Chapter 6.

CONCLUSION
Conclusion

6. CONCLUSION

The present investigations were undertaken to develop certain methods for the standardization of Mahamrutyunjaya rasa in context with the marker as well as mineral components.

Ayurvedic literature records the formula of Mahamrutyunjaya rasa as: 1 part each of powdered purified Aconitum ferox, Solanum indicum, Piper nigrum and Piper longum, with 1 part each of purified sulphur, purified sodium metaborate and 2 parts of purified cinnabar.

The plant materials were studied as per the WHO guidelines. The macroscopical and microscopical examination of the crude intact drugs and powdered drugs was done. The determination of ash values, extractive values, moisture content, volatile matter, pesticide residues, heavy metals, microbial content was also performed. All these parameters showed that the plant material complied with the limits prescribed in the WHO guidelines. The petroleum ether, methanol and aqueous extracts revealed the presence of alkaloids, steroids, carbohydrates, saponins, phenolics and amino acids.

The standardization of procedure for formulation preparation was done and it was concluded that a number of physicochemical changes occur in the raw materials when traditional methods are applied and thus they should be carried out exactly as per the reported procedures.

The physicochemical analysis of aconite alkaloids demonstrated that the processing helped in conversion of the toxic alkaloids to other innocuous alkaloids. The HPTLC studies showed a clear change in the concentration of various alkaloids. The IR studies also depicted the loss of a C=O group and C-O group. This change may be attributed to the loss of ester group from the diester alkaloids. However, due to the presence of a large number of alkaloids in the roots, the exact chemical change cannot be predicted. It has been reported that the monoester alkaloids have similar pharmacological activity with lower toxicity as compared to the diester alkaloids. The toxic effects of the diester alkaloids is presumably due to the induction of arrhythmias by increase in the ectopic impulse formation, making representative triggered activities due to early as well as delayed after-depolarization. The monoester alkaloids have a slow increase in the ectopic impulse formation. It has also been reported that
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the diester alkaloids are prone to hydrolysis and convert to monoester alkaloids easily. Thus, for the quality control of aconite roots as the raw materials, HPTLC finger prints and IR spectra can be used as a standard.

Sulphur was found to be in the orthorhombic crystalline form with small amounts of monoclinic crystalline forms, displayed by two sharp endothermic peaks in the differential thermogram. The physicochemical changes were observed which may be due to the purification 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. Thus, it can be concluded that the processing of sulphur using the traditional methods brings about purification of sulphur, reducing the toxic nature of sulphur. Further, the unwanted components are reduced in the final product. Thus the XRD and DSC data may be used as a standard reference for the quality control of the raw materials.

The physicochemical changes in cinnabar were also observed on purification. The d-spacing values of the final product matched with the reference data showing high purity of cinnabar in the trigonal trapezohedral crystalline form. The purification of cinnabar may be done in order to remove all the extraneous material so that mercury has the desired therapeutic effect without any side effects due to increased bioavailability. Metals, even in their physically and chemically pure form might produce adverse effects because they are inorganic in nature and they are heterogeneous to the body tissue. By impregnating and triturating with organic material, like juices, decoctions of herbs etc., they are made homologous to the tissue cells and their toxicity is reduced and acceptability to the cell is increased. Thus, for the quality control of cinnabar as raw materials, the XRD pattern can be used as a standard.

From the XRD and DSC data of sodium metaborate, it was observed that their was some kind of physicochemical change, however in the absence of reference data of different compounds of boron, the exact changes could not be predicted. The gradual increase in sharpness of the endothermic peak at 137.54°C with loss of other peaks showed that purification of sodium metaborate occurred by shodhana. The differential thermograms also showed an extra peak at 74.71 °C in the raw material which disappeared in the final product which may be due to
the release of hydrates. However, for the quality control of sodium metaborate as raw material, the XRD pattern and DSC thermograms can be used as a standard.

The prepared laboratory formulation (FORM-1), and two proprietary formulations (FORM-2 and FORM-3) were subjected to physical, chemical and biological standardization.

The official tests performed for the marketed formulations showed that the physical properties of both the formulations were different. The disintegration time of FORM-2 was very high as compared to FORM-3. The hardness value was also high for FORM-2. However, the friability test showed higher value in FORM-3 formulation. The deviation in size was not observed in both the formulations. FORM-2 was found to be very hard with slow rate of disintegration which would release the active ingredients slowly, where as FORM-3 was found to be prone to breakage due to high friability. Thus, both the tablets did not comply with all the limits prescribed in IP.

