C H A P T E R - II

MATERIAL AND METHODS

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As discussed in the first chapter of Introduction; male albino rats were used for experimental work, CCl_4 hepatic toxicity was used as the hepatotoxic model. Mandur bhasma on Ayurvedic preparation was the drug used for the testing hepatocurative property.

Mandur bhasma was prepared in the laboratory according to the procedure described in Rasa Ratna Samucchaya (Sharma, 1981) with some modifications.

Mandur Bhasma

Mandur is the ore of iron in the form of ferric oxide obtained from the 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 Garcinea indica for 24 hrs. and washed thoroughly with water. One kg of powdered mandur was titurated with the five litres of juice of leaves of Aloe vera and dried. The bhasma was prepared by giving a Gajaputa.

The quality test for Mandur bhasma was conducted as described in the ayurvedic and sidha tests.

The quality test :

Take

1 gm	-	Mandur Bhasma
200 gm	-	Ghee
200 mg	-	Jauggary
200 mg	-	Abrus precatorius - seeds
200 mg	-	Honey,
200 mg	-	Borax.

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

Animals used for experiments :

Albino rats originally derived from Haffkine strain were bred and maintained in departmental animal house providing rat feed (Lipton India Ltd. Calcutta, India) and water ad libitum. Male albino rats of 110-120 days old weighing 150 to 175 gms were used for experiments.

Selection of CCl_4 dose and Mandur Bhasma dose :

To study the curative effects CCl_4 was given earlier so

that hepatic injury can be induced. Since we have already tested the dose (3 ml/kg body weight) that induce the necrosis in liver along with liquid paraffin (1 ml/kg body weight) as vehicle. (Devarshi et al., 1986). The hepatoprotective dose of mandur bhasma (10 mg/kg body weight) which resulted in active regeneration of liver (Devarshi et al., 1986). In Ayurvedic therapy Mandur Bhasma is prescribed to take orally and therefore treatment of this drug was given with food.

In the present project same dose of CCl_4 was used to induce necrosis. Same dose of Mandur Bhasma was used to test curative effects.

In preliminary experimental work it was observed that on 15th day after Mandur Bhasma treatment to CCl_4 + liquid paraffin / treated rats showed total histological recovery of liver.

In the interest to study the transitory histophysiological observations in liver; it was decided to select half the time of total recovery i.e. 7 days. Therefore in present thesis interval of 7 days is used to study the histological recovery of the liver.

In reference to hepatic recovery only the histological data is considered and therefore in reference to recovering effects in liver the histological alterations in kidney are considered.

Experimental Protocol :(Table - 1)

The male albino rats of above described specifications were maintained in eight groups. Each group contained six animals

The groups were as described below :

Group I - Normal rat.

These rats were normal not treated with any drug and were served as control.

All the treatments were given at 9.00 a.m. before feeding.

Group II - CCl_4 treated rats

CCl_4 (3 ml/kg body weight/day) was administered subcutaneously to these rats from day 1 to day 11. From day 12 to day 18 CCl_4 treatment was ceased.

Group III - Liquid treated rats

Liquid paraffin (heavy fraction of IP grade) was injected subcutaneously from day 1 to day 11 (1 ml/kg body weight/day). The treatment was terminated on day 12 onwards. No treatment was given from day 12 to day 18.

Group IV - CCl_4 + Liquid Paraffin treated rats

(Rats of these groups indicated the hepatic necrosis on day 11 of treatment). To these rats CCl_4 (3 ml/kg body weight/day)

Table 1 - Experimental Protocol

Group	CCl_4 Treatment	Liquid Paraffin treatment	Mandur bhasma Treatment	Day of sacrification
Group I (Normal)	-	-	-	Day 19
Group II (CCl_4)	day 1 to 11 daily (3 ml/kg body wt/day	-	-	Day 19
Group III (Liquid paraffin)	- -	day 1 to 11 daily (1 ml/kg body wt/day)	-	Day 19
Group IV (CCl_4 + Liquid paraffin)	dayl 1 to 11 daily (3 ml/kg body wt/day)	day 1 to 11 daily (1 ml/kg body wt/day)	- -	Day 19
Group V (Mandur bhasma)	- -	- -	day 12 to 18 daily (10 mg/kg body wt/day)	Day 19
Group VI (CCl_4+Mandur bhasma)	day 1 to 11 daily (3 ml/kg body wt/day)	-	day 12 to 18 daily 10 mg/kg body wt/day	Day 19
Group VII (Liquid Paraffin + Mandur bhasma)	-	day 1 to 11 (1 ml/kg body wt./day)	day 12 to 18 daily (10 mg/kg body wt./day)	Day 19
Group VIII Liquid paraffin + Mandur bhasma + CCl_4	day 1 to 11 daily (3 ml/kg body wt./dy)	Day 1 to 11 (1 ml/kg body wt./day)	day 12 to 18 daily (10 mg/kg body wt./day)	Day 19

+ liquid paraffin (1 ml/kg body weight/day) was given from day 1 to day 11. The treatment was stopped from day 12 and no treatment was given from day 12 to day 18.

