Chapter 4.

MATERIALS and METHODS

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4.1) Plant material

Dried roots of *Aconitum ferox,* were purchased from the local stores along with dried fruits of *Piper nigrum* and *Piper longum.* Fresh roots of *Solanum indicum* were collected from the local gardens of Vadodoara. All the plant materials were identified by NISCAIR, Delhi. The voucher specimens of the herbs have been deposited in the Pharmacy department, The M.S. University of Baroda.

4.2) Preparation of powdered material

The selected plant materials were collected, cleaned to remove any adhering material and then dried in shade. The dried plant materials were then subjected to size reduction to coarse powder and used for further studies.

4.3) Standardization of the plant materials as per the WHO guidelines[197].

4.3.1) Macroscopic evaluation[198]

The size, color, surface characteristics, texture, fracture characteristics and odor of the crude drugs were studied and compared with the standard monographs.

4.3.2) Microscopic evaluation[199]

The intact crude drugs as well as powdered drugs were studied under the microscope to analyze the cellular characteristics of the drugs.

4.3.2.1) Study of Transverse section:

The selected drug samples were taken in a test tube and 5 % potassium hydroxide in methanol was added so that the sample remained submerged. The samples were boiled for few minutes. Transverse sections of the drugs were taken in a watch glass containing water with the help of a brush. The sections were then transferred to a watch glass containing Phloroglucinol-Hydrochloric acid and allowed to stain for 2-3 minutes. The sections were again transferred to watch glasses containing water, so as the excess stain was washed away. The sections were then placed on clean glass micro-slides, with the help of brush. Few drops of water were added and a clean cover-slip was placed on the slide. The slides were mounted for study on the microscope.

4.3.2.2) Study of Powder characteristics:

The microscopic structures of powdered drugs were also studied using the slides prepared by above method using powdered drugs in place of sections.

4.3.3) Determination of ash[198]

The ash remaining following ignition of medicinal plant materials was determined by three different methods which measure total ash, acid-insoluble ash and water-soluble ash.

4.3.3.1) Total ash:

About 2 gm of the ground drug was weighed accurately in a previously ignited and tarred crucible. The material was spread in an even layer and ignited by gradually increasing the heat to 500-600°C until it was white, indicating the absence of carbon. The ash was cooled and weighed. The total ash was calculated in % of air-dried material.

4.3.3.2) Acid-insoluble ash:

About 25 ml 70% Hydrochloric acid was added to the total ash and covered with a watch glass. The mixture was then boiled for 5 minutes. The insoluble matter was collected on an ashless filter-paper and transferred to the crucible and ignited to constant weight. The residue was weighed and the acid-insoluble ash was calculated in % of air-dried material.

4.3.3.3) Water-soluble ash:

About 25 ml of water was added to the total ash and boiled for 5 minutes. The insoluble matter was collected on an ashless filter-paper. The crucible was ignited to constant weight. The weight of the residue was subtracted from the weight of total ash and the content of water-soluble ash in % of air-dried material was calculated.

4.3.4) Determination of Extractable matter[198]

The amount of the active constituents extracted with different solvents from a given amount of plant material was determined. The three main solvents used were water, methanol and petroleum ether. The method of hot extraction was used for this purpose. About 4.0 gm of coarsely powdered material was accurately weighed in a glass-stoppered conical flask. 100ml of the specified solvent was added and the final weight was recorded as the total weight. The

flask was shaken and allowed to stand for 1 hour. A reflux condenser was attached to the flask and boiled gently for 1 hour, cooled and weighed. The total weight was readjusted to the original weight with the solvent specified. It was then filtered rapidly through a dry filter. 25 ml of the filtrate was transferred to a tarred flat-bottomed dish and evaporated to dryness on a water-bath. The extract was dried at 105°C for 6 hours, cooled and then weighed. The content of the extractable matter in % of air-dried material was calculated.

4.3.5) Determination of water and volatile matter[198]

About 2 gm of plant material was accurately weighed in a previously dried and tarred flat weighing bottle. The sample was dried in an oven at 100-105°C until there was no difference in the consecutive weighing. The loss of weight in % of air-dried material was calculated.

4.3.6) Determination of pesticide residues[200]

The presence of organo-phosphorus and organo-chlorinated pesticides was analyzed using TLC studies. An aqueous extract of the formulations were prepared and spotted on silica gel G plates. The following solvent systems and detecting reagents were then sprayed. The observations were recorded. Two mobile phases used were DMF: Ether (4:6) and Methylcyclohexane. The detecting reagent for organochlorinated pesticides was Tetrabromophenolphthalein, and for organophosphorus pesticides was 0.5% Silver nitrate in water and acetone.

4.3.7) Determination of heavy metals[198]

Heavy metals like arsenic, mercury, lead and cadmium were analyzed using atomic absorption spectrophotometer in the ash of the powdered drug material. The elements detected were quantified by using standard calibration plots and expressed in PPM.

4.3.8) Determination of microorganisms[198]

The plant materials were analyzed for bio-burden present as per the reported methods. The tests were performed to determine the amount of aerobic bacteria

and presence of yeasts and moulds, *E.coli, Enterobacteria* and *Salmonellae* using the reported culture media.

4.3.9) *Qualitative Phytochemical Screening*

The aqueous, methanol and petroleum ether extracts were studied to determine the chemical constituents in the plants.

4.3.9.1) Extraction:

Petroleum ether, Methanol and water, in any case are good solvents for the preliminary extraction and most classes of compounds show solubility in them. Hence the coarse powder of shade-dried plant was extracted .The extracts were concentrated and air-dried.

4.3.9.2) Chemical Tests[199]*:*

The extracts were subjected to chemical tests for presence of following phytochemical classes like Carbohydrates, Alkaloids, Anthraquinones and Saponin glycosides, Phytosterols, Phenolics, Tannins, Flavonoids, Proteins and amino acids using reported methods.

4.3.9.3) Thin Layer Chromatographic studies[201]*:*

The extracts obtained were subjected to thin layer chromatographic studies using reported methods to determine the presence of various phyto-constituents. The results were compared with the results obtained in the qualitative tests. The mobile phases and detecting reagents of various classes of compounds are mentioned in Table 4.1.

Table 4.1: Detection conditions of plant constituents using TLC.

