Preparation and Physicochemical Characterization of Ingredients of Indian Traditional Medicine, *Mahamrutyunjaya Rasa*.

Pallavi Lavhale M.Pharm. PhD.
Associate Professor and Head (Pharmacognosy Department)
RamEesh Institute of Vocational and Technical Education
Greater Noida UP India

Introduction

- Ayurvedic medicine originated in India more than 2000 years ago.
- It makes use of herbs, metals and minerals for curative effects.
- In former days *Vaidyas* (Ayurvedic practitioner) dispensed medication to the patients in the fresh form.
- Quality control of traditional medicines is important in assuring the therapeutic efficacy, safety and to rationalize their use in the health care.
- No information is documented about the likely impact of changes in the manufacture techniques.

- Ayurveda uses *shodhana* technique to render them fit for human consumption.

- It is believed that the *shodhana* process converts the metal into its specially desired chemical compound which eliminates the toxicity of the metal and has the necessary medicinal benefits.

- Since these preparations are sustaining themselves since centuries in clinical use, therefore one cannot exclude its use.

- The purification procedures are well documented but none of the methods have been studied in detail to determine the structural and chemical changes taking place in the ingredients.

- It is essential requirement to discuss the non-toxicity and therapeutic value of such formulations.

Mahamrutyunjaya Rasa, **is a formulation containing a mixture of herbs and minerals used as a cardio-active formulation prescribed in the *A.S.S. Rasa Rasayana Prakarana*.**

Ingredients		Quantity
Visa	*Aconitum ferox*	1Part
Brihati	*Solanum indicum*	1Part
Pippali Kana	*Piper longum*	1Part
Marica	*Piper nigrum*	1Part
Gandhaka	Sulphur	1Part
Tankana	Sodium metaborate	1Part
Hingula	Cinnabar	2Part
Jambira	Lemon juice	Q.S

The adult dose of the formulation is 125 mg twice a day.

Aconite Root

Aconitum ferox also known as *Aconitum virorum* is a species of monkshood, in the family Ranunculaceae.

Chemical Constituents: Norditerpenoid alkaloids based on a hexacyclic C_{19} skeleton, and those based on the C_{20} skeletons. The principal active ingredients are alkaloids, including aconitine, mesaconitine and hypaconitine.

Pharmacological activities: The cardiac action of the alkaloids is due to their effect on the voltage-sensitive sodium channels of the cell membranes of the myocardium.

The alkaloids are toxic with a very narrow safety range, because they easily induce ventricular tachycardia and fibrillation even at therapeutic dose levels.

Solanum Roots

A number of ayurvedic formulations contain Solanum indicum Like: *Dasamularista , Mahamrutyunjaya rasa, Brhatyadi kvatha, Brhati mula siddha ghrta*

Chemical constituents: Alkaloids solanine and solanidine. Disogenin, lanosterol, sitosterol, solasonnine, solamargine and solasidine.

Pharmacological Activities: Anti-Hypertensive, Immunostimulant and Cytotoxic.

Piper Species

The two species of piper used in the present formulation were *Piper nigrum* and *Piper longum*. Piperine is the active principle of both the drugs.

Cinnabar

Mercury is used in many ayurvedic formulations as red sulphide(HgS) of mercury as drug of longetivity. Cinnabar is insoluble, has very low bioavailability and its long-term use is major cause of mercury intoxication.

Sulphur

Sulphur is used in ayurvedic formulations widely. It increases bile, acts as a laxative and alterative. Improperly purified sulphur medicine if consumed over a long period causes toxic effects like dyspepsia, flatulence.

Sodium metaborate

In ayurveda, it is given internally in acidity of the stomach, amenorrhoea and to promote uterine pains during labour. Consumption over a long duration of time may cause gastrointestinal distress including nausea and diarrhea.

Study Objectives

The present study was planned in the following manner:

- Preparation of laboratory formulation.
- Standardization of the procedure for formulation preparation.
- Biological Standardization of the formulations

❑ Toxicity studies

❑ *In vitro* cell viability studies

❑ Studies on the cardio-protective activity using Isoproterenol induced myocardial infarction in rats.

Selection of Formulations

Mahamrutyunjaya rasa was prepared in the laboratory as per the standard text and named as FORM1. Two marketed formulations were selected for the study, FORM2 and FORM3.

Preparation of the Ayurvedic formulation as per the standard text

The ingredients had to be purified as per the traditional procedure. 1 part each of processed *Aconitum ferox*, *Solanum indicum*, *Piper nigrum* and *Piper longum* powdered and sieved through 85 mesh sieve. It was then mixed with 1 part purified sulphur, 1 part purified sodium metaborate and 2 parts of purified cinnabar.

S. Govind, S. Brahma Shankar, S. Ambika dutt shastra, *Bhaishjaya Ratnavali, Chowkhambh Sanskrita sthan, Varanasi, 549*.

Standardization of formulation procedure

- It is believed that the *shodhana* process converts the poisonous raw material into its specially desired chemical compound which eliminates the toxicity of the substance and has the necessary medicinal benefits.

- *Aconitum ferox*, Sulphur, Cinnabar and Sodium metaborate have to be processed before internal administration as per the ayurvedic literature.

- The structural and chemical changes taking place in the ingredients are not reported, which is essential requirement to ascertain their non-toxicity and therapeutic value.

- **An attempt was made to derive certain standard data which may form the basis of quality control of the raw materials present in the formulation.**

- **The standard methods as per traditional text were followed and the physicochemical changes were investigated by collecting samples at different steps of purification. The samples were analyzed using various techniques, viz. FTIR, XRD, DSC and HPTLC.**

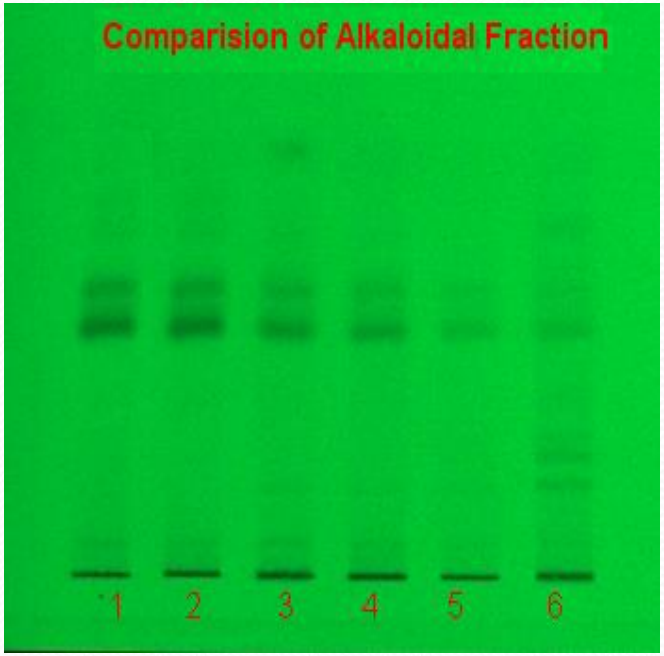
Aconite alkaloids

The aconite roots were processed and samples for in process quality check were collected. The alkaloidal fractions were subjected to HPTLC and IR studies. The purification procedure was as follows:

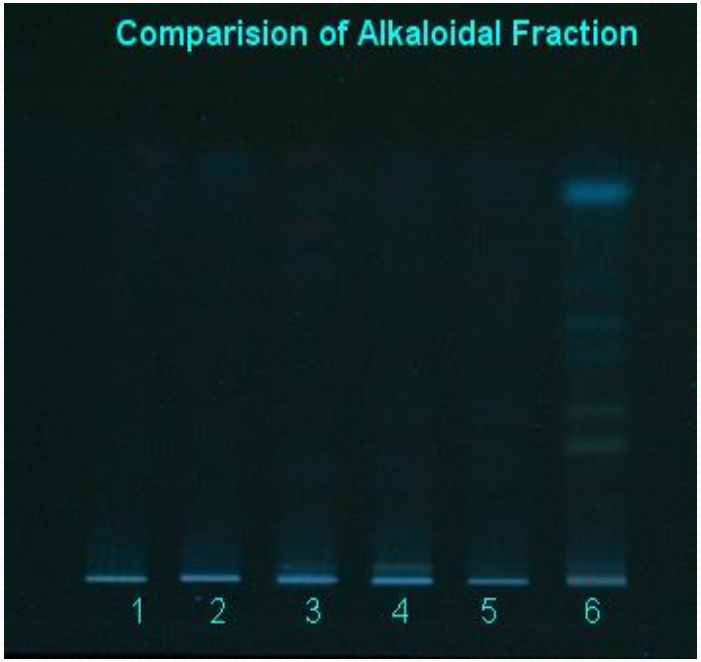
- Crude Drug (A-1)
- Washed with water (A– 2)
- Soaked in cow urine for 24 hrs. (A – 3)
- Soaked in cow urine for 48 hrs. (A- 4)
- Washed with water and boiled with milk. (A -5)
- Washed with water and dried. (A-6)

HPTLC Studies

- Stationary phase: Pre-coated Silica gel HPTLC plates. F-254nm.
- Mobile Phase: Toluene: Ethyl Acetate: Diethyl amine (7:2:1 v/v)
- Detection: The plate was scanned 254 nm and 366 nm.