The inorganic content in all the formulations was found to be very high due to presence of a number of minerals. The total ash and acid insoluble ash of FORM-3 was much higher as compared to the two other formulations. The high values signify presence of higher quantities of inorganic contents which may prove to be toxic in nature. Thus, the ash value should be determined to perform the preliminary standardization.

The extractive values of the formulations, FORM1 and FORM2 were found to be higher than that of FORM3 depicting the presence of phytochemicals in the particular solvents in higher quantity. The reason may be higher concentration of mineral components in FORM3.

FORM2 was found to contain organochlorinated pesticides which may be due to the presence of pesticides in the raw materials. Thus, the results signify the importance of the tests for pesticides residues on the raw materials before incorporation in the formulation. It should become mandatory for the manufacturers to perform the tests for pesticides residues.

Heavy metals were found to be absent in all the formulations. It is an important test as heavy metals are toxic in nature. The test for microbial contamination was also found to comply with the prescribed limits. The natural organic
components present in the formulation lead to presence of high microbial contamination. Thus, the test becomes important for the quality control of the formulations.

The qualitative tests showed the presence of carbohydrates, alkaloids, anthranol glycosides, saponins, phenolics, tannins, proteins, amino acids and essential oils in all the formulations. The qualitative tests are important to determine the presence of the active ingredients in the formulations. They form an important standardization parameter for the poly herbal formulations where a large number of ingredients are present.

The chemical standardization of the formulations were performed by developing new analytical methods for the determination of the marker compounds and the minerals in the three formulations.

The chemical marker aconitine was used for the standardization of Aconitum roots present in the formulation. Three methods using HPLC, HPTLC and spectroflourimetric were developed for the determination of aconitine. The methods were found to be applicable to Ayurvedic formulations. The results of analysis performed by the developed methods showed that the content of aconitine in the three formulations varied considerably. It was observed that the content of aconitine varied in the three formulations, with the % RSD values of higher than 10 %, which would significantly influence the quality and safety because it is the target toxic component for the quality control of Mahamrutyunjaya rasa. The concentration of aconitine in FORM-3 was found to be considerably high as compared to FORM-1 and FORM-2 in all three assays. The validation parameters showed that the methods were precise, accurate, sensitive and robust.

A stability indicating HPLC method for aconitine was also developed. Aconitine degraded in the alkaline conditions, in oxidative conditions, in photolytic and in humid conditions. It however degraded slowly in higher concentrations of HCl and at high temperature. The method was validated and found to be precise, accurate, sensitive and robust. The method was also applied to all the three formulations and the assay results were similar to the results obtained by other developed methods. The degradation profile of the marker and formulation was also found to be similar. However, the formulation samples kept for degradation were...
in humid conditions showed significantly higher rate of degradation. The reason being presence of biological components in the formulation. The results of stress testing undertaken revealed that the method is selective and stability indicating.

Solanine was used for the standardization of Solanum roots in *Mahamrutyunjaya rasa*. Two methods using HPLC and HPTLC were developed for the estimation of solanine in *Mahamrutyunjaya rasa*. The solanine content in all the formulations also showed slight variation and the method can be applicable to different formulations containing solanine.

The HPLC- and HPTLC-methods developed showed similar results of the assay performed in the formulations. Thus both the methods can be applied for assay of solanine in *Mahamrutyunjaya rasa*.

A HPTLC method was also developed for the estimation of piperine in *Mahamrutyunjaya rasa*. The content of piperine in the formulations also varied to some extent. The method was found to be precise, accurate, sensitive and robust.

A stability indicating HPLC method was developed for piperine. Piperine degraded in the alkaline and photolytic conditions and in the presence of water. It however degraded slowly in higher concentrations of hydrochloric acid and hydrogen peroxide. The method was validated and found to be precise, accurate, sensitive and robust. The degradation pattern in the formulations was also found to be similar to that of marker and the peak of the marker could be differentiated from the peaks of the other components as well as degradation products. The method was also applied to the formulations. The results of stress testing undertaken revealed that the method is selective and stability indicating.

The HPTLC and stability indicating HPLC methods developed showed similar results of the assay performed in the formulations. Thus both the methods can be applied for assay of piperine *Mahamrutyunjaya rasa*.

