Trichloroacatic acid

0.375% TBA- Thiobarbituric acid

0.25 N HCl-Hydrochloric acid

Solution may be heated to dissolve TBA

Bioassay-

Sample	Blank
1 ml sample	1ml water
2ml TCA - TBA - HCL Reagent	2 ml TCA -TBA-_HCL Reagent

Heat for 15' in a water bath at 80 C

Cool

Centrifuge at 1000 g for 10'.

Supernatent	Supernatent

Read at 535 mu

Calculations-

Concentration of malondialdehyde is calculated using an extinction coefficient 1.56 x 10^5 M^{-1} cm^{-1} (Wills,1971)

Protein Oxidation (Levin *et al*, 1990)

Chemicals :

1] 2, 4, dinitrophenylhydrazine (DNPH) Reagent [200 mg of 2-4-Dinitro phenyl hydrazine-10 mM + 100 ml; 0.2 M HCl].

2] 20 % TCA

3] Ethanol+ ethyl acetate in equal volume

4] Guanidine hydrochloride [15 g *i.e.* 6 M in 20 mM potassium phosphate ml D.W.]

Bioassay-

Sample	Blank
1.5 ml homogenate	1.5 ml water
add 1.5 ml of 20% TCA	1.5 ml of 20% TCA

Wait for 15 min. Centrifuge and drain. Take the precipitate. Add 0.5 ml of DPNH reagent to each of the tubes and wait at RT for 1hr. Stir intermittently. Add 0.5 ml of 20% TCA to each of the

tubes and wait for 15-20 minutes and centrifuge and discard the supernatant. Wash the precipitate with ethanol-ethyl acetate mixture and wait for 15 min and add 0.6 ml of guanidine hydrochloride. Wait for 15 min. and add 2.5 ml DPNH Reagent. Read at 360-390. Calculate taking molar absorption coefficient 22000 M^{-1} cm^{-1}

Glutathione assay : (Grunert and Phillips 1951).

Chemicals: 0.067 M- Sodium nitroprusside,

0.067 M- Sodium cyanide in 1.5 M Na_2CO_3 (Sodium Carbonate).

Saturated Sodium Chloride.

Bioassay -

Sample	Blank
6 ml - Sample	6 ml- D. water
1 ml Sodium Nitroprusside.	1 ml Sodium Nitroprusside.
1 ml Sodium Cyanide in Na_2CO_3	1 ml Sodium Cyanide in Na_2CO_3

Read at 520 nm (O. D.)

Calculations:

Glutathione concentrations are measured using formula O.D.X 6200/Protein.

Formaldehyde Assay: (Werringloer 1978).

Chemicals:

12.5% TCA

NASH Reagent- 6 nm Ammonium Acetate

60 nm Acetyl Acetone

1.5 nm Acetic Acid.

Bioassay :

Sample	Blank
1 ml Sample	1 ml water
1.5 ml 12.5% TCA	1.5 ml 12.5% TCA

Centrifuge at 4000 g for 10'

2 ml supernatent	2 ml supernatent
1 ml NASH reagent	1 ml NASH reagent

Heat at 60 ^{0} C for 10'

Read at 412 nm

Calculations: Formaldehyde concentration was determined using formula O. D. X 471.70 μmoles/Protein.

Estimation of Rifampicin (Krishna *et al*, 1984):

All the rats from different groups were killed on day 31st of the treatment. Different organs salivary glands, oesophagus, stomach, duodenum, ileum, pancreas, liver, thyroid, kidney and testes were removed and immediately weighed and homogenized in Teflon homogeniser in phosphate buffer pH 7.2 and used for rifampicin extraction. The organic phase was used to read the rifampicin content. Rifampicin levels were estimated spectrophotometrically using butanol-heptane mixture (4:1). The absorbance of organic extract was read at wavelength 482 nm against the blank and the concentrations of the rifampicin in the unknown samples were obtained from a standard graph.

Faecal matter was collected daily during 8 - 30 to 9 - 00 am and spread on glass petri dishes and dried at 37.5 ^{0}C. Rifampicin was extracted and estimated from dried faecal matter as per Sunahara and Nakagawa (1972). Rifampicin content was

determined in salivary glands, oesophagus, stomach, duodenum, ileum, liver, pancreas, kidney, thyroid and testis. The tissues were dissected out from the experimental animals and weighed. The tissues were homogenized in phosphate buffer and used for the extraction of rifampicin as described above and concentrations of rifampicin were calculated using calibration curve of rifampicin prepared by dissolving known amount of rifampicin in butenol and hexane (4:1) mixture. For the calibration curve different concentrations of rifampicin were used.

Rifampicin levels were estimated spectrophotometrically using butanol-heptane mixture (4:1). The absorbance of organic extract was read at wavelength 482 nm against the blank and the concentrations of the rifampicin in the unknown samples were obtained from a standard graph.

Estimation of total proteins :

Total proteins were estimated using Folin-Ciocalteu phenol reagent as described by Lowry *et al* (1951).

Preparation of chemicals-

I. Lowry's A –

2 % sodium carbonate in 0.1 N NaOH.

II. Lowry's B –

0.5 % copper sulphate in 1 % sodium tartarate at pH 7.0.

III. Lowry's C –

50 ml Lowry's A + 1 ml Lowry's B. Prepare this reagent fresh just prior to use.

IV. Folin-Ciocalteu phenol reagent-

Sodium tungstate [$Na_2WO_4.2H_2O$]	100 g
Sodium molybdate [$Na_2MoO_4.2H_2O$]	25 g
Distilled water	700 ml
Conc. HCl	100 ml
Phosphoric acid	50 ml

Reflux the above mixture for 10 hr in glass apparatus

Then add to this mixture

Lithium sulphate	150 g
Distilled water	50 ml

Add five drops of bromine water and boil this to remove excess bromine. Cool and dilute to 1.0 N.

Assay of proteins

Solution	Blank ml	Sample ml
H_2O	1.5	1.3
Homogenate	0.0	0.2
Lowry's C	3.0	3.0
	Wait for 15 minutes	
Folin-Ciocalteu reagent	0.5	0.5

Wait for 60 minute and take readings at 660 nm.

Convert optical density to protein value from the standard graph plotted as optical density verses concentration of standard albumin. Calculate the amount of protein per g tissue.

Statistical analysis :

The results of various experiments were analysed statistically. The statistical calculations were carried out according to the method given in the textbook of statistics (Agarwaal, 1990). The following abbreviations and formulae were used:

X – independent variable

N – number of observations

Formulae –

2. $\bar{x}$ = arithmatic mean of x

3. $$\bar{x} = \frac{x}{n}$$

SD – standard deviation

$$SD = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\quad}\,\overline{n - 2}}$$

$$SE = \frac{SD}{\sqrt{n}}$$

SE – Standard error for a mean of n variables.

Students t – test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{E_1 - E_2}$$

Where $\bar{x}_1 - \bar{x}_2$ is mean difference of two variables

and

$(SE_1 - SE_2)$ = Error of the mean difference

$$= \sqrt{\frac{SD_1^2}{n_1} + \frac{SD_1^2}{n_2}}$$

df = Degree of freedom for a differences of two means of n_1 and n_2 variables.

$$df = n_1 + n_2 - 2$$

Significance – The probability *p* of obtained 't' value at least as calculated for a given number of degree of freedom is given in Fischer's table (Fischer, 1938). The *p* values are signified according to the following conventions-

P > 0.05 the difference is said to be insignificant

P < 0.05 the difference is said to be almost significant

P < 0.02 the difference is said to be significant

P < 0.001 the difference is said to be highly significant