(a)



(b)

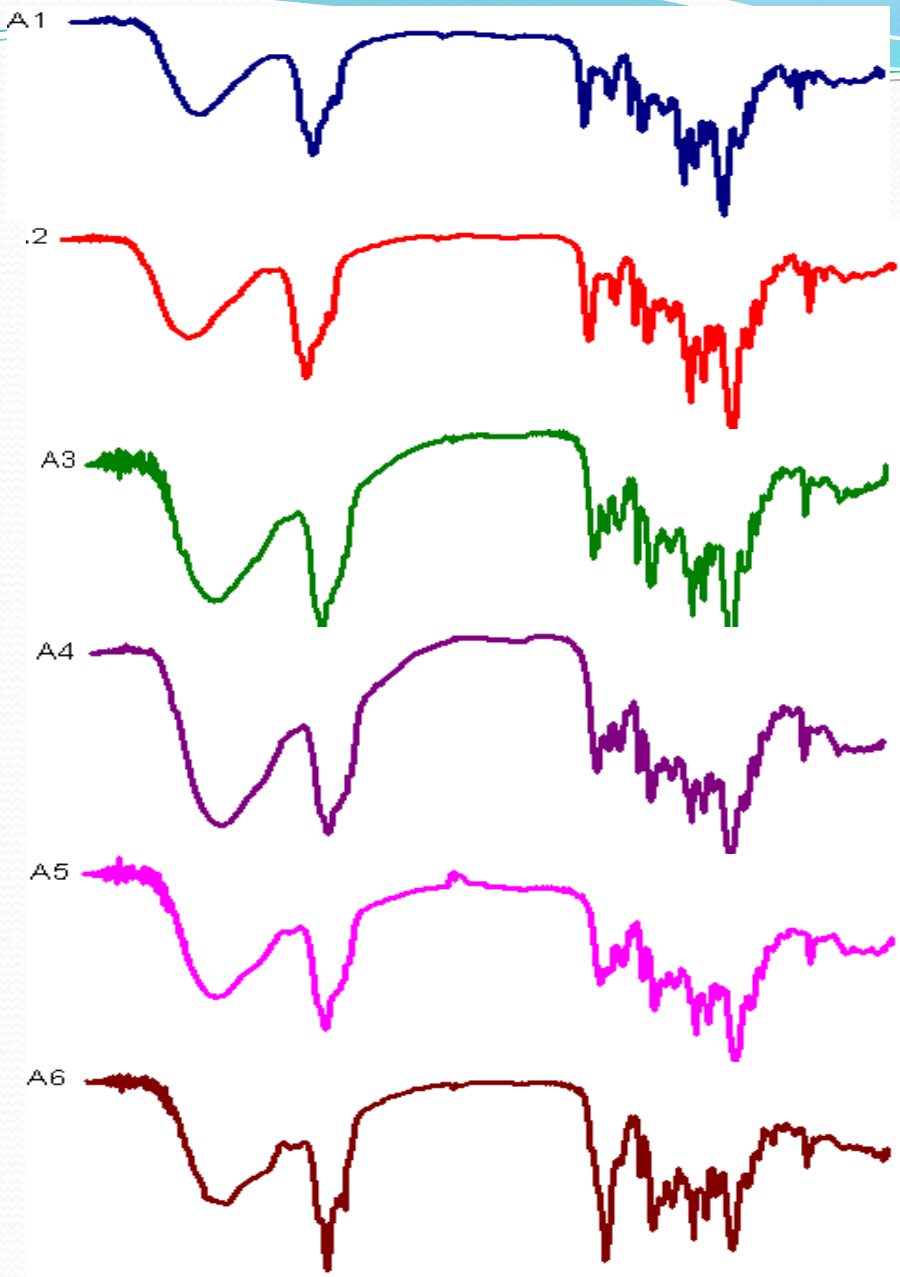
1-Crude Drug (A-1), 2-Washed with water (A– 2) , 3-Soaked in cow urine for 24 hrs. (A – 3), 4-Soaked in cow urine for 48 hrs. (A- 4), 5- Washed with water and boiled with milk. (A -5) , 6-Washed with water and dried. (A-6)

Sample	Concentration µg per spot	Peak	Rf value	Height	Area	% Height	% Area
A-1	100	AG-1	0.42	210.3	6295.1	**44.77**	**41.28**
	100	AG-2	0.48	149.7	5618.0	**31.86**	**38.58**
A-2	100	AG-1	0.42	160.6	6092.4	41.12	40.21
	100	AG-2	0.48	144.3	5294.0	29.26	32.79
A-3	100	AG-1	0.42	148.9	5748.1	30.00	35.40
	100	AG-2	0.48	135.3	5070.5	26.25	33.28
A-4	100	AG-1	0.42	129.9	5309.5	24.88	28.74
	100	AG-2	0.48	126.8	5142.2	24.01	32.98
A-5	100	AG-1	0.42	71.5	2048.1	20.30	25.22
	100	AG-2	0.48	79.7	2296.7	21.04	29.14
A-6	100	AG-1	0.42	114.6	4974.6	**16.39**	**22.06**
	100	AG-2	0.48	100.3	5325.6	**14.89**	**24.72**

The tracks from 1-6 show that there was a gradual chemical degradation with decrease in the concentration of two alkaloids, and increase in concentration of other alkaloids.

FTIR Studies

%Transmittance

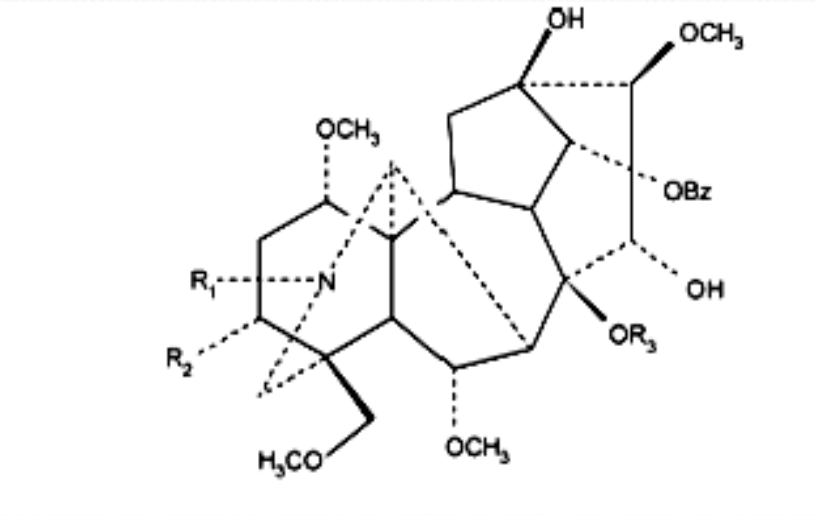


IR Spectra of samples of Aconitum extracts

A1	A2	A3	A4	A5	A6
Peaks, cm^{-1}					
3435	3436	3417	3420	3422	3372,2958
2929	2930	2930	2928	2929	2924
2819	2821	-------	-------	2851	2852
1720	**1717**	**1713,1659**	**1710,1655**	**1708,1670**	**1676**
1601	1601	1601	1601	1602	1597
1514	1515	1515	1515	1514	1514
1462	1463, 1419	1463,1455	1461	1459	1455
1368	1369	1372	1370	1376	1378
1294	**1295**	-------	--------	--------	------
1271	1271	1271	1271	1271	1271
1224	1224	1223	1224	1223	1223
1177	1177	1177	1177	1177	1176
1096	1097	1101	1101	1101	1101
1024	1024	1037	1037	1036	1036
985	985, 803	984, 878	984,935, 878	985,927, 874,	915
765	765	765	764	765	765
-------	689	---------	---------	611	612

1720	C=O stretching of esters
1676	C=O stretching of Ketones.
1294	C-O or O-H stretch

It is observed from the IR spectra that during the purification there was loss of an ester group (peak at 1720 cm^{-1}), which may have been replaced with a Keto-group (1676 cm^{-1}).



Name	R1	R2	R3
Benzoylaconine	C_2H_5	OH	H
Aconitine	C_2H_5	OH	Acetyl
Benzoylmesaconine	CH_3	OH	H
Mesaconitine	CH_3	OH	Acetyl
Benzoylhypaconine	CH_3	H	H
Hypaconitine	CH_3	H	Acetyl

Chemical structures of benzoylaconine, aconitine, benzoylmesaconine, mesaconitine, benzoylhypaconine and hypaconitine.

The **HPTLC and IR studies**, show that there were significant differences in alkaloid contents between the processed and unprocessed aconite roots.

The purification procedure may thus be responsible for the chemical degradation of the di-ester alkaloids.

It has been reported in earlier studies that the diester alkaloids of Aconitum alkaloids are prone to hydrolysis.

Toxicological studies have demonstrated that the toxicity of diester alkaloids (aconitine, hypaconitine and mesaconitine) is almost the same with LD_{50} values for mice per injection about 0.15 mg/kg body weight, while the hydrolyzed monoester alkaloids (benzoylaconine, benzoylhypaconine and benzoylmesaconine) show much lower toxicity.

The processing of the aconitum alkaloids thus involve the hydrolysis of acetyl group which is depicted in the IR spectra.

However, due to the presence of a number of alkaloids in the alkaloid fraction, the exact chemical changes cannot be predicted.

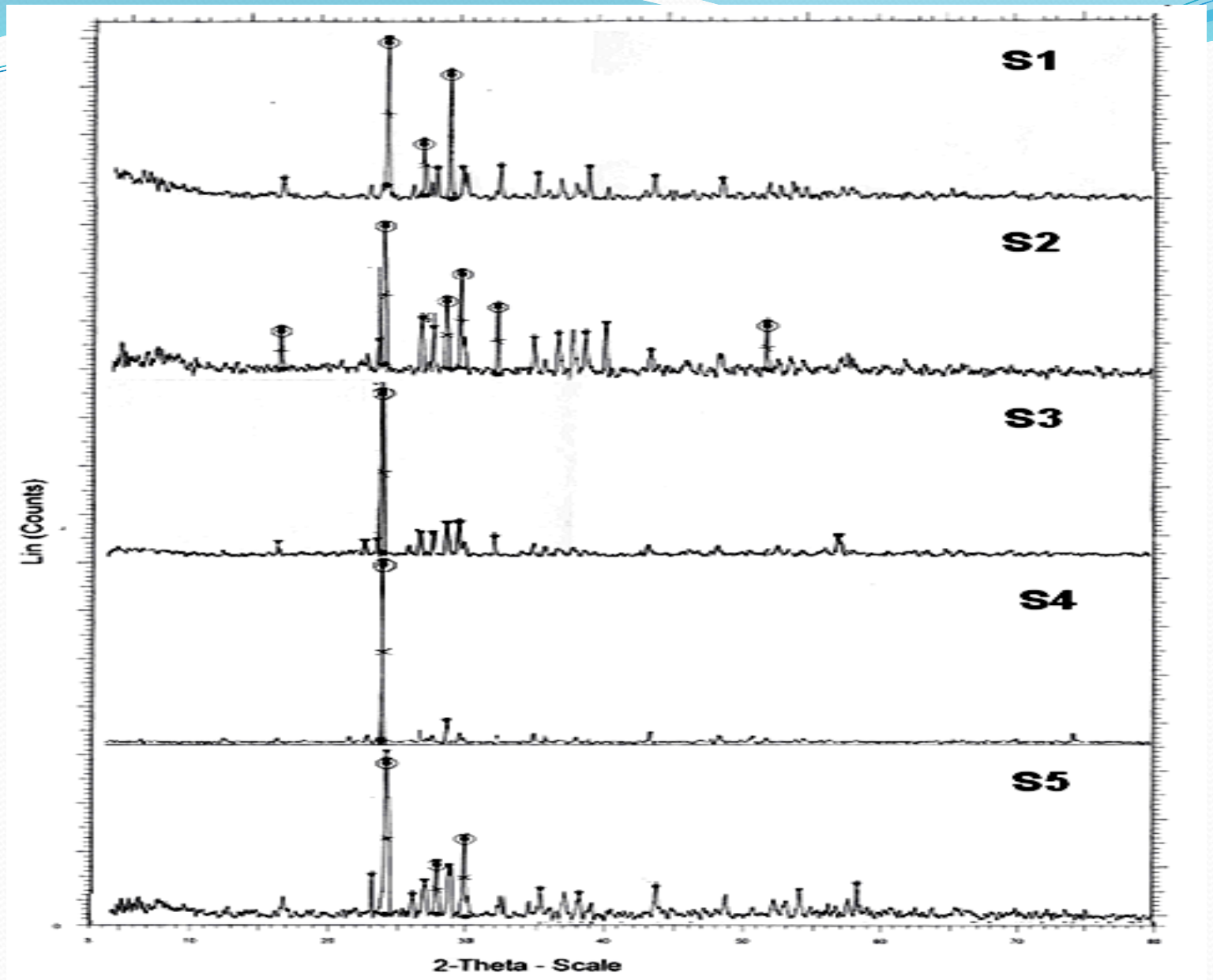
The IR spectras may be used as reference for comparison of the raw materials as a quality control parameter. The changes in the processed roots can be checked using the standard IR spectras.