Mahamrutyunjaya rasa is a multicomponent preparation with a number of active chemical constituents. In such formulations, the development of methods for the simultaneous estimation of various components is of immense value.
An HPLC method for the simultaneous estimation of Aconitine, Solanine and Piperine in *Mahamrutyunjaya rasa* was also developed and validated. The method was applied for the simultaneous determination of aconitine, solanine and piperine in the Ayurvedic formulations (FORM-1, FORM-2, FORM-3). It was observed that the content of aconitine varied in the three formulations, with the %RSD values of higher than 10 %, which would significantly influence the quality stability because it is the target toxic component for the quality control of the formulation. The content of piperine and solanine also varied in all the formulations to some extent. Method validation data indicated that the method was reliable, reproducible and accurate.

Two HPLC methods for simultaneous estimation of Aconitine and Solanine in combination and Solanine and Piperine in combination in *Mahamrutyunjaya rasa* was also developed and validated. The methods were changed in such a way so as to perform faster analysis as compared to the method developed for simultaneous estimation of the three components. The results of assay obtained were similar to those obtained by above methods.

Various methods for the estimation of the inorganic components in the formulations were also developed. A method for the estimation of sulphur by colorimetry was developed. The results were reproducible with low %RSD values. The method was validated and found to be precise, accurate, reliable, robust and sensitive. Further the step of ashing in sample preparation was not required making the method faster and simple. The results of analysis of the formulations and the recovery study of drug suggested that there is no interference from the other components, which are present in the formulation. The results were close to the probable concentration present in the formulation, thus proving the utility of the method.

Three methods were used for the estimation of boron in *Mahamrutyunjaya rasa*. A reported simple and sensitive spectrofluorimetric method was used for the determination of boron with Alizarin Red S. The method was applied satisfactorily to the determination of boron in the ash of the formulations. The labelled claim was calculated and the results were close to the expected concentration, thus the method was applied successfully for the estimation of boron in herbo-mineral formulation.
A novel voltammetric method for boron determination was developed. The label claim of Boron in the formulation is about 4.3 mg per tablet. The results of the analysis showed that the method was successful in determining the correct concentration of boron. The method was also validated and found to be precise, accurate, reliable, robust and sensitive.

The third method for the estimation of Boron in Mahamrutyunjaya rasa was by ICP-AES. The content found was comparable with the content estimated by the spectrofluorometry method and voltammetric method.

The estimation of Mercury in Mahamrutyunjaya rasa was done using ICP-AES. The results showed a lot of variation in the concentration of mercury in the three formulations. The marketed formulations (FORM2 and FORM3) had very high concentrations of mercury as compared to the formulation prepared in the laboratory (FORM-1).

The analytical results showed the variations in the concentration in the marker components and thus the biological standardization was performed so as to compare the effects of the three formulations in the biological system.

The biological evaluation of Mahamrutyunjaya rasa was performed by acute toxicity studies, invitro cell viability studies and the therapeutic potential was studied using isoproterenol induced myocardial infarction in rats.

Toxicity study was carried out on formulations as per the OECD guidelines in female albino mice. Histopathological examination of the liver, heart and kidney of the mice treated with 550 mg/kg. b.w. of FORM1 and FORM2 appeared normal suggesting lack of any hepatotoxicity, cardiotoxicity or nephrotoxicity.

While mice treated with 550 mg/kg. b.w. of FORM3 showed cardiac injury and hepatic injury on the liver surface to some extent.

Effect of FORM1, FORM2 and FORM3 treatments on viability of rat embryonic cardiac cells (H9c2) were studied in comparison to the untreated (control) cells. The data obtained from the cell viability studies on the formulations indicated that FORM1, showed its effect in concentration and time dependent manner on the viability of H9c2 cells. As both the concentration and time of incubation increased, cell viability was found to be decreased. The data for FORM2 formulation indicates that the formulation had similar effect as that of FORM1.

The cell viability assay results indicated that FORM3 is highly toxic even at low
doses when incubated for 12 h. With the increase in concentration and time of incubation, significant decrease in cell viability was observed. The cell viability studies thus revealed that FORM3 is highly toxic also at low doses.