Group V Mandur bhasma treated rats.

The rats were not given any treatment from day 1 to day 11. From day 12 to day 18 they were given Mandur bhasma (10 mg/kg body weight/day) orally with food.

Group VI - CCl_4 + Mandur bhasma treated rats.

These rats were given CCl_4 (3 ml/kg body weight/day subcutaneously) from day 1 to 11. This treatment was terminated on day 12. From day 12 to day 18 Mandur bhasma (10 mg/kg body weight/day orally) was given to the same rats with food.

Group VII - Liquid Paraffin + Mandur bhasma treated rats.

From day 1 to day 11 liquid paraffin (1 ml/kg body weight/day subcutaneously) was administered. This treatment was ceased from day 12 onwards. From day 12 to day 18 mandur bhasma (10 mg/kg body weight/day orally) was given to same rats with food.

Group VIII - CCl_4 + Liquid Paraffin + Mandur bhasma treated rats.

Six rats from this group were subcutaneously administered CCl_4 (3 ml/kg body weight/day) + liquid paraffin (1 ml/kg body

weight/day) from day 1 to day 11. This treatment was terminated from day 12 onwards. These rats were treated with mandur bhasma (10 mg/kg body weight/day; orally) from day 12 to day 18.

On day 19 all the animals from Group I to VIII were killed by giving ether anaesthesia.

Liver and kidneys were removed immediately in proper pieces (0.5 cm X 0.5 cm) and transferred to Bouin's fluid, 10 % Buffered formalin, 10 % formalin for histological studies.

Preparation of Fixatives :

A. Bouin's Fluid: (Thompson, 1966)

Solutions - All day reagents should be weighed out on a torsion balance.

I - Saturated Aqueous Picric Acid

Picric acid [$(HO\text{-}C_6H_2)(NO_2)_3$]	
Nitrophenol U.S.P.	20 gm
Distilled water	1000 ml

Dissolve the picric acid in the water with the acid of heat; allow to cool and decant the supernatant fluid.

II - Bouin's stock solution

Solution I	750 ml
Formaldehyde solution (36 to 40 %) HCHO by assay)	250 ml

III - Bouin's Fixative fluid

Solution II

Glacial acetic acid (CH_3COOH)	95 ml
(99.7 % acetic acid)	5 ml

Add the acetic acid to the stock Bouins' solution just before the fixative is to be used. The pH of this fluid should be approximately 1.5 to 1.7.

B. 10 % Buffered Formalin :

Solution - All dry reagents should be weighed out on a torsion balance.

Diabasic anhydrous sodium phosphate.

(Na_2HPO_4)	6.5 gm
Monobasic acid potassium phosphate KH_2PO_4	4 gm.
Formaldehyde solution (36 to 40 %	
HCHO by assay)	100 ml
Distilled water	900 ml

pH of this solution should be between 6.8 to 7.0.

C. 10 % Formalin :

Solutions - All dry reagents should be weighed out on a torosion balance.

Formaldehyde solution (36 to 40 %	
HCHO by assay)	100 ml
Distilled water	900 ml

Mix and Use

The tissues were processed for paraffin sectioning as described by Pears (1969) Thompson (1966). Sections of 5 µ were cut on spencor microtome and were used for staining.

The sections were stained with double staining method - Eosin - hematoxylin. Triple staining method of Acid-Fuschin-Aniline blue was also used.

I - Eosin - hematoxylin staining (Thompson, 1969)

Harris' Alum hematoxylin was used for staining.