Sulphur

Sulphur was purified as follows:

- Crude Sulphur (S– 1)
- Sulphur was treated with cow ghee (S – 2)
- Mixture was heated in Iron vessel and melted (S – 3)
- Mixture was washed with cow milk (S – 4)
- The mixture was washed with water and powdered. (S – 5)

The samples were analyzed using XRD and DSC.



X-ray diffraction spectra of Sulphur samples.

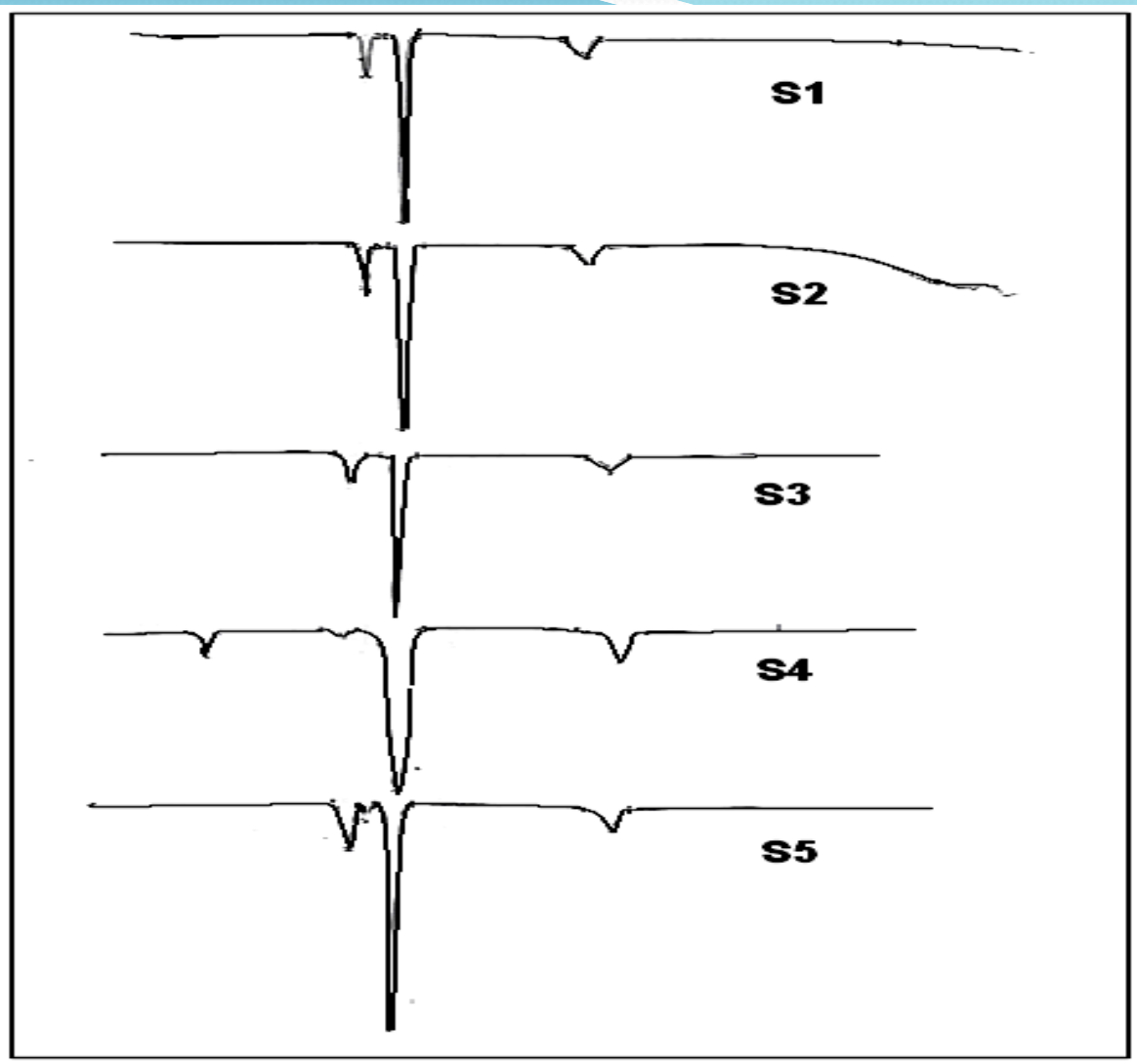
d- spacing values of Sulphur

d-Spacing values	
Orthorhombic Sulphur d(Å)	**S-1 d(Å)**
3.85 (a=23.083)	3.825 (a=23.235)
3.219 (a= 27.690)	3.198 (a=27.871)
3.447 (a=25.826)	3.422 (a= 26.016)
Orthorhombic Sulphur d(Å)	**S-2 d(Å)**
3.85 (a=23.083)	3.844 (a=23.114)
3.113 (a=28.653)	3.102 (a=28.757)
3.219 (a=27.690)	3.215 (a=27.721)
3.447 (a=25.826)	3.443 (a=25.851)
3.336 (a=26.315)	3.331 (a=26.743)
2.848 (a=31.385)	2.838 (a= 31.499)

Orthorhombic Sulphur d(Å)	**S-3 d(Å)**
3.85 (a=23.083)	3.834 (a=23.178)
3.084 (a=28.653)	3.099 (a=28.77)
3.336 (a=26.701)	3.321 (a=26.823)
Monoclinic Sulphur d(Å)	**S-4 d(Å)**
3.803 (a=23.372)	3.781 (a=23.505)
3.168 (a=28.145)	3.166 (a=28.156)
Orthorhombic Sulphur d(Å)	**S-5 d(Å)**
3.85 (a=23.083)	3.853 (a=23.174)
3.084 (a= 28.928)	3.084 (a=28.927)
3.336 (a= 26.701)	3.312 (a=26.891)
3.219 (a=27.690)	3.205 (a=27.81)

XRD studies of sulphur

- The d-spacing values of Sulphur samples were compared with coinciding values of the reference standards of various allotropes of Sulphur.
- The pattern of S-1 shows that the raw material *gandhaka* (Sulphur) has a number of peaks coinciding with the reference orthorhombic Sulphur in the Fddd space group .
- The diffraction pattern of S-2 shows that the number of peaks coinciding with orthorhombic Sulphur increase, depicting the increase in that form of Sulphur.
- **The pattern of S-4 shows the presence of only two intense peaks which are found to be present in monoclinic type of Sulphur crystal.**
- **Again in S-5, the peaks coincide with the orthorhombic Sulphur. However, the peaks are even sharper which reflect higher purity of the final product.**



Differential thermograms of Sulphur samples.

DSC data:

Sr. No.	S-1	S-2	S-3	S-4	S-5
1	----------	----------	----------	75.97°C	----------
2	108.60°C	110.93°C	110.04°C	108.49°C	111.55°C
3	----------	----------	----------	----------	115.61°C
4	119.97°C	121.10°C	121.29°C	121.01°C	121.31°C
5	186.83°C	174.17°C	174.73°C	173.96°C	174.74°C

Thermal Studies of Sulphur

- The peaks displayed for S-1, S-2 and S-5 are similar with changes in the number and sharpness of the peaks.