In in vivo studies, isoproterenol treatment in rats resulted in a marked elevation of CK-MB level. Pretreatment of rats with FORM1 and FORM2 prevented the maximum increase of the enzyme during the peak infarction in the tissues. Moreover, the activities of other cardio specific enzyme markers like LDH, GOT and ALKP in the serum were also found to be reduced in the FORM1 and FORM2 pretreated MI rats. The heart tissue of FORM1 and FORM2 pretreated animals showed no changes in cardiac structure and were similar to that of the control group. This indicated that both FORM1 and FORM2 were free of toxicity. Further, administration of FORM1 and FORM2 to the MI rats reduced the damage of cardiac muscle induced by isoproterenol. Pretreatment of rats with FORM1 and FORM2 for 15 days almost completely protected the isoproterenol-induced cardiac muscle damage with little necrotic areas. The FORM1 and FORM2 rats showed minimal myocardial degeneration and were completely devoid of any cardiotoxicity.

A significant increase in the serum marker enzymes levels in FORM3 treated rats was observed when compared to the control group which depicts the highly toxicity nature of the formulation. The reason for high toxicity of FORM3 formulation may be the increased intracellular aggravation of Ca²⁺ which may be due to the high concentration of aconitine. Conspicuous damage of the myocardium was present in all FORM3 treated animals. Strips of intensely eosinophilic cells and rather large groups of necrotic cells accompanied by mild mononuclear infiltrate were observed. Myocardial necrosis was located with large areas of necrotic myofibers. The FORM3 +ISO treated rats also showed acute myocardial necrosis and consisted of variably sized areas of contracted and/or fragmented myofibers with inflammatory infiltrate.

It is evident from the results of the study that Mahamrutyunjaya rasa has significant cardio-active property when compared with the control groups.
### Table 6.1 Results of the Standardization of the three formulations.

<table>
<thead>
<tr>
<th>SR NO</th>
<th>STANDARDIZATION PARAMETER</th>
<th>FORM1</th>
<th>FORM2</th>
<th>FORM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Uniformity of Weight, mg</td>
<td>63 ± 3.12</td>
<td>65 ± 4.67</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Disintegration test, minutes</td>
<td>13.5 ± 0.5</td>
<td>8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Hardness, kg/cm²</td>
<td>5.6 ± 0.01</td>
<td>4.7 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Diameter, mm</td>
<td>5.1 ± 0.09</td>
<td>5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Thickness, mm</td>
<td>5.2 ± 0.1</td>
<td>4.4 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Friability, %</td>
<td>0.45-0.51</td>
<td><strong>0.64-0.72</strong></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Total ash, %</td>
<td>41.34 ± 1.87</td>
<td>43.16 ± 1.34</td>
<td>46.78 ± 0.97</td>
</tr>
<tr>
<td>8.</td>
<td>Acid insoluble ash, %</td>
<td>32.98 ± 0.78</td>
<td>31.89 ± 1.12</td>
<td><strong>36.78 ± 1.54</strong></td>
</tr>
<tr>
<td>9.</td>
<td>Water soluble ash, %</td>
<td>11.98 ± 1.76</td>
<td>13.87 ± 2.09</td>
<td>12.06 ± 1.78</td>
</tr>
<tr>
<td>10.</td>
<td>Water soluble extractive value, %</td>
<td>23.87 ± 1.34</td>
<td>21.67 ± 1.89</td>
<td><strong>18.97 ± 1.78</strong></td>
</tr>
<tr>
<td>11.</td>
<td>Alcohol soluble extractive value, %</td>
<td>24.35 ± 1.54</td>
<td>23.89 ± 1.89</td>
<td><strong>19.08 ± 1.90</strong></td>
</tr>
<tr>
<td>12.</td>
<td>Loss on Drying, %</td>
<td>2.13 ± 0.09</td>
<td>1.87 ± 0.15</td>
<td>1.78 ± 0.11</td>
</tr>
<tr>
<td>13.</td>
<td>Pesticide Residue</td>
<td>Absent</td>
<td><strong>Organochlorinated Pesticides present</strong></td>
<td>Absent</td>
</tr>
<tr>
<td>14.</td>
<td>Heavy metals</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>15.</td>
<td>Microbial Contamination</td>
<td>Within limits</td>
<td>Within limits</td>
<td>Within limits</td>
</tr>
<tr>
<td>16.</td>
<td>Qualitative tests (Active constituents)</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>17.</td>
<td>Concentration of Aconitine (μg) per tablet</td>
<td>0.121± 0.02</td>
<td>0.14 ± 0.01</td>
<td><strong>0.52 ± 0.03</strong></td>
</tr>
<tr>
<td>18.</td>
<td>Concentration of Solanine (μg) per tablet</td>
<td>0.13 ± 0.02</td>
<td>0.145 ± 0.02</td>
<td><strong>0.078 ± 0.01</strong></td>
</tr>
</tbody>
</table>
19. Concentration of Piperine (µg) per tablet  
   - 0.441± 0.03  
   - 0.572± 0.05  
   - 0.40± 0.02