Class of compound	**Solvent system**	**Detection**	**Identification**
Anthraglycosides	Ethyl acetate: methanol: water (100:13.5:10)	KOH Reagent	Red: Anthraquinone Yellow: Anthrones
Alkaloids	Ethyl acetate: methanol: water (100:13.5:10)	Dragendorff's Reagent	Orange Brown
Arbutin like compounds	Ethyl acetate: methanol: water (100:13.5:10)	Berlin Blue	Blue
Bitter Principles	Ethyl acetate: methanol: water (100:13.5:10)	Vanillin – Sulphuric Acid	Red/Yellow-Brown/Blue-green

Saponins	Ethyl acetate: methanol: water (100:13.5:10)	A.S. Reagent	Blue
Essential Oils	Toluene: Ethyl acetate (93:7)	V.S. Reagent	Red/Yellow/Blue/ Brown (visible)

4.4) Isolation of marker compounds

4.4.1) *Aconitum alkaloids*[202]:

The finely pulverized roots (2Kg) was defatted by petroleum ether(60-80 °C) using soxhlet apparatus. The defatted drug was dried and treated with 25% ammonium hydroxide in water for 24 hours. The pH of the slurry was adjusted to 9.00 with ammonia. The alkaloid fraction was extracted using 500 ml chloroform three times. The chloroform extract was then concentrated and evaporated to dryness in a tarred dish. The alkaloids in the fraction were separated by preparative TLC using ethyl acetate: methanol (9:1) as the mobile phase. The compounds were extracted in chloroform and concentrated. The separate constituents were then characterized and analyzed using reference standards. Standard Aconitine at Rf value of 0.45 was matched with the alkaloid isolated from the alkaloidal fraction.

4.4.2) Solanum alkaloids[203]:

The powdered roots (2Kg) were macerated with 90% alcohol and 2% acetic acid. The extract was then concentrated under vaccum. Equal volume of 10% Acetic acid was added to the extract and filtered. The filtrate was defatted using benzene and ether (1:1) using a separating funnel. The defatted solution was then heated and the pH was adjusted to 10 using ammonia. The solution was kept overnight to obtain precipitates. The whole procedure was repeated by addition of 5 % acetic acid.

4.4.3) Piper alkaloids[204]:

The finely powdered fruits of *Piper nigrum*(1Kg) were extracted by soxhletion in methanol. The methanol extract was cooled and concentrated below 60 °C. 10% alcoholic potassium hydroxide was added to the concentrated extract and the solution was decanted. The residue was cooled in ice bath and water was added

dropwise to precipitate piperine. The precipitates were re-crystallized using acetone and hexane.

4.5) Selection of Formulations

Mahamrutyunjaya rasa was prepared in the laboratory as per the standard text[205] and named as FORM1. Two marketed formulations were selected for the study. FORM2 was *Mahamrutyunjaya rasa* manufactured by Baidyanath, Nagpur, Maharashtra, India and FORM3 was *Mahamrutyunjaya rasa* manufactured by Pune Rasashala, Pune, Maharashtra, India.

4.6) Preparation of *Mahamrutyunjaya Rasa* as per the standard text

The ingredients of *Mahamrutyunjaya rasa* are mentioned in Table.3.2:

Table 3.2 Formula of *Mahamrutyunjaya rasa.*

Ingredients	Source	Amount
Brihati	*Solanum indicum*	1 part
Bachnag	*Aconitum ferox*	1 part
Marica	*Piper nigrum*	1 part
Pippali	*Piper longum*	1 part
Gandhaka	Sulphur	1 part
Hingula	Cinnabar	2 part
Tankana	Sodium biborate	1 part
Jambira	*Citrus limon*	As per requirement

The ingredients had to be purified as per the traditional procedure. The purified ingredients were then pulverized to fine powder and passed through 85 mesh sieve. The powders of Aconitum, Solanum, *Piper longum* and *Piper nigrum* were then mixed by sieving. Further sulphur and sodium borate were added. This mixture was then mixed with cinnabar. Vatis of this powder were prepared using lemon juice.

4.7) Standardization of procedure for formulation preparation[206-215]:

The formulation contains a number of poisonous constituents which have to be purified before incorporation in the formulation. Aconite, cinnabar, sulphur and sodium metaborate were purified, whereas *Solanum indicum* and both the *Piper*

species were not purified as they are directly incorporated in *Mahamrutyunjaya rasa* without any preliminary processing.

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 also investigated by collecting samples at different steps of purification and were analyzed.

4.7.1) Aconite alkaloids:

The aconite roots were washed with water and soaked in cow urine for 48 hours. The drug was then washed with water and boiled in milk. The drug was then washed with water and dried. The samples of crude drugs were collected and the alkaloid fraction was subjected to HPTLC and IR studies. The samples were collected at the following steps of purification.

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

4.7.2) Sulphur:

The crude sulphur was mixed with cow ghee and the mixture was heated in an iron vessel and melted. The melted mixture was cooled and wrapped in a muslin cloth and washed with cow milk. The mixture was further washed with water, dried and powdered. The samples at different steps were collected and subjected to XRD and DSC studies. The samples were collected at the following steps.

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

4.7.3) Cinnabar:

The crude cinnabar was treated with lemon juice about 7 times until the color of the powder became dark red. After each treatment the cinnabar was washed with water. After the final treatment the cinnabar was dried. The samples were collected at different steps and subjected to XRD. The samples were collected at the following steps.

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

4.7.4) Sodium metaborate:

The crude sodium metaborate was heated till the constant weight was obtained. The moisture free sodium metaborate was stored in air tight container. The samples were collected at the following steps and analyzed using XRD and DSC.

1) Crude sodium metaborate (Sample – 1).
2) Sodium metaborate was heated for 2 hrs at 80°C (Sample – 2).
3) Sample weight was constant in three consecutive weighing (Sample – 3).

4.8) Physical Standardization of the three Formulations[198, 207]:

All the three formulations were subjected to physical testing as per the WHO guidelines in order to set the standards for the analysis of the formulations. Although the methods for chemical and biological standardization of the formulations have been developed and are much more sophisticated, the physical standardization proves to be equally important. The appearance of the formulation, the amount of the dose administered, the time required for the disintegration and release of active constituents are all important factors which affect the physiological action of the formulation. Also there are a number of toxic components in the formulation and overdosage may lead to serious side effects and at times may prove to be fatal. The marketed formulations are therefore supplied in the form of tablet to ascertain that a fixed dose is administered. Thus, the marketed tablets should be analysed as per the IP monographs for the following parameters:

4.8.1) Uniformity of Weight:

The weight variation of the marketed formulations was studied as per the IP. Twenty tablets of each formulation were weighed and the average weights and maximum deviation of average weights were calculated. The uniformity in weight is important parameter for quality control of ayurvedic formulations because a number of small scale manufacturers use the traditional manual method of tablet preparation. Thus, weight variation is bound to occur and may lead to variation in the amount of formulation administered.