- **A small endothermic peak is observed at 115.61°C in the S-5 sample which may be due to a different type of allotrope of Sulphur formed during the heating procedure.**

Inference

- The sulphur used as the crude raw material is a mixture of α-orthorhombic Sulphur and small amounts of β-sulphur.
- Significant change is observed in DSC of S-4 wherein additional endothermic peak is observed at 76°C which may be due to the removal of the hydrates. The XRD of the sample tends to display the presence of monoclinic sulphur.
- The XRD and DSC patterns of S-5, display that the structure of S-4 reverts back to the S_8 orthorhombic sulphur.
- The unwanted components are also reduced which may be observed by the sharpness of the peaks in the X-ray diffraction pattern of S-5.
- **Thus, from the above study it can be concluded that the processing of sulphur brings about purification, reducing the toxic nature of sulphur.**
- **By impregnating with organic material, like ghee, sulphur is made homologous to the tissue cells and their toxicity is reduced and acceptability to the cell is increased.**

Cinnabar:

The purification of cinnabar was performed as follows:

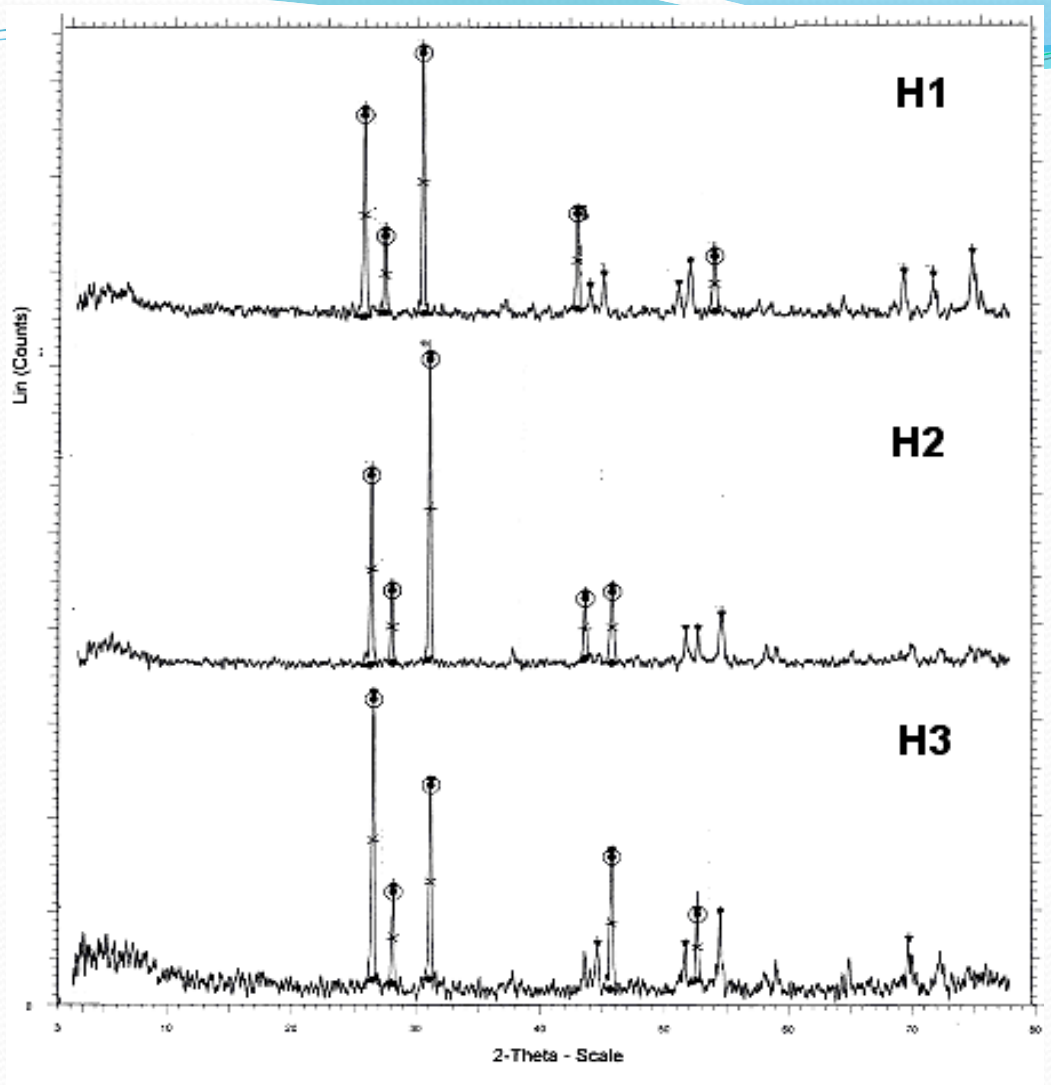
- Crude cinnabar (H- 1)
- Cinnabar was treated with lemon juice-three times (H -2)
- Cinnabar was treated with lemon juice-seven times (H -3).

The samples were analyzed using XRD.

XRD studies on Cinnabar

The d-spacing values of H-3 matched with the reference data showing high purity of cinnabar in the **trigonal trapezohedral cystalline form.**

HgS d(Å)	H-1 d(Å)	H-2 d(A)	H-3 d(A)
3.36 (a= 26.507)	3.337 (a= 26.363)	3.370 (a=26.422)	3.358 (a= 26.517)
3.181 (a= 28.218)	3.181 (a= 28.024)	3.171 (a=28.109)	3.162 (a= 28.199)
2.85 (a= 31.362)	2.877 (a= 31.060)	2.874 (a=31.089)	2.862 (a= 31.224)
2.06 (a= 43.917)	2.078 (a= 43.50)	2.030 (a= 44.593)	2.071 (a= 43.665)
1.67 (a= 54.937)	1.682 (a= 54.508)	1.682 (a= 54.490)	1.677 (a= 54.677)



X-ray diffraction spectra of Cinnabar samples.

Inference

- When compared with reference values of trigonal trapezohedral cystalline form of cinnabar, it is clear that the final product is of very pure nature.

- Further, on treatment with lemon juice the organic material make cinnabar more homologous to the body for its assimilation and the therapeutic effect.

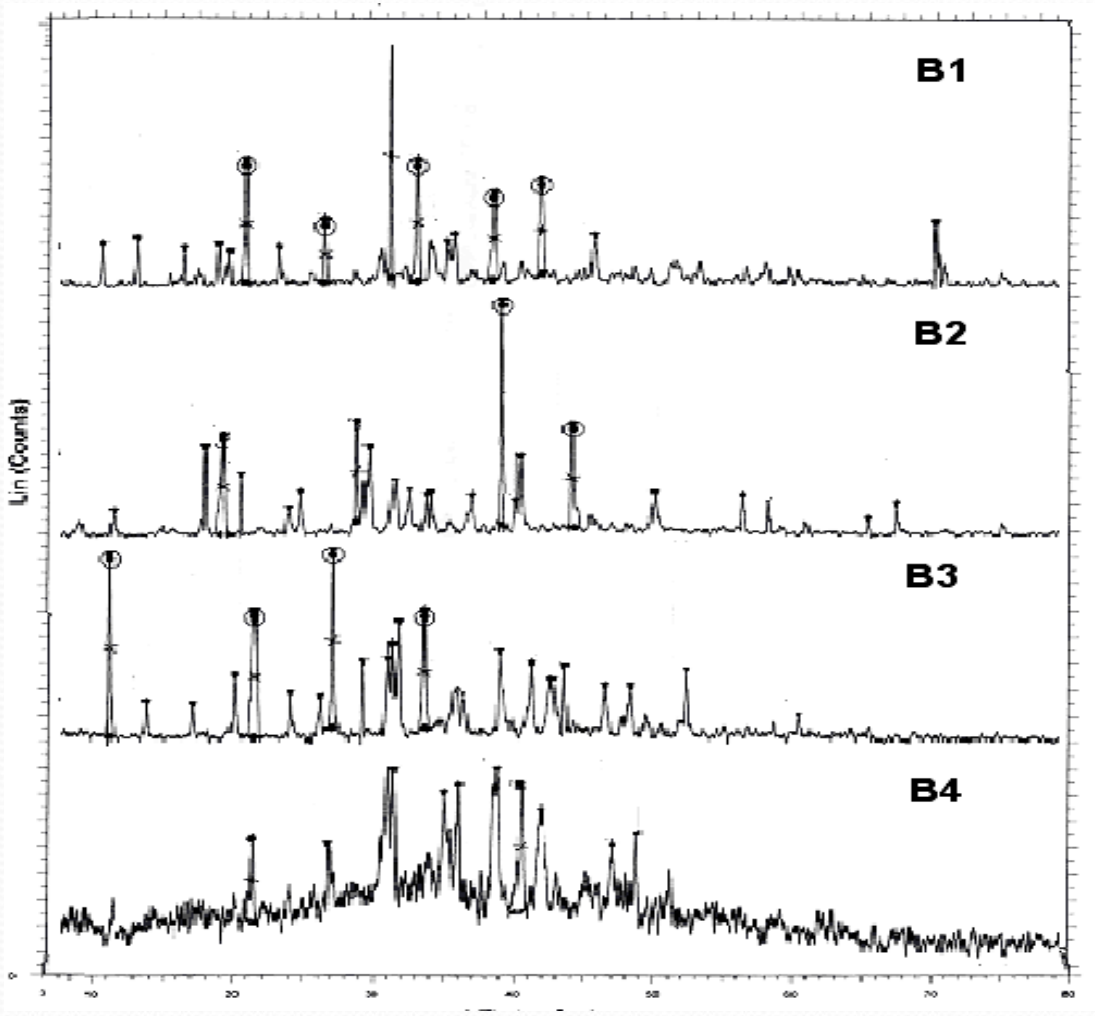
Sodium metaborate

The purification of sodium metaborate was performed as follows:

- Crude sodium metaborate (B – 1)
- Heat sodium metaborate for 10 minutes (B – 2)
- Heat sodium metaborate for 20 minutes (B-3)
- Remove moisture completely (B – 4).

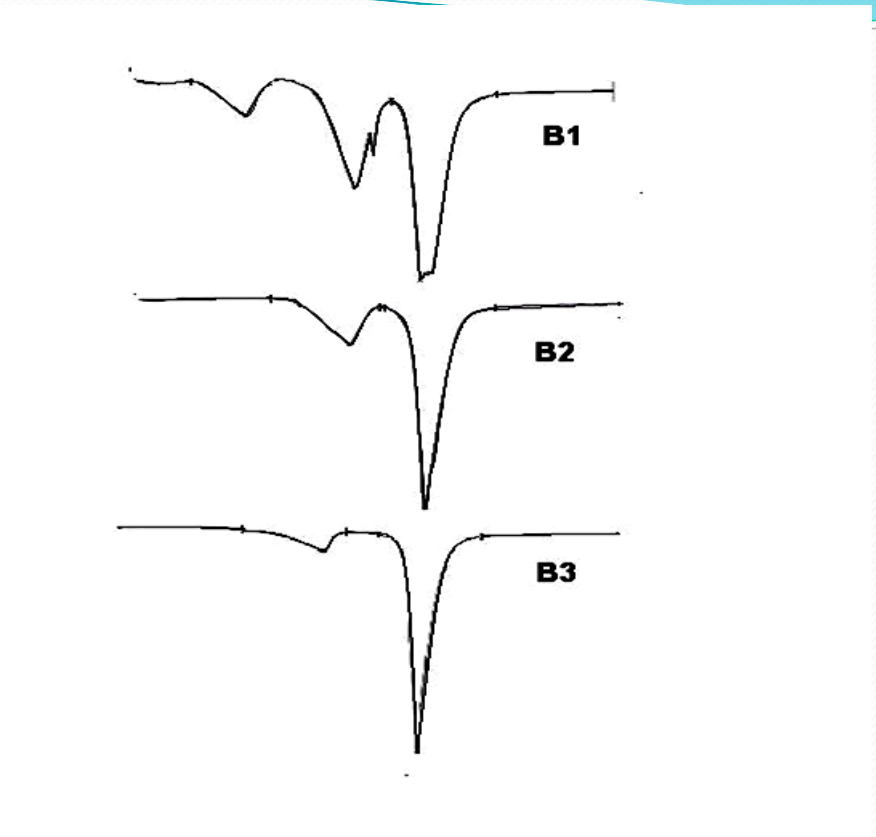
The samples were analyzed using XRD and DSC.