20. Concentration of Sulphur, mg per tablet  
   - 10.06± 0.72  
   - 11.09 ± 1.02  
   - 11.94 ± 0.83

21. Concentration of Boron, mg per tablet  
   - 4.25±0.52  
   - 4.15±0.42  
   - 5.1±0.39

22. Concentration of Mercury, mg per tablet  
   - 4.50 ± 0.70  
   - 5.95 ± 0.63  
   - 5.94 ± 0.55

23. Acute Toxicity Studies (550 mg/kg b.w., Histopathology)  
   - No Toxicity  
   - No Toxicity  
   - Minor changes

24. Cell viability Studies (200 µg/ml in 24 hrs.)  
   - Decreased 47.49 %  
   - Decreased 65.08 %  
   - Decreased 94.98%

25. Effect on Isoproterenol induced myocardial infarction rats. (25mg/kg and 50 mg/kg b.w)  

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<table>
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<tbody>
<tr>
<td>A.</td>
<td>Levels of serum markers in comparison to negative control</td>
<td>Significant decrease</td>
</tr>
<tr>
<td>B.</td>
<td>Heart Weight/Body weight</td>
<td>Significant decrease</td>
</tr>
<tr>
<td>C.</td>
<td>Histopathology</td>
<td>Normal Architecture</td>
</tr>
</tbody>
</table>

Thus, in the present study a number of methods for the standardization of *Mahamrutyunjaya rasa* have been developed in terms of physical, chemical and biological standardization. The results of all the tests were compared and significant variations were observed. The FORM1 prepared by the traditional method was found to comply with the tests performed for physical standardization. The assay results of all the markers and minerals showed that the concentration of the active components were within acceptable limits. The stability indicating methods when applied to the formulation did not show any deviation from the standard substances. However, aconitine in the samples in
humid conditions (75 % RH at 30°C) was found to degrade at a faster rate. The safety and efficacy of FORM1 were further confirmed by the toxicity studies, cell viability studies and pharmacological activity. FORM1 qualified all the tests and was found to be safe as well as effective for use as a cardioactive formulation. The reason being the standardization of the crude plant materials used as raw materials and proper purification of toxic components using standardized methods as per the traditional text.

The FORM2 from Baidyanath showed few deviations from the standard qualify. In terms of hardness and disintegration, the values of FORM2 were above the acceptable limits. The presence of organochlorinated pesticides was also observed. The assay results of the markers did not show large deviation in the concentration of aconitine, solanine and piperine when compared to FORM1. The results of the stability studies were also similar to FORM1. However, the concentration of mercury was found to be high as compared to FORM1. The results of biological activity showed similar results as FORM1 and thus was found to be effective cardioactive formulation with less toxicity.

The FORM3 from Pune Rasashala showed significant variations in the results of all the methods of standardization. The formulation was found to have high friability in the friability test and was thus prone to breakage. The assay results showed that aconitine was present in very high concentration as compared to other two formulations. The variation in the concentration of aconitine may be due to the improper processing of the raw material. The standardization of the raw material is therefore important when such poisonous components are present in the formulations. Solanine and Piperine concentrations also varied but the % RSD was lesser as compared to aconitine. The stability indicating assay methods showed similar degradation pattern as FORM1. The concentration of sulphur and boron were also found to be acceptable with less variation. However, the concentration of cinnabar in the FORM3 was high as compared to the label claim. The results of the biological standardization showed that FORM3 is toxic. The formulation when administered for 15 days produced infarcts in the cells themselves and were not at all effective as cardioprotective. The results of toxicity was further confirmed from the cell viability studies. Thus FORM3 plays no role as a cardioprotective.
The results of complete standardization studies showed significant variation in the three medicines (Table 6.1). Therefore, from the above study one can conclude that for the preparation of *Mahamrutyunjaya rasa*, standardized traditional methods and raw materials should be used. Further, the concentration of the markers should be assayed which have been observed to have direct effect on the safety and efficacy.

So, it is highly recommended that the standardization of this Ayurvedic medicine must be done as a routine measurement, so as to provide a safe application to patients in clinics, and good manufacture practices. Thus reliable standardization tools for effective utility of these traditional medicines were developed.