4.8.2) Disintegration test:

The disintegration test of the marketed formulations was studied as per the IP. The disintegration of a number of vatis has been questionable in the past as their hardness is very high. Therefore, in order to ascertain that the tablet has been disintegrated for the release of active constituent, the disintegration and the duration of disintegration were determined.

4.8.3) *Hardness:*

Hardness is an important parameter which should be determined for tablets as per the IP. The official limit of hardness of a tablet is 3-5 kg/cm^2. If the tablet is

below the limit then the tablet may be prone to breakage or loss of the uniformity in weight. If the hardness is above the limit then the tablet will not disintegrate in the required period of time. Thus the marketed tablets were subjected to hardness test using Pfizer hardness tester as per the IP.

4.8.4) Diameter:

The diameter of the marketed tablets was measured using vernier calliper as per the reported method.

4.8.5) Thickness:

The thickness of the marketed tablets was measured using vernier calliper as per the reported method.

4.8.6) Friability:

The friabilty test was performed using the Roche friabilitor as per the reported method in IP.

4.8.7) Determination of Ash:

The procedure prescribed in WHO guidelines was used for the determination of ash value for all the three formulations.

4.8.8) Determination of extractable matter:

The procedure prescribed in WHO guidelines was used for the determination of extractable matter of all three formulations.

4.8.9) Determination of pesticide residues:

All the three formulations were subjected to tests for pesticide residues as per the reported methods.

4.8.10) Determination of heavy metals:

The concentration of heavy metals in all the formulations was analyzed using Atomic absorption spectroscopy.

4.8.11) Determination of microorganisms:

The tests for microbial contamination were done as per the reported methods for all the three formulations.

4.8.12) Chemical tests:

The qualitative phyto-chemical screening of the methanol extract of all three formulations was performed.

4.8.13) TLC studies:

The qualitative phyto-chemical screening of the methanol extract of all three formulations was performed.

4.9) Chemical Standardization of the three Formulations

The chemical standardization of herbal drugs requires different analytical methods depending on the number of markers and instruments available. This section of the study deals with the development and validation of various analytical methods of the markers available which play a major role in monitoring the therapeutic potential of the drug and at the same time are responsible for the narrow therapeutic index.

The following is a brief and condensed description of the methods developed which can offer new analytical possibilities for the determination of content and identity of drug in their combination dosage form.

Different spectrophotometric, spectrofluorimetric, HPLC, HPTLC and voltammetric methods were developed for analyzing the biomarkers in the formulations. Apparatus and materials requirement for all the methods were similar and are described in the following section.

4.9.1) Instrumentation

4.9.1.1) Apparatus and software for HPLC measurement

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of Shimadzu LC-20 AT Prominence solvent delivery module, a manual Rheodyne injector with a 20 µl fixed loop and a SPD-20A Prominence UV–visible detector. The separation was performed on a Phenomenex C_{18} column (particle size 5 µm; 250 mm X 4.6 mm ID; Phenomenex Torrance, USA) preceded by an ODS guard column (10 µm, 10 mm × 5 mm ID) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.193 (Spinchrom Pvt. Ltd., Chennai, India).

4.9.1.2) Apparatus and software for HPTLC measurements

A Camag microlitre sample (Hamilton, Bonaduz, Switzerland) syringe was used for sample application on pre-coated silica gel aluminium plate 60F-254, (20 cm x 10 cm with 0.2 mm thickness, (E. Merck, Darmstadt, Germany) using a Camag Linomat-V (Switzerland). The linear ascending development was carried out in 20 cm X 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). Dimensions: length X width X height =12 cm X 4.7 cm X 12.5 cm. Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode for all measurements and operated by CATS software (V1.4.3 Camag).

4.9.1.3) Apparatus for Spectrofluorimetric measurement

A Shimadzu spectrofluorophotometer (Tokyo, Japan Model RF-540 with DR-3 data recorder), and fused fluorescence free quartz square 10 mm cell having capacity of 5 ml was employed for all spectral and fluorescence measurements.

4.9.1.4) Apparatus and software for spectrophotometric measurement

Spectrophotometric measurements were carried out on a Shimadzu 1700 double beam UV Visible spectrophotometer with a fix slit width of 1 nm coupled HP7540 computer loaded with Shimadzu UV PC software of version 2.10. The spectral bandwidth was 1 nm and the wavelength scanning speed was 2800 nm/min Matched quartz cuvettes (1cm) were used for all the spectral measurements.

4.9.1.5) Apparatus and software for voltammetric measurement

Voltammetric measurements were performed using a 757 VA computrace, a PC controlled system made up of a VA Computrace stand. The analyzer was controlled with a VA Computrace software 1.0. The central element of this stand was the Multi-mode electrode (MME) with a hanging mercury electrode (HMDE) as a working electrode, a Ag/AgCl with saturated KCl as a reference electrode and a platinum wire as an auxillary electrode were used. Pure mercury was used throughout the study for HMDE.

Ultrasonic bath (Ultrasonics Selec, Vetra, Italy) was used in the study wherever required.

The pH was measured using LabIndia pH-meter (TICO^{+}), Mumbai.

4.9.1.6) Markers and Formulations

Standard substances including aconitine (95% by HPLC), solanine (97% from potato sprouts) and piperine (97%) were all purchased from Sigma Aldrich Pvt. Ltd. (USA). The proprietary Ayurvedic medicines (Baidyanath, Nagpur, India (FORM2) and Pune Rasashala, Pune, India (FORM3)) were procured from local drug store.

4.9.2) Development of analytical methods for estimation of Aconitine in Mahamrutyunjaya rasa

Three different methods, HPLC, HPTLC and Spectrofluorimetry were developed for the estimation of aconitine.