XRD Studies of Sodium metaborate



X-ray diffraction spectra of Sodium metaborate samples.

Thermal studies on Sodium metaborate



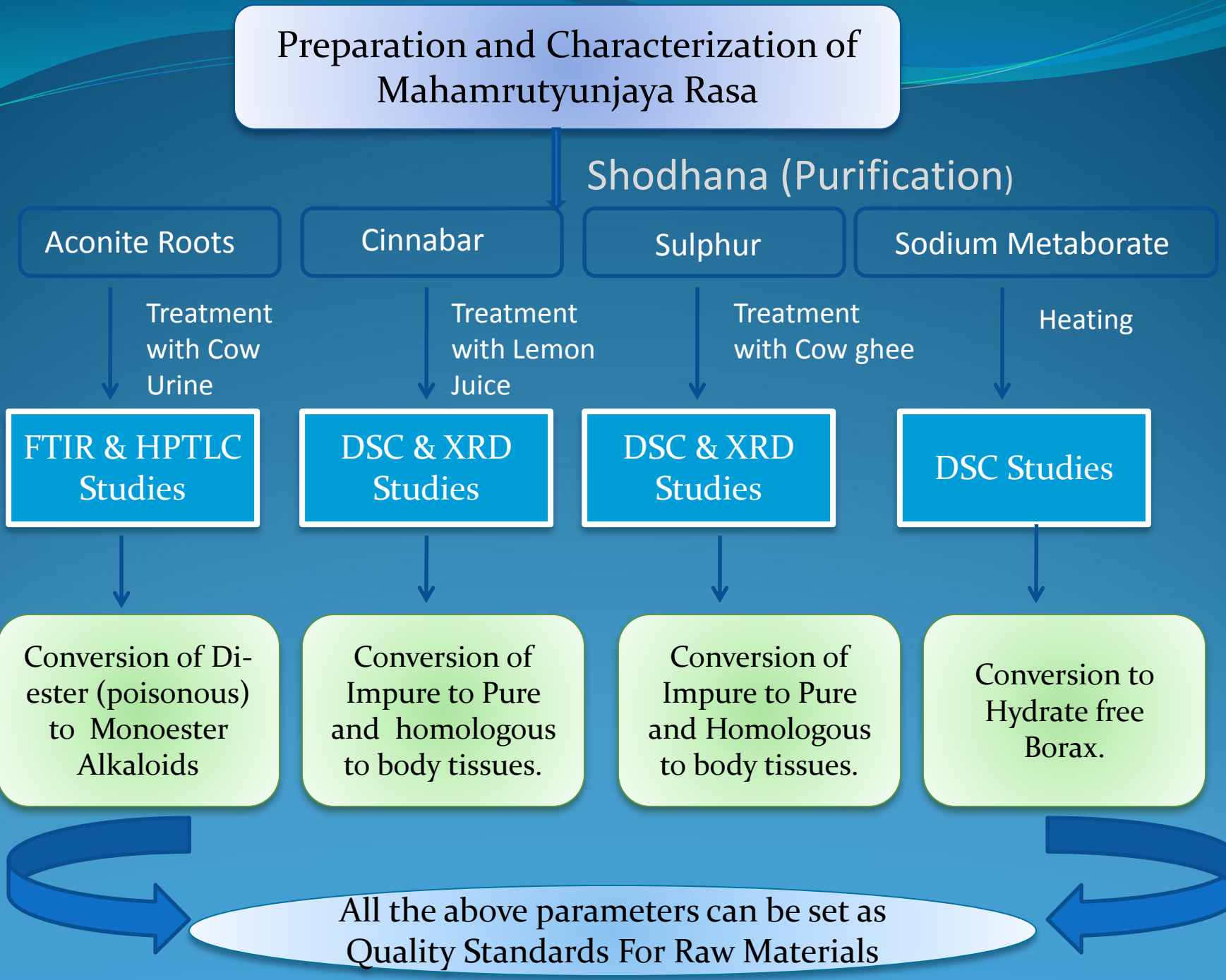
Differential thermograms of Sodium metaborate samples.

Sr. No.	B-1	B-2	B-2
1	74.71ºC	---------	---------
2	114.65 ºC	110.62 ºC	104.22 ºC
3	138.67 ºC	138.39 ºC	137.54 ºC

- The Differential thermograms of B-1 show an extra peak at 74.71 ºC which may be due to the presence of solvates of water, while the peak is missing in the differential thermogram of B-2.

- The purity of the raw material can be ascertained from the DSC study as the sharpness of the endothermic peak at 137ºC increased markedly even when low concentration of sample was analyzed.

- However, the slight shift from 110.62 ºC of B-2 to 104.22 ºC in the final product shows certain changes taking place in the crystal structure which further needs to be studied.


Preparation and Characterization of Mahamrutyunjaya Rasa
Shodhana (Purification)
Aconite Roots
Cinnabar
Sulphur
Sodium Metaborate
Treatment with Cow Urine
Treatment with Lemon Juice
Treatment with Cow ghee
Heating
FTIR & HPTLC Studies
DSC & XRD Studies
DSC & XRD Studies
DSC Studies
Conversion of Di-ester (poisonous) to Monoester Alkaloids
Conversion of Impure to Pure and homologous to body tissues.
Conversion of Impure to Pure and Homologous to body tissues.
Conversion to Hydrate free Borax.
All the above parameters can be set as Quality Standards For Raw Materials

Biological evaluation of Formulations

1. Cell Viability studies
2. Toxicity studies
3. Isoproterenol induced MI in rats

Sample Preparation

The tablets were powdered and suspended in water using 1% tween 80 for the *in vivo* studies.

Animals

Female Balb/c mice weighing between 20-25 g and male albino rats of Wistar strain weighing 250 to 280 g were used for the studies.

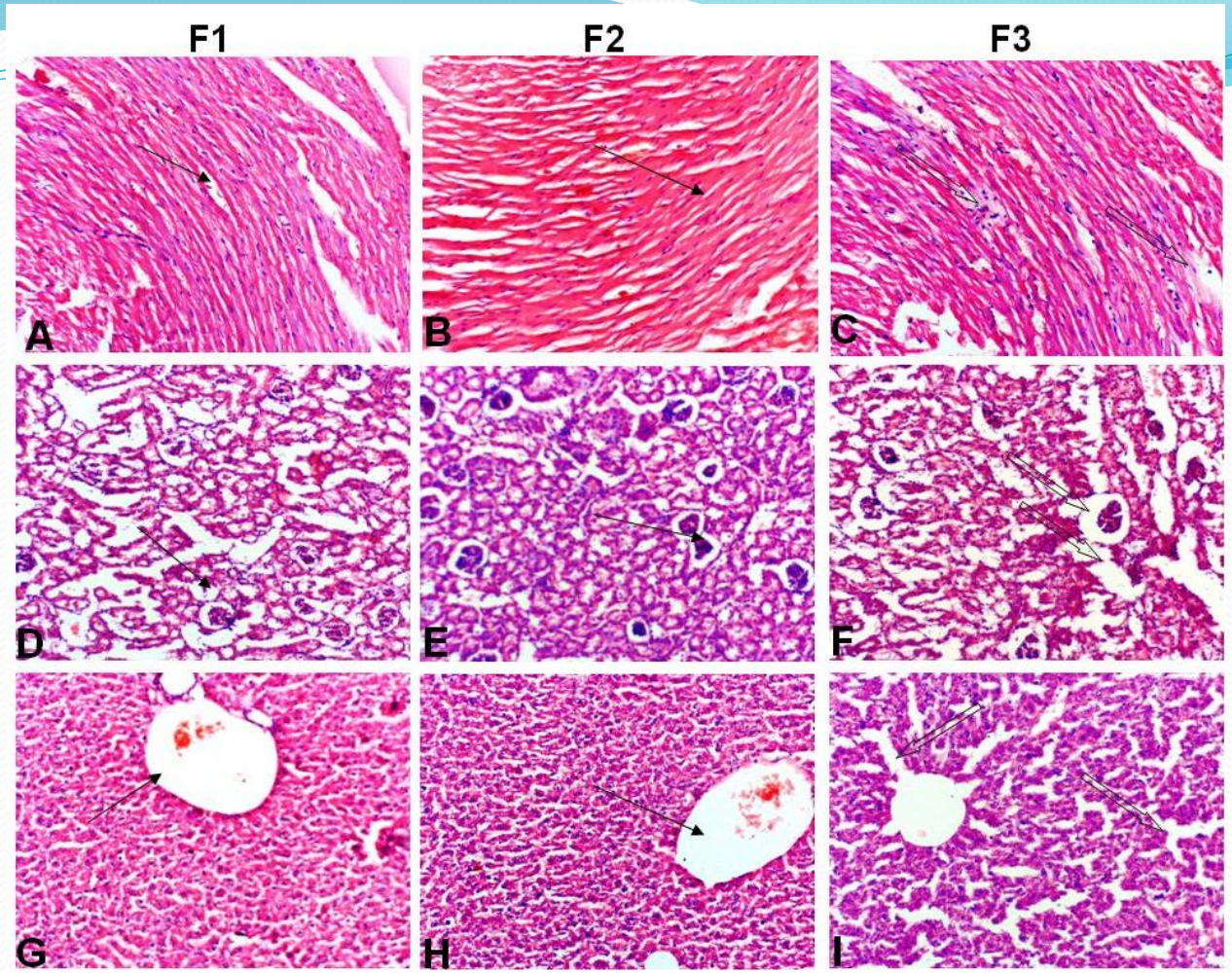
Determination of toxicity of formulations in mice

Toxicity study with single oral doses of formulations was carried out as per the OECD guidelines using mice.