Preparation :

A - Hematoxylin (National Aniline No. 564)	5 gm
B - Ethyl alcohol absolute	50 ml
C - Aluminium ammonium sulfate $(Al_2(SO_4)_2SO_4 - 24H_2O)$	100 gm
D - Distilled water	1000 ml
E - mercuric oxide (Red)	2.5 gm

Dissolve the aluminium ammonium sulfate in the water with heating in 2000 ml capacity corning beaker, Dissolve hematoxylin in alcohol. Mix the two solutions and bring it to rapid boiling. Remove from the heat and add the mercuric oxide (red) very slowly in a small amount at a time shake well and take the container in cold water for cooling.

B. Eosin (Thompson,1966).

Eosin Y (National Aniline, No. 516) - 2 gm

Absolute alcohol 200 ml

II Mallory's Aniline Blue Stain (Thompson, 1966).

A - 1 % alcoholic Iodine.

B - 5 % sodium Thiosulfate

C - 0.5 % Aqueous Acid Fuschin (National Aniline No-402

D - Aniline Blue - Orange G.

a. Aniline Blue (water soluble National Aniline No. 426) 0.5 gm

b. Orange G (National Aniline No.696) 2.0 gm

c. Phosphotungstic acid 1.0 gm

d. Distilled water 100 ml.

Mix all the above chemicals and use.

Staining Methods : 1 (Eosin + Hematoxylin)

i) Dewax for 1/2 hr in xylene.

ii) Hydrate through descending alcohol grades.

iii) Wash with distilled water.

iv) Treat with Harris' hematoxylin for 8 minutess at room temperature.

v) Wash with distilled water respectively.

vi) Dehydrate throgh alcohol grades.

vii) Bring slides to absolute alcohol.

viii) Stain with Eosin for 1 minute.

xi) Wash with absolute alcohol.

x) Clear in xylene.

xi) Mount in DPX.

2. (Mallory's Aniline Blue Stain)

i) Dewax the slides for 1/2 hr.

ii) Bring slides to absolute alcohol.

iii) Treat with 1 % alcoholic iodine for 5 minutes.

iv) Wash with water.

v) Treat with 5 % sodium thiosulfate for 5 minutes.

vi) Wash with running water (20 minutes).

vii) Treat with 0.5 % Acid fuschin - for 5 minutes.

viii) Treat with Aniline blue - Orange G for 50 to 60 minutes.

ix) Wash with 95 % alcohol.

x) Wash with alcohol.

xi) Clear in xylene.

xii) Mount in DPX.

Using above techniques the histological architecture of liver and kidney was studied.

In addition to histological structure lipid peroxidation was studied in liver and kidney as described below as per Ohkawa and Ohishi (1978).

Reagent required :

TCA - TBA - HCl reagent.

15 % W/V Trichloro Acetic Acid.

0.375 % W/V Thiobarbituric Acid.

0.25 N Hydrochloric Acid.

This solution may be heated to dissolve Thiobarbituric Acid.

Method

1 ml - Biological Sample (2 mg of Liver/kidney/ml. in 0.25 M sucrose).

2 ml - TCA - TBA - HCl reagent

Mix thoroughly.

Heat for 15' in a water bath adjusted at $80^{o}C$.

Cool - Centrifuge to remove precipitate.

Read at 535 nm adjusting zero with blank.

Blank -

10 ml - Distilled water/0.25 M sucrose

2 ml - TCA - TBA - HCl

Heat in water bath at $80c^{o}$ for 15'.

Malon dialdehyde concentration of the sample is calculated using an extinction coefficient of $1.56 \times 10^{5-1}$ M cm^{-1}.

Since the main damage of liver is by increased lipid peroxidation due to liberated CCl_3^+ free radical in present project lipid peroxidation is also assayed.

Results of various kinetic studies were analysed statistically.

The statistical calculations had been peroformed according to routine formulae given in text book of statistics used for biological works (Fisher, 1936; Aggarwal, 1990).

The following abbreviations and formulae were used :

x - independent variable

n - number of observations

Formulae -

1. $\bar{x}$ = Arithmetic mean of X

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

2. SD - standard deviation

$$S.D. = \sqrt{\frac{(\bar{x}-x)^2}{n-1}}$$

3. SE - Standard error for a mean of n variables

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

4. Student's t-test

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

where $\bar{x}_1 - \bar{x}_2$ = Mean difference of two variables.

O $SE_1 - SE_2$ = Error of the mean difference

$$= \sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}$$

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

$df = n_1 + n_2 - 2$

6. Significance - The probability p of obtaining 't' value at least as the calculated for a given number of df, is found in fisher's tables (Fisher, 1936). 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 = <0.01 The difference is said to be significant.

P = < 0.001 The difference is said to be highly significant.

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