4.9.2.1) HPLC method

4.9.2.1.1) Chemicals and Reagents

Acetonitrile and methanol were of HPLC grade (Qualigens, Mumbai). Potassium di-hydrogen phosphate (KH_2PO_4), Tri-ethylamine and 85% *ortho*-phosphoric acid (H_3PO_4) of analytical-reagent grade were also purchased from the same supplier. Triple distilled water was used throughout the study. All the other solvents and reagents used were of analytical grade and were filtered through a 0.2 µm Ultipor ® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

4.9.2.1.2) Preparation of Standard Solutions

Standard stock solutions of aconitine (1000 µg/ml) were prepared by dissolving 10 mg of pure drug in 10 ml methanol. Appropriate and accurate aliquots of the stock solutions were transferred to 10 ml calibrated flasks and diluted up to the volume with methanol in the range of 10-100 µg/ml.

4.9.2.1.3) Preparation of sample solutions

Twenty tablets of all the three formulations were powdered and about 1gm each of the three formulations was accurately weighed and extracted in 25 ml of 0.1N HCl by sonication for 10 minutes at room temperature which was then fractionated with 10 ml ethyl acetate thrice to remove the non-alkaloid components. The acidic aqueous solutions were basified using ammonia to pH 11 and further extracted with 10 ml chloroform thrice. Chloroform was

evaporated under reduced pressure. The residue was dissolved in methanol by sonication and further dilutions were made in acetonitrile.

4.9.2.1.4) Analytical conditions

Analysis was isocratic at 1.0 ml/min flow rate with ammonium bicarbonate buffer (15mM, pH 7.5 was adjusted using ammonia): acetonitrile (40:60 v/v) as mobile phase. The mobile phase was prepared freshly everyday. The mobile phase was filtered through a 0.2 µm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. The absorbance of aconitine was good at 223 nm and further it was free from any interference. Hence, the eluted peak was detected at 223 nm. The sensitivity of the detector was set at 0.01 AUFS. Prior to injecting solutions, the column was equilibrated for at least 60 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (RSD) was required to remain below 1.0% on peak area basis.

4.9.2.1.5) Optimization of Chromatographic conditions

Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers. Therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, mobile phase compositions, and flow rate to check the retention time, shape, resolution, and other system suitability parameters of all the peaks.

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase pH varied at 7, 7.5, and 8 keeping the composition of ammonium bicarbonate buffer (15 mM): acetonitrile (40:60:10 v/v) and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 35:65, 40:60 and 45:65 (v/v) with pH and flow rate kept constant at 8 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 40:60 (v/v) and 8, respectively. The effects of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the buffer system with the organic solvent in the desired proportions.

4.9.2.1.6) Method Validation:

In order to verify that the proposed method is applicable to formulation analysis, validation was performed as per the ICH Guidelines[216].

a) Calibration curve (linearity)

Six different concentrations of aconitine were analyzed and their calibration curve was constructed in the specified concentration range (10-100 µg/ml). The calibration plots were generated by replicate analysis ($n = 3$) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

b) Repeatability, precision and stability

The injection repeatability was determined by the analysis of six continuous injections using the same sample, while the analysis repeatability was examined by the injection of six different samples prepared by the same procedure. The standard solution (10, 40, 100 µg/ml) was used for the test of injection repeatability and analysis repeatability.

The instrument precision was examined by performing the intra-day and inter-day assays of six replicate injections of the standard solutions at three concentration levels (10, 40, 100µg/ml). The intra-day assay precision was performed at the interval of 4 h in 1 day, while the inter-day assay precision was performed over 6 days.

c) Limit of detection and limit of quantification

LOD and LOQ were determined by kSD/s where k is a constant (3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

d) Specificity

Specificity is the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix.

e) Robustness

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 223 nm to 221 nm and 225 nm

2) Column: Using another column (Hypersil ODS, particle size 5 µm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

Standard solution was injected 6 times for each change. System suitability parameters like resolution, peak asymmetry, theoretical plates, capacity factor, and RSD were calculated for each peak. Recoveries and % RSDs were calculated during each change.

f)Accuracy

The accuracy of the method was determined by calculating the recoveries of aconitine by the method of standard additions. Known amount of standard (80%, 100% and 120 %) was added to the pre-analyzed sample solution, and the amounts of the standard was estimated by measuring the peak area and by fitting the value to the straight-line equation of calibration curve.

4.9.2.2) HPTLC method

4.9.2.2.1) Chemicals and Reagents

Chloroform, Toluene, Ethyl Acetate, Diethyl amine and Ammonia of analytical grade were purchased from Qualigens (Mumbai). Draggendorff's Reagent was prepared as per reported method (Wagner).

4.9.2.2.2) Preparation of standard solutions

A 100 µg/ml solution of aconitine was prepared in chloroform.

4.9.2.2.3) Preparation of sample solutions

The alkaloid fraction was prepared by treating 1gm of powdered formulation with ammonia and extracting with ethyl acetate. The ethyl acetate extract was concentrated and evaporated under vacuum. A 10 mg/ml solution of alkaloid fraction was prepared in chloroform. 100 mg of dry extract was weighed and dispersed in chloroform in 10 ml volumetric flask. The volume was made up to 10 ml.

4.9.2.2.4) Application of test samples

The samples were spotted in the form of bands of width 6 mm on the pre-coated silica gel G plates. The plates were pre-washed by methanol and activated at

110° C for 5 min prior to chromatography. A constant application rate of 0.1 µl/s was employed and space between two bands was 6mm. For Calibration Curve of aconitine the stock solution was used. Different volumes from 2-10 µl of stock solution were applied, which gave different concentration 200-1000 ng per spot respectively.

4.9.2.2.5) Development

The mobile phase consisted of Toluene: Ethyl Acetate: Diethyl amine (7:2:1 v/v). Linear ascending development was carried out in trough chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 15 mins at room temperature. The length of chromatogram run was 85 mm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air-dryer.

4.9.2.2.6) Detection

The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from intensity of diffusely reflected light. The slit dimension was kept at 5 mm x 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light.

The plate was scanned and quantified at 235 nm. The plate was sprayed with Dragendorffs reagent immediately scanned and quantified at 500 nm using the Camag TLC Scanner-3. Data of peak area of each band were recorded .A calibration curve was obtained by plotting peak area Vs. concentration and peak height Vs. concentration of aconitine. Spectra of the samples and standard aconitine were matched.

4.9.2.2.7) Method Validation

a) Calibration Curve of aconitine.