Effect of single oral dose administration of formulations in mice

Mortality was observed in mice administered with single oral dose (2000 mg/kg) of F1, F2 and F3, while with **550 mg/kg dose no mortality was seen**.

OECD. 1998. Test Guideline 408. Single Dose 90-day Oral Toxicity Study in Rodents. In: OECD Guidelines for the Testing of Chemicals. Organization for Economic Cooperation & Development, Paris.



Microscopic images of mice heart, kidney and liver illustrating effect of single oral dose administration of formulation F1, F2 and F3 (Staining: Hemotoxylin and Eosin). Microscopic images of mice **heart** from (A) F1, (B) F2, (C) F3 groups; images of **mice kidney** from (D) F1, (E) F2, (F) F3, images of **mice liver** from (G) F1, (H) F2, (I) F3 groups.

Cell culture and treatment

The H9c2 cell line derived from embryonic rat heart tissue was purchased from America Tissue Type Collection (Manassas, VA; catalog # CRL – 1446). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 µg/ml of streptomycin in 75 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2.

Sample Preparation

Alkaloid fractions of the formulations (FORM1, FORM2 AND FORM3) were prepared and fractions were dried under high vacuum for several hours to remove any traces of solvents used during their processing.

The fractions dissolved in dimethyl sulfoxide (DMSO) were used for *in vitro* studies.

The final concentration of DMSO was not more than 0.2 % during the experiments.

Determination of cell viability

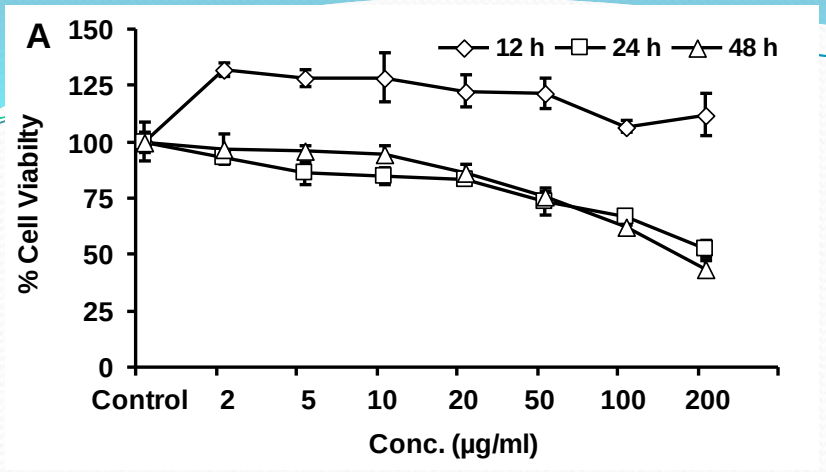
The viability of H9c2 cells after treatment with alkaloidal fractions of formulations F1, F2 and F3 was assayed by the reduction of MTT to formazan.

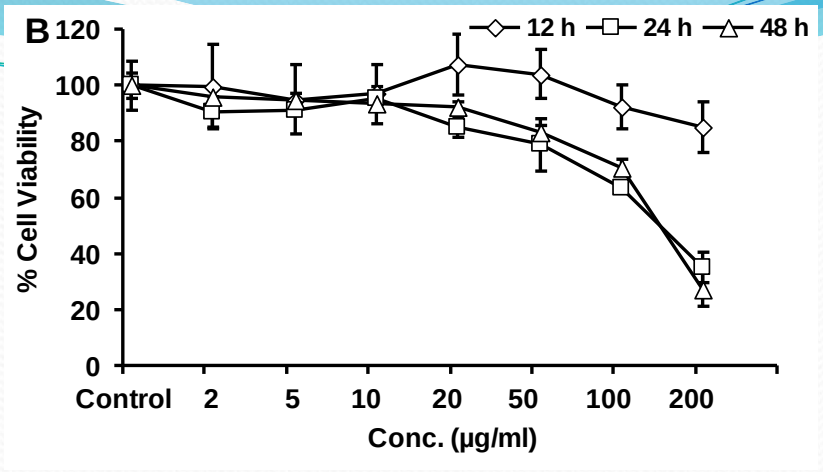
The cells (5×10^3) were cultured in 96- well microtiter plates, and left overnight in an incubator at 37°C with 5% CO_2 before being exposed to different concentrations of formulations.

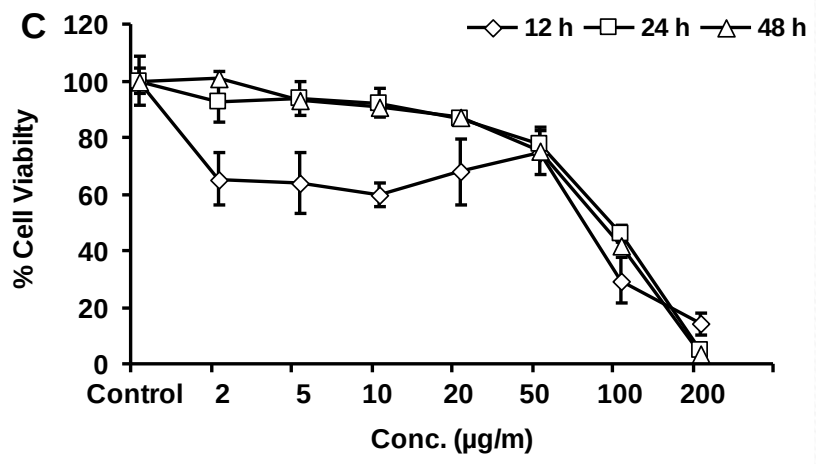
Cells were treated with different concentrations (2, 5, 10, 20, 50, 100 and 200 µg/ml) of FORM1, FORM2 and FORM3 for 12, 24 and 48 h and their viability was determined using 10 µl of 5 mM MTT with additional incubation for 4 h.

Thereafter, the medium was removed, the formazan crystals were dissolved in 100 µl of DMSO and the absorbance was measured at 570 nm using microplate reader (Molecular Devices, Spectra MAX 250). The data of the survival curves were expressed as the percentage of untreated controls .

•Plumb J, Milroy R, Kaye S. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res*. 1989; 49: 4435-4440.







Effect of formulations FORM1 (A), FORM2 (B) and FORM3 (C) on viability of H9c2 cells after 12, 24 and 48 h of treatment. Values are expressed as mean ± SD and are average of three determinations. c

Effect of Formulations on viability of H9c2 cells

Cells incubated with **FORM1 (2 to 200 µg/ml) for 12 h showed increased cell viability**.

Significant decrease in cell viability was observed after 24 and 48 h of treatment with maximum decrease of 47.49% at 24 h and 56.34% at 48 h with 200 µg/ml concentration.

Formulation FORM2 showed **14.85% decrease in cell viability at 200 µg/ml** concentration after 12 h of treatment, while treatment for 24 and 48 h decreased cell viability by 65.08% and 72.66% with 200 µg/ml concentrations respectively.

Treatment with formulation **FORM3 decreased cell viability** by 34.71, 36.08, 40.42, 31.97, 25.35, 70.55, and **85.55% after 12 h** of treatment, similarly 24 h treatment decreased cell viability by 7.68, 6.49, 7.94, 13.63, 22.09, 53.84, and 94.98% at 2, 5, 10, 20, 50, 100 and 200 µg/ml concentrations respectively.

Cell viability was decreased by 7.23, 9.23, 13.01, 24.48, 58.51, and **96.15%** with 5, 10, 20, 50, 100 and 200 µg/ml concentrations respectively, **after 48 h of treatment.**

Determination of protective effect of formulations against ISO-induced myocardial infarction (MI) in rats

Rats were administered with 25 and 50 mg/kg doses of formulations for 15 days and at the end of treatment MI was induced by injecting 25 mg/kg s.c. dose of ISO twice at the interval of 24 h.

Serum levels of cardiac marker enzymes like lactate dehydrogenase (LDH), creatine kinase isoenzymes (CK-MB), glutamate-oxaloacetate transaminase (GOT), and alkaline phosphatase (ALK-P) along with uric acid were determined in order to evaluate the cardiac injury.

Heart was dissected out, washed in ice-cold saline and weighed accurately to determine heart weight/ body weight ratio (HW/BW).

Histopathological studies on heart were carried out in order to assess any changes in cellular architecture. The body weights of the animals were recorded throughout the experimental period.

Hearse DJ, De Leiris J, Loisance J, editors. ***Enzymes in cardiology***. A wiley- Interscience publication, London, John Wiley and sons Ltd, 1979.

Manjula TS, Geetha A, Shyamala Devi CS. Effect of aspirin on isoproterenol induced myocardial infarction- a pilot study. ***Ind J Biochem Biophys 1992;29:378-9.***

Iriama. Uric acid in ischaemic tissues. ***Jikeikai Med J 1987;34:145-68.***

Experimental design

Animal were divided into different groups, containing six animals each.

Group-I: Served as a control, administered orally with 1% solution of tween 80 in water for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 h);

Group-II: administered orally with 1% tween 80 for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 h);

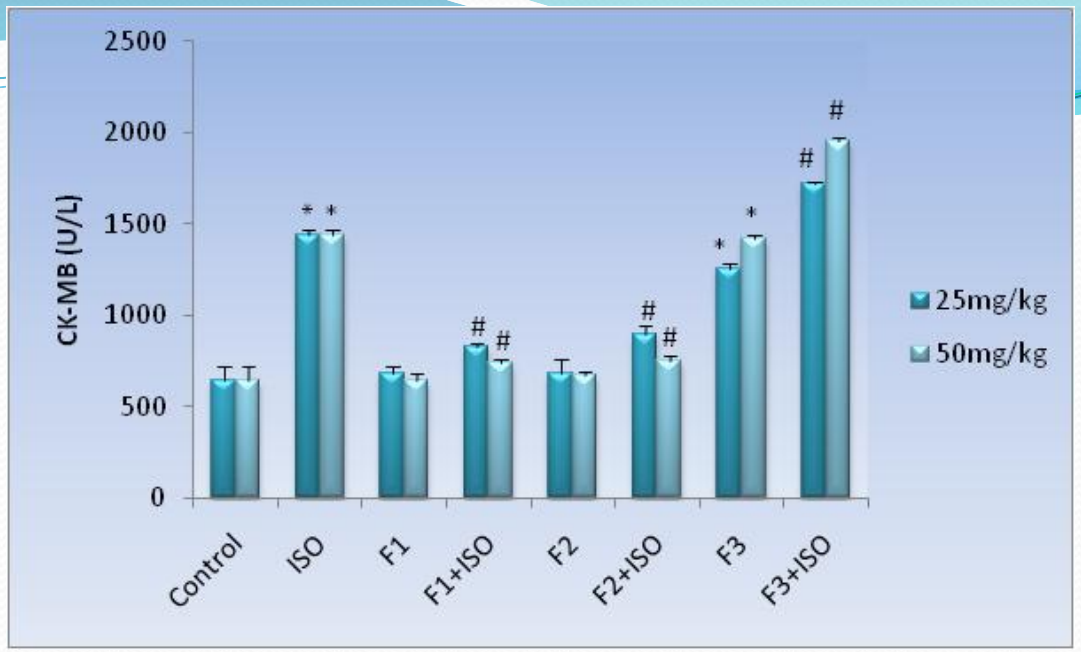
Group-III, -VI, -IX: Served as formulation control, administered orally with 50 mg/kg dose of F1, F2 and F3 respectively for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 h);

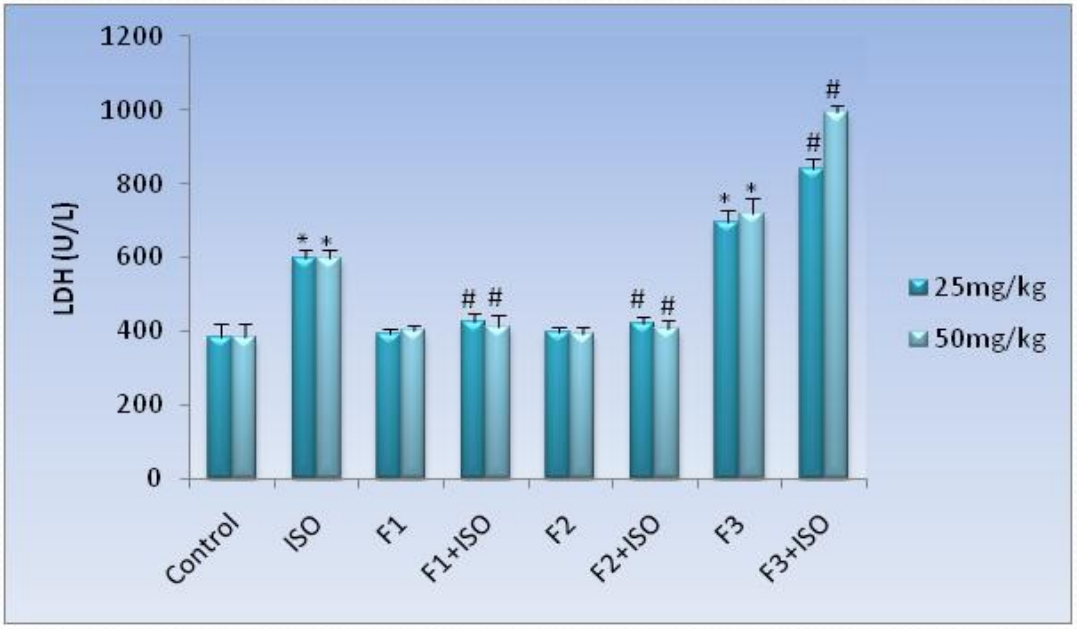
Group-IV, -VII, -X: administered orally with 25 mg/kg dose of FORM1, FORM2 and FORM3 respectively for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 h);

Group-V, -VIII, -XI administered orally with 50 mg/kg dose of FORM1, FORM2 and FORM3 respectively for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 h).

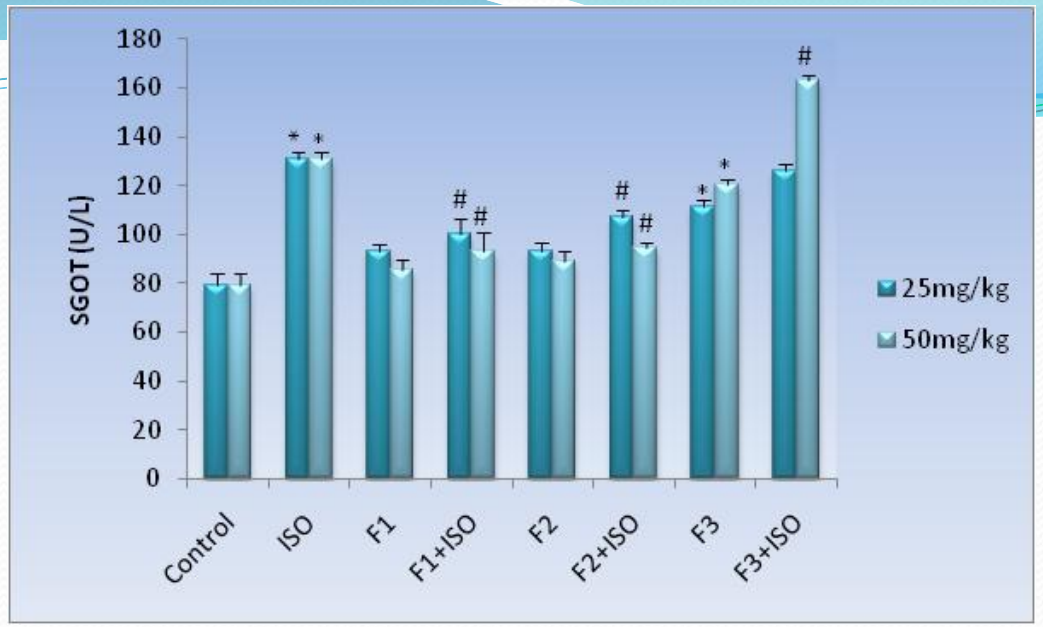
Statistical Analysis of Data

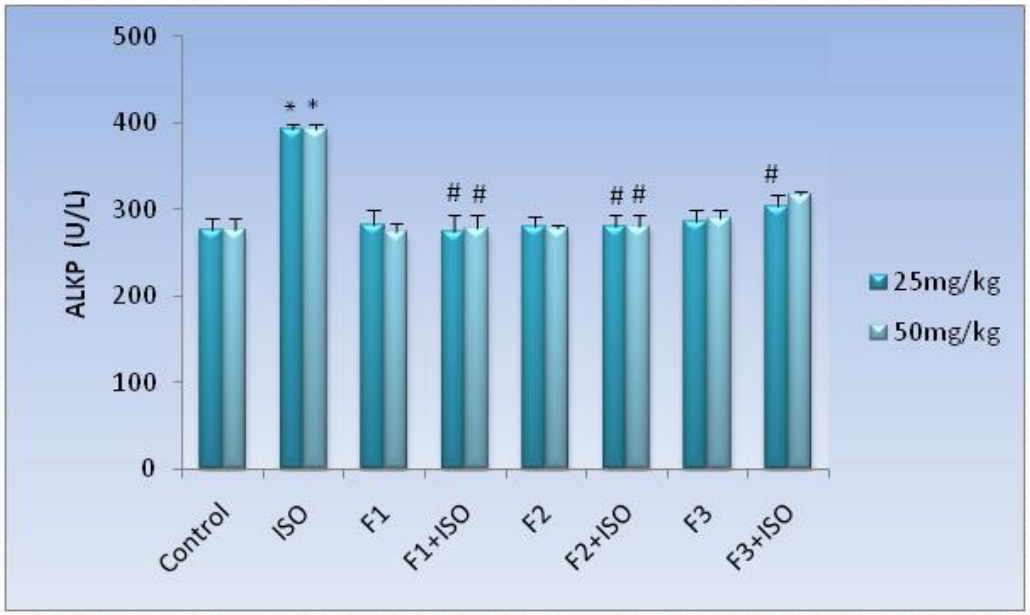
Data are presented as Mean ± S.E.M. The significance of differences was estimated by one-way analysis of variance followed by application of the Dunnett's Multiple Comparisons and Tukey-Kramer Multiple Comparisons test. A *P* value of less than 0.05 was considered to be significant.



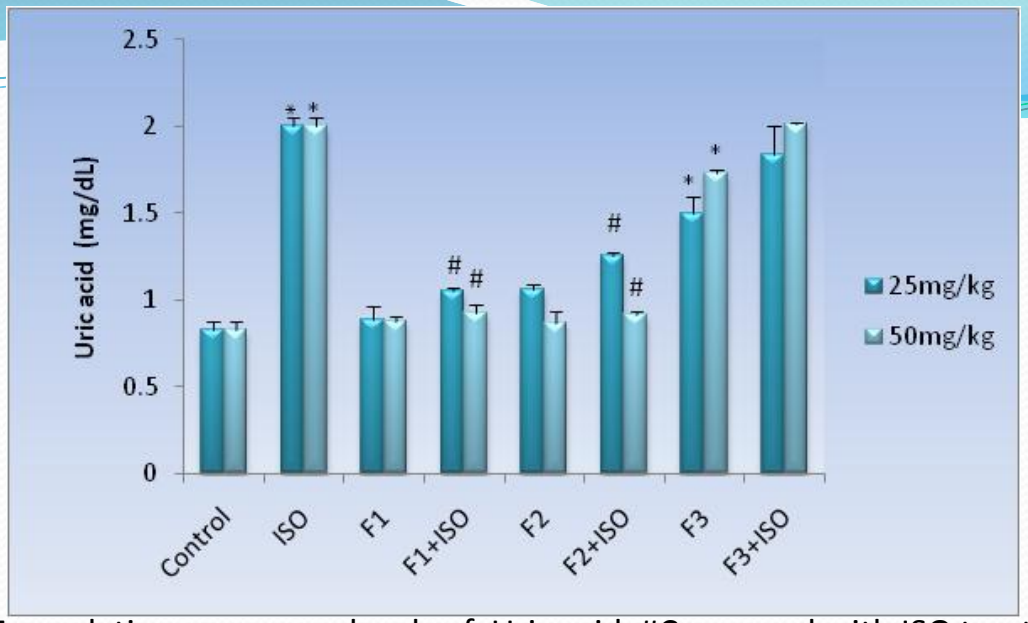


Effect of Formulations on serum levels of CK-MB and LDH. #Compared with ISO treated group, *Compared with saline treated (control) group