A stock solution of aconitine (100 µg/mL) was prepared in chloroform and bands in the range of 200-1000 ng per spot were applied. The drug was spotted in duplicate on TLC plate to obtain concentrations of 200-1000 ng per spot of

aconitine. The data of peak height/area versus drug concentration were treated by linear least-square regression.

b) Precision

The intra-day and inter-day variation for the determination of aconitine was carried out at two different concentration levels 200 and 1000 ng per spot.

c) Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions were tried at two different concentration levels of 200 and 1000 ng per spot.

d) Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times. The signal-to-noise ratio was determined. An LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were experimentally verified by diluting known concentrations of aconitine until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

e) Recovery studies

The analyzed samples were spiked with extra 80, 100 and 120% of the standard aconitine and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different levels in the formulations.

f) Spot Stability

The time the sample is left to stand on the solvent prior to chromatographic development can influence the stability of separated spots and is required to be investigated for validation. Two-dimensional chromatography using same solvent system was used to find out any decomposition occurring during spotting and development. In case, if decomposition occurs during development, peak(s) of decomposition product(s) shall be obtained for the analyte both in the first and second direction of the run.

4.9.2.3) Spectrofluorimetric method.

4.9.2.3.1) Preparation of standard solution

A stock solution (100 µg/ml) of aconitine was prepared in methanol.

4.9.2.3.2) Preparation of sample solution

Twenty tablets of all the three formulations were powdered and about 1gm each of the three formulations was accurately weighed and extracted in 25 ml of 0.1N HCl by sonication for 10 minutes at room temperature which was then fractionated with 10 ml ethyl acetate thrice to remove the non-alkaloid components. The acidic aqueous solutions were basified using ammonia to pH 11 and further extracted with 10 ml chloroform thrice. Chloroform was evaporated under reduced pressure. A 10 µg/ml solution of the alkaloid fraction in chloroform was prepared. With the use of a marked capillary, 100 µL of the resultant solution was applied on chromatographic plate as a band along with a reference spot of standard aconitine. Preparative TLC was run using silica gel G as a stationary phase and a mobile phase consisting of ethyl acetate: methanol (9:1v/v). Visualization of aconitine was performed under UV chamber, having Rf value 0.52. The bands of aconitine were scrapped off using sharp blade, extracted with methanol (3 X 10 mL) and filtered through Whatmann filter paper No.42 (Whatmann, London), the residue on the filter paper was washed with methanol and final volume of the solution was made up to 50 mL. It was considered as a sample stock solution. 5 mL of this solution was adjusted to 10 ml and used as working sample.

4.9.2.3.3) Instrumental Parameters:

Scan speed: Medium, Abscissa scale: 2, Ordinate scale: 2, Slit-widths: 5 nm for excitation and 3 nm for emission; Excitation wavelength: 270 nm; and Emission wavelength: 505 nm.

4.9.2.3.4) Effect of solvent:

Different solvents such as methanol, ethanol, acetonitrile, chloroform, dichloromethane and ethyl acetate were studied spectrofluorimetrically for aconitine.

4.9.2.3.5) Procedure:

Suitable aliquots from the stock solutions were transferred into 5 ml flask in the range of 100-600 ng/ml. Standard solution of aconitine was scanned in the range of 200-600 nm using Spectrophotometer and the excitation wavelength was determined to be 270 nm. Same solution was scanned for emission wavelength using the spectrofluorimeter in the range of 271-600 nm keeping 270 nm as the excitation wavelength and it was found to be 505 nm. Each time the solvent blank was also scanned.

4.9.2.3.6) Method Validation

a) Linearity and range

The linearity of aconitine complex was evaluated by analyzing a series of different concentrations of aconitine. In this study, seven different concentrations of aconitine were chosen within the linearity range, and each was repeated three times. A linear relationship was found between the fluorescence intensity and the concentration of the aconitine in the range 100-600 pg/ml. The optical characteristic such as linearity range and regression equation (slope, intercept and correlation coefficient) were determined for the method.

b) Detection and Quantitation Limits

The LOD and LOQ values for aconitine were calculated from the calibration curves as kSD/b where k=3 for LOD and 10 for LOQ, SD is the standard deviation of the intercept and b is the slope of the calibration curve.

c) Precision

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels (10, 25 and 50 ng/ml) for Aconitine. The experiments were repeated six times a day for intra-day precision and on three different days for inter-day precision.

d) Accuracy

To confirm the accuracy of proposed method, recovery study was performed by the standard addition technique. Three different levels (80,100 and 120%) of standards were added to pre-analyzed tablet samples, and each level was repeated three times.

e) Robustness

The robustness of the method was checked by examining the reflection of the slight changes to the results in methodological parameters. The solvent brand used in the analysis was purposely altered in order to determine the robustness of the method.

4.9.2.4) Stability indicating HPLC method for determination of Aconitine[217-218].

This official definition of stability indicating assay methods is: *'the validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference.'*

With the advent of International Conference on Harmonisation (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products.

The stability-indicating assay methods are deveoped for the analysis of stability samples of the formulations. Thus, an attempt was made to develop stability indicating methods for the marker compounds and apply the method to the formulation samples.

4.9.2.4.1) Reagents

Acetonitrile, methanol and water (HPLC grade) were purchased from Qualigens. Ltd.(Mumbai, India). Hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide solution (all analytical reagent grades) were purchased from Qualigens (Mumbai, India) and ortho phosphoric acid was also purchased from Qualigens (Mumbai, India).

4.9.2.4.2) Preparation of standard solution

Standard stock solution of aconitine (1000 µg/mL) was prepared by dissolving 25 mg of pure drug in 25 mL methanol in volumetric flask. Suitable dilutions were made from above stock solution to obtain the 20 and 500 µg/mL of drug concentration for degradation study.

4.9.2.4.3) Preparation of sample solution

Twenty tablets from each brand of one batch were accurately weighed, their mean weight determined and powdered in a glass mortar. An amount of the tablet mass equivalent to 1 gm was transferred to a 25 ml volumetric flask and sonicated with about 20 ml of methanol using an ultrasonic bath for 10 minutes, further diluted to volume with same solvent and this solution was used for the degradation studies.

4.9.2.4.4) Forced degradation studies of Aconitine and formulations

In order to establish whether the developed HPLC method was stability-indicating or not, tablets sample solution and standard solution of pure aconitine were stressed under various conditions to conduct forced degradation studies. From the above stock solutions of standard drug and sample, 0.5 mL of aliquots were diluted separately upto 10 mL with 0.6% H_2O_2, distilled water, 1M HCl and 0.01M NaOH, to achieve a concentration of 50 µg/mL of aconitine. Solutions in water, 1M HCl and 0.01M NaOH were heated at 80 °C for 24 h. For oxidative degradation, drugs were stored at room temperature (r.t.) in 0.6% H_2O_2 for 24 h. Degradation was also carried out in solid state by exposing pure drugs and drug product to dry heat at 80 °C for 48 h. Photolytic studies were carried out by exposing a thin layer of solid Aconitine placed in a Petri-dish, also the solutions of drugs and samples in 1M HCl, 0.01M NaOH and water to sunlight for 24 hrs. Suitable controls were maintained under dark conditions. Samples were withdrawn initially and subsequently at prefixed time intervals. Samples were neutralized by acid or alkali, wherever necessary and were diluted with acetonitrile to yield starting concentrations of 20 and 50 µg/ml of aconitine. Appropriate blanks were injected before analysis of forced degraded samples.

4.9.2.4.5) Chromatographic conditions

The chromatographic separation was achieved on an Hypersil BDS C_{18} (250 mm × 4.6 mm, 5 µm) column using a mobile phase containing mixture of acetonitrile-KH_2PO_4 buffer solution (10 mM, adjusted to pH 8.0 ± 0.1 with 1% Triethylamine) (50:50, v/v). The mobile phase was filtered through a nylon filter (pore size 0.2 µm) and degassed before use. The flow rate of the mobile phase was 1 mL/min and the wavelength was monitored at 223 nm.

4.9.2.4.6) Method validation

Linearity was studied by injecting seven concentrations of the standard Aconitine (10—100 µg/ml) in triplicate into the HPLC system. The peak area versus concentration data was performed by least-squares linear regression analysis. The LOD and LOQ values were calculated from kSD/b where k=3 for LOD and 10 for LOQ. SD is the standard deviation of the responses of the minimum detectable drug concentration and b is the slope of the calibration curve. For intra-day precision, 3 different concentrations of sample solutions of Aconitine (10, 40 and 100 µg/mL) were analyzed six times on the same day whereas for inter-day precision same drug concentrations were analyzed on six different days and the percentage RSD of area was calculated. Accuracy was evaluated by fortifying the degraded solutions of aconitine with three different levels of standard solution of aconitine (80%, 100% and 120%) and calculating the percent recovery from the differences between the peak areas obtained for the fortified and unfortified solutions. The specificity of the method was established through study of resolution factors (R_s) of the drug peaks from the nearest resolving peak, and also among all other peaks. Specificity of the method towards the drugs was also established through determination of purity of aconitine peak in stressed samples through study of purity plots using a PDA detector.

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 223 nm to 221 nm and 225 nm.

2) Column: Using another column (Phenomenex, particle size 5 µm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile and methanol supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

The assay was carried out in triplicate (n=3) at three different concentration levels of Aconitine (10, 40 and 100 µg/mL). In the system suitability tests, six replicate injections of freshly prepared working standard solutions of Aconitine (100 µg/mL) and two injections of the solutions prepared for the specificity procedure were injected into the chromatograph, and the % relative standard

deviation of peak areas, resolution factor, tailing factor and theoretical plates were determined.

4.9.3) Development of analytical methods for estimation of solanine in Mahamrutyunjaya rasa

4.9.3.1) HPLC method

4.9.3.1.1) Chemicals and Reagents

Acetonitrile and methanol used were of HPLC grade (Qualigens, Mumbai). Potassium di-hydrogen phosphate (KH_2PO_4) and 85% *ortho*-phosphoric acid (H_3PO_4) of analytical-reagent grade were also purchased from the same supplier. Triple distilled water was used for the study. All the other solvents and reagents used were of analytical grade and were filtered through a 0.2 µm Ultipor ® Nylon 66 membrane filter (Pall Life Sciences, USA) prior to use.

4.9.3.1.2) Preparation of Standard Solutions

Standard stock solutions of solanine (1000 µg/ml) were prepared by dissolving 10 mg of pure solanine in 10 ml methanol. Appropriate and accurate volume aliquots of the stock solutions were transferred to 10 ml calibrated flasks and diluted to volume with methanol in the range of 10-100 µg/ml.

4.9.3.1.3) Preparation of Sample solutions

Twenty tablets of all the three formulations were powdered and about 1gm each of the three formulations was accurately weighed and extracted in 25 ml of 0.1N HCl by sonication for 10 minutes at room temperature which was then fractionated with 10 ml ethyl acetate thrice to remove the non-alkaloid components. The acidic aqueous solutions were basified using ammonia to pH 11 and further extracted with 10 ml chloroform thrice. Chloroform was evaporated under reduced pressure. The residue was dissolved in methanol by sonication and further dilutions were made in acetonitrile.

4.9.3.1.4) Analytical conditions

Analysis was isocratic at 1.0 ml/min flow rate with Tris buffer (10 mM, pH 6.00): acetonitrile (60:40 v/v) as mobile phase. The mobile phase was prepared freshly everyday. The mobile phase was filtered through a 0.2 µm membrane filter to remove any particulate matter, mixed and degassed by sonication

before use. The absorbance of solanine was good at 218 nm and further it was free from any interference. Hence, the eluted peak was detected at 218 nm. The sensitivity of the detector was set at 0.01 AUFS. Prior to injecting solutions, the column was equilibrated for atleast 60 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (RSD) was required to remain below 1.0% on peak area basis.

4.9.3.1.5) Optimization of Chromatographic conditions

Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers. Therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, mobile phase compositions, and flow rate to check the retention time, shape, resolution, and other system suitability parameters of all the peaks.

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase pH varied at 5,6, and 7 keeping the composition of Tris buffer (10 mM, pH 6.00): acetonitrile (60:40 v/v) and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 55:45, 60:40 and 65:35 (v/v) with pH and flow rate kept constant at 6 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 60:40 (v/v) and 6, respectively. The effects of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the buffer system with the organic solvent in the desired proportions.

4.9.3.1.6) Method Validation:

In order to verify that the proposed method is applicable to formulation analysis, validation was performed as per the ICH Guidelines.

a) Calibration curve (linearity)

Seven different concentrations of solanine were analyzed and their calibration curve was constructed in the specified concentration range (1-100 µg/ml). The calibration plots were generated by replicate analysis (n = 3) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

b) Repeatability, precision and stability

The injection repeatability was determined by the analysis of six continuous injections using the same sample, while the analysis repeatability was examined by the injection of six different samples prepared by the same procedure. The standard solution (10, 40, 100 µg/ml) was used for the test of injection repeatability and analysis repeatability.