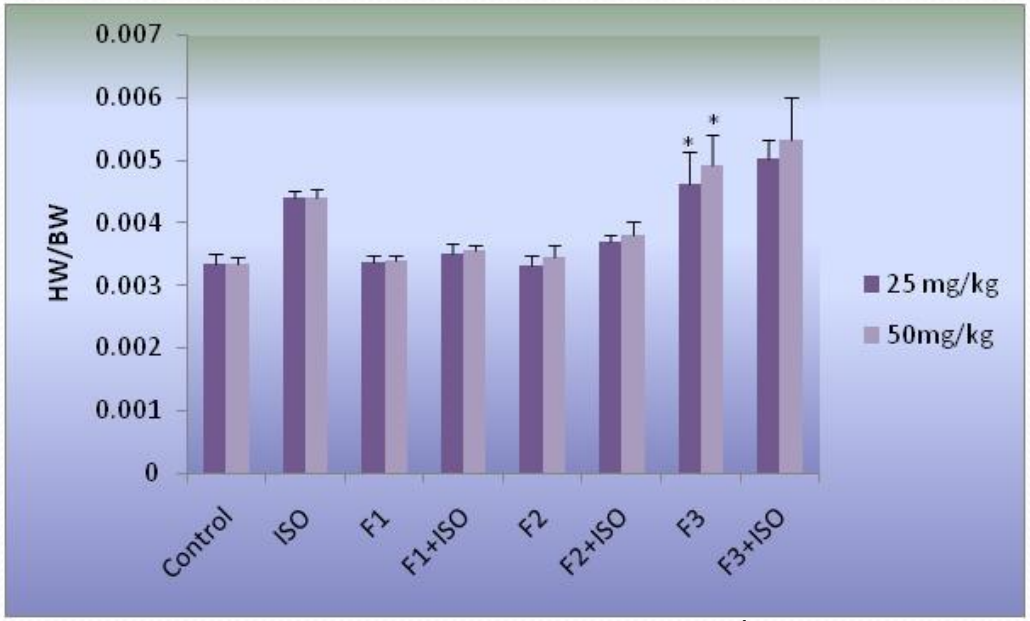




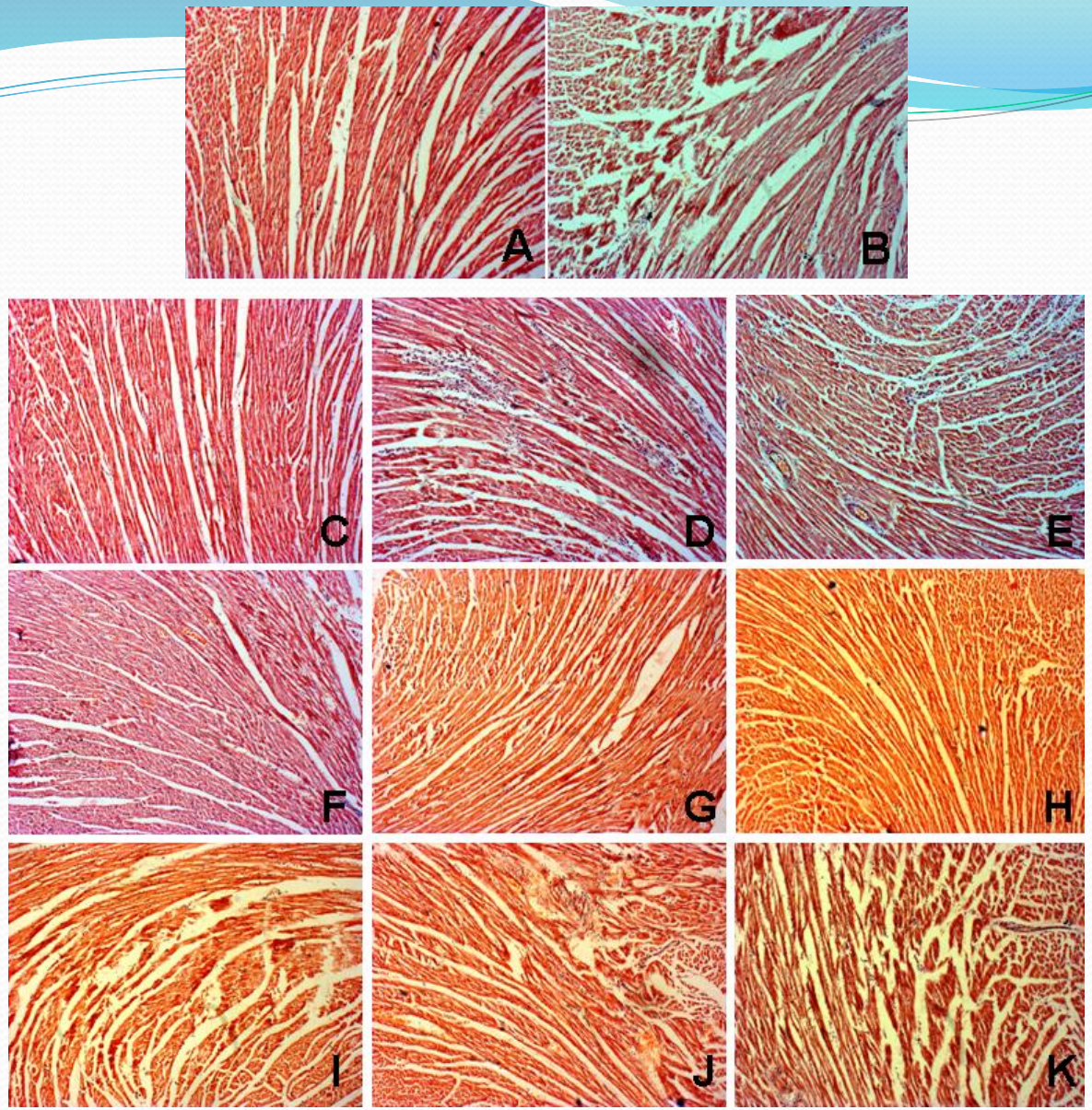
Effect of Formulations on serum levels of GOT and ALKP . #Compared with ISO treated group, *Compared with saline treated (control) group



Effect of Formulations on serum levels of Uric acid. #Compared with ISO treated group, *Compared with saline treated (control) group



Effect of formulations F-1, F-2 and F-3 on Heart weight/body weight ratio (HW/BW). *Compared with control group.



Microscopic images of rat heart illustrating the effect of formulation F-1, F-2 and F-3 in ISO induced MI rats (Staining: Haematoxylin and Eosin). Microscopic images of rat heart from Group-I (A), Group-II (B), Group-III (C), Group-IV (D), Group-V (E), Group-VI (F), Group-VII (G), Group-VIII (H), Group- IX (I), Group-X (J), Group-XI (K).

Myocardial necrosis induced by ISO is probably due to a primary act on the sarcolemmal membrane, followed by stimulation of adenylate cyclase, activation of Ca^{2+} and Na^{+} channels, exaggerated Ca^{2+} inflow, excess of excitation-contraction coupling mechanism, energy consumption and cellular death.

Increased serum levels of CK-MB, LDH, ALK-P, GOT and uric acid are the diagnostic indicators of ISO induced MI.

An increase in the activity of these enzymes in serum is due to their leakage from myocytes as a result of necrosis induced by ISO.

Increase in serum uric acid could be due to excessive degradation of purine nucleotides and proteolysis.

Cardioprotective effects of formulations were assessed by analyzing the levels of serum marker enzymes like GOT, ALK-P, CK-MB, LDH, and uric acid.

Formulations FORM1 and FORM2 treatment prevented the maximum increase of CK-MB, LDH, GOT and ALK-P in the serum during the peak infarction in the tissues.

The cardioprotective action of MHR may be attributed to a number of components present in the formulation.

Aconitum roots containing the diterpenoid alkaloids like *aconitine* can be one of the ingredients recognized for the cardiac action.

Aconitine has been reported to have a positive inotropic effect by elevating the intracellular Ca^{2+} concentration through different sub-cellular mechanisms which lead to an increase in Ca^{2+} transients in myocardial cells.

A significant increase in the serum marker enzymes levels in rats treated with Form3 alone shows its cardiotoxic nature.

The reason for cardiotoxicity of FORM3 may be aggravation of intracellular Ca^{2+} occurring due to the presence of high *aconitine* content.

Conclusion

SR. NO	STANDARDIZATION PARAMETER	FORM1	FORM2	FORM3
1	Acute Toxicity Studies (550 mg/kg b.w., Histopathology)	No Toxicity	No Toxicity	**Minor changes**
2	Cell viabilty Studies (200 µg/ml in 24 hrs.)	Decreased 47.49 %	Decreased 65.08 %	**Decreased 94.98%**
3	Effect on Isoproterenol induced myocardial infarction rats. (25mg/kg and 50 mg/kg b.w)			
4	Levels of serum markers in comparison to negative control	Significant decrease	Significant decrease	**Significant Increase**
5	Heart Weight/Body weight	Significant decrease	Significant decrease	**Significant Increase**
6	Histopathology	Normal Architecture	Normal Architecture	**Damaged Myocardium**

Therefore, from the above study one can conclude that for the preparation of *Mahamrutyunjaya rasa*, standardized traditional methods and raw materials should be used.

The characterization techniques like FTIR, XRD, DSC, HPTLC which have been used in the present studies can be used as a physico-chemical fingerprint for characterization of the raw materials in industry not only to check uniformity but also to ensure that each step is been followed as per the standard text.

A routine use of such scientific techniques will lead to standardization of the product to a certain extent and would definitely help in building confidence in use of such products for medication.

My due acknowledgements are to:

1) Indian Council of Medical Research
2) Sir Ratan Tata Trust
3) University Grants Commission
4) National Centre for Cell Sciences, Pune
5) Anchrom Laboratory, Mumbai
6) Zydus Cadila, Ahmedabad

Our Team

- Dr. Sadhana J. Rajput

  Professor, Pharmacy Department,
  The M.S. University of Baroda,
  Gujarat India

- Dr. Manish S. Lavhale

  Associate Director,
  Pharmazz India Private Limited,
  Greater Noida, UP, India

THANK YOU