The instrument precision was examined by performing the intra-day and inter-day assays of six replicate injections of the standard solutions at three concentration levels (10, 40, 100µg/ml). The intra-day assay precision was performed with the interval of 4 h in 1 day, while the inter-day assay precision was performed over 6 days.

c) Limit of detection and limit of quantification

LOD and LOQ were determined by kSD/s where k is a constant (3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

d) Specificity

Specificity is the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix. It was checked by determining the standards in laboratory prepared ternary mixtures. Moreover, the proposed method was applied to the proprietary formulation also.

e) Robustness

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 218 nm to 216 nm and 220 nm

2) Column: Using another column (Hypersil ODS, particle size 5 µm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile and methanol supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

Standard solution was injected 6 times for each change. System suitability parameters like resolution, peak asymmetry, theoretical plates, capacity factor, and RSD were calculated for each peak. Recoveries and % RSDs were calculated for each component during each change.

f) Accuracy

The accuracy of the method was determined by calculating the recoveries of solanine by the method of standard additions. Known amounts of standard (80%, 100% and 120%) was added to the pre-analyzed sample solution, and the amount of the standard was estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

4.9.3.2) HPTLC method

4.9.3.2.1) Chemicals and Reagents

Chloroform, Methanol and Ammonia of analytical grade were purchased from Qualigens (Mumbai). Draggendorff's Reagent was prepared as per reported method.

4.9.3.2.2) Preparation of standard solutions

A 100 µg/ml solution of solanine was prepared in methanol.

4.9.3.2.3) Preparation of sample solutions

The methanol extract of the powdered formulation was prepared by sonication for 20 minutes. The extract was concentrated and evaporated under vacuum. 100 mg of dry extract was weighed and dispersed in methanol in 10 ml volumetric flask. The volume was made up to 10 ml.

4.9.3.2.4) Application of test samples

The samples were spotted in the form of bands of width 6 mm on the pre-coated silica gel G plates. The plates were pre-washed by methanol and activated at 110° C for 5 min prior to chromatography. A constant application rate of 0.1 µl/s was employed and space between two bands was 6 mm. For Calibration Curve of solanine the stock solution was used. Different volumes from 1.6-4.8 µl of stock solution were applied, which gave different concentration 1600-4800 ng per spot respectively.

4.9.3.2.5) Development

The mobile phase consisted of Chloroform: Methanol: Ammonia (7:3:0.5 v/v) Linear ascending development was carried out in trough chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 15 minutes at room temperature. The length of chromatogram run was 85 mm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air-dryer.

4.9.3.2.6) Detection

Concentrations of the compound chromatographed were determined from intensity of diffusely reflected light. The slit dimension was kept at 5 mm x 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. The plate was sprayed with Dragendorffs reagent immediately scanned and quantified at 500 nm using the Camag TLC Scanner-3. Data of peak area of each band were recorded. A Calibration curve was obtained by plotting peak area Vs. concentration and peak height Vs. concentration of solanine. Spectra of the samples and standard solanine were matched.

4.9.3.2.7) Method Validation

a) Calibration Curve of solanine.

A stock solution of solanine (1000 µg/mL) was prepared in methanol and bands in the range of 1600-4800 ng per spot were applied. The drug was spotted in duplicate on TLC plate to obtain concentrations of 1600-4800 ng per spot of solanine. The data of peak height/ area versus drug concentration were treated by linear least-square regression.

b) Precision

The intra-day and inter-day variation for the determination of solanine was carried out at two different concentration levels 1600 and 4800 ng per spot.

c) Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions were tried at two different concentration levels of 1600 and 4800 ng per spot.

d) Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times. The signal-to-noise ratio was determined. An LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were experimentally verified by diluting known concentrations of solanine until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

e) Recovery studies

The analyzed samples were spiked with extra 80, 100 and 120 % of the standard solanine and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different levels in the formulations.

4.9.4) Development of analytical methods for estimation of piperine in Mahamrutyunjaya rasa

4.9.4.1) HPTLC Method

4.9.4.1.1) Chemicals and Reagents

Toluene, ethyl acetate and methanol of analytical grade were purchased from Qualigens (Mumbai). Draggendorff's Reagent was prepared as per reported method.

4.9.4.1.2) Preparation of standard solutions

A stock solution of 1000 µg/ml was prepared in methanol. A 20 µg/ml solution was prepared in methanol using the stock solution.

4.9.4.1.3) Preparation of sample solutions

The methanol extract of the powdered formulation was prepared by sonication for 20 minutes. The extract was concentrated and evaporated under vacuum. 100 mg of dry extract was weighed and dispersed in methanol in 10 ml volumetric flask. The volume was made up to 10 ml.

4.9.4.1.4) Application of test samples

The samples were spotted in the form of bands of width 6 mm on the pre-coated silica gel G plates. The plates were pre-washed by methanol and activated at 110 °C for 5 min prior to chromatography. A constant application rate of 0.1 µl/s was employed and space between two bands was 6 mm. For calibration curve of Piperine the 20 µg/ml solution was used. Different volumes from 2-10 µl of the solution were applied, which gave different concentration 40-200 ng per spot respectively.

4.9.4.1.5) Development

The mobile phase consisted of Toluene: Ethyl acetate (7:3 v/v). Linear ascending development was carried out in trough chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 15 minutes at room temperature. The length of chromatogram run was 85 mm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air-dryer.

4.9.4.1.6) Detection

The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum. Concentrations of the compound chromatographed were determined from intensity of diffusely reflected light. The slit dimension was kept at 5 mm x 0.45 mm and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut off filter, each track was scanned thrice and baseline correction was used. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. The plate was scanned at 343 nm. The plate was sprayed with Dragendorffs reagent immediately scanned and quantified at 500 nm using the Camag TLC Scanner-3. Data of peak area of each band were recorded. A Calibration curve was obtained by plotting peak area Vs. concentration and peak height Vs. concentration of piperine. Spectra of the samples and standard piperine were matched.

4.9.4.1.7) Method Validation

a) Calibration Curve of piperine.

A solution of piperine (20 µg/mL) was prepared in methanol and bands in the range of 40-200 ng per spot were applied. The drug was spotted in duplicate on TLC plate to obtain concentrations of 40-200 ng per spot of piperine. The data of peak height/area versus drug concentration were treated by linear least-square regression.

b) Precision

The intra-day and inter-day variation for the determination of piperine was carried out at two different concentration levels 40 and 200 ng per spot.

c) Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions were tried at two different concentration levels of 40 and 200 ng per spot.

d) Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times. The signal-to-noise ratio was determined. An LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were experimentally verified by diluting known concentrations of piperine until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

e) Recovery studies

The analyzed samples were spiked with extra 80, 100 and 120 % of the standard piperine and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different levels in the formulations.

4.9.4.2) Stability indicating HPLC method for determination of Piperine.

4.9.4.2.1) Reagents

Acetonitrile and water (HPLC grade) were purchased from Qualigens. Ltd., (Mumbai, India). Hydrochloric acid, sodium hydroxide pellets, ortho phosphoric acid and hydrogen peroxide solution (all analytical reagent grades) were purchased from Qualigens Fine chemicals (Mumbai, India).

4.9.4.2.2) Preparation of standard solution

Standard stock solution of piperine (1000 µg/mL) was prepared by dissolving 25 mg of pure drug in 25 mL water in volumetric flask. Suitable dilutions were made from above stock solution to obtain the 20 and 500 µg/mL of drug concentration for degradation study.

4.9.4.2.3) Preparation of sample solution

Twenty tablets from each brand of one batch were accurately weighed, their mean weight determined and powdered in a glass mortar. An amount of the tablet mass equivalent to 1 gm was transferred to a 25 mL volumetric flask and sonicated with about 20 mL of methanol using an ultrasonic bath for 10 min, further diluted to volume with same solvent and this solution was used for the degradation studies.

4.9.4.2.4) Forced degradation studies of Marker and formulations

In order to establish whether the analytical method was stability-indicating, tablets sample solution and standard solution of pure piperine were stressed under various conditions to conduct forced degradation studies. From the above stock solutions of standard drug and sample, 2 mL of aliquots were diluted separately upto 10 mL with 3% H_2O_2, distilled water, 1M HCl and 1M NaOH, to achieve a concentration of 200 µg/mL of Piperine. Solutions in water, 1M HCl and 1M NaOH were heated at 80 °C for 24 h. For oxidative degradation, drugs were stored at room temperature (r.t.) in 3% H_2O_2 for 24 h. Degradation was also carried out in solid state by exposing pure drugs and drug product to dry heat at 80 °C for 48 h. Photolytic studies were carried out by exposing a thin layer of solid aconitine placed in a petri-dish, also the solutions of drugs and samples in 1M HCl, 1M NaOH and water to sunlight for 24 hrs. Suitable controls were maintained under dark conditions. Samples were withdrawn

initially and subsequently at prefixed time intervals. Samples were neutralized by acid or alkali, wherever necessary and were diluted with mobile phase to yield starting concentrations of 50 and 100 µg/mL of piperine. Appropriate blanks were injected before analysis of forced degraded samples.

4.9.4.2.5) Chromatographic conditions

The chromatographic separation was achieved on an Hypersil C_{18} (250 mm × 4.6 mm, 5 µm) column using a mobile phase containing mixture of acetonitrile-KH_2PO_4 buffer solution (10 mM, adjusted to pH 7.0 ± 0.1 with 1% Triethylamine) (35: 65, v/v). The mobile phase was filtered through a nylon filter (pore size 0.45 µm) and degassed before use. The flow rate of the mobile phase was 0.7ml/min and the wavelength was monitored at 343 nm.

4.9.4.2.6) Method validation

Linearity was studied by injecting seven concentrations of the standard piperine (10-100 µg/mL) in triplicate into the HPLC system. The peak area versus concentration data was performed by least-squares linear regression analysis. The LOD and LOQ values were calculated from kSD/b where k= 3 for LOD and 10 for LOQ. SD is the standard deviation of the responses of the minimum detectable drug concentration and b is the slope of the calibration curve. For intra-day precision, 3 different concentrations of sample solutions of piperine (10, 40 and 100 µg/ml) were analyzed six times on the same day whereas for inter-day precision same drug concentrations were analyzed on six different days and the percentage RSD of area was calculated. Accuracy was evaluated by fortifying the degraded solutions of Piperine with three different levels of standard solution of Piperine (80%, 100% and 120%) and calculating the percent recovery from the differences between the peak areas obtained for the fortified and unfortified solutions. The specificity of the method was established through study of resolution factors (R_s) of the drug peaks from the nearest resolving peak, and also among all other peaks. Specificity of the method towards the drugs was also established through determination of purity of Piperine peak in stressed samples through study of purity plots using a PDA detector.

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 343 nm to 341 nm and 345 nm.
2) Column: Using another column (Phenomenex, particle size 5 µm; 250 mm X 4.6 mm ID)
3) Solvent Brand: Acetonitrile and methanol supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

In the system suitability tests, six replicate injections of freshly prepared working standard solutions of piperine (50 µg/mL) and two injections of the solutions prepared for the specificity procedure were injected into the chromatograph, and the % relative standard deviation (% RSD) of peak areas, resolution factor, tailing factor and theoretical plates were determined.

4.9.5) HPLC method development for simultaneous estimation of Aconitine, Solanine and Piperine.

4.9.5.1) Preparation of Standard solution

Approximately 5 mg of standard Aconitine, 5 mg of standard Solanine and 5 mg of standard Piperine were weighed precisely and dissolved in 5 ml of acetonitrile obtaining stock concentrations of 1000 µg/ml. Aliquots of each standard were mixed to obtain solutions in the range of 10-100 µg/ml in acetonitrile. The stock solutions were refrigerated and were found to be stable for few weeks.

4.9.5.2) Preparation of sample solution

About 1gm each of the three formulations were powdered and extracted in 25 ml of 0.1N HCl by sonication for 10 minutes at room temperature which was then fractionated with 10 ml ethyl acetate thrice to remove the non-alkaloid components. The acidic aqueous solutions were basified using ammonia to pH 11 and further extracted with 10 ml chloroform thrice. Chloroform was evaporated under reduced pressure. The residue was dissolved in methanol by sonication and further dilutions were made in acetonitrile.

4.9.5.3) Analytical conditions

Analysis was isocratic at 1.0 ml/min flow rate with acetonitrile-KH_2PO_4 buffer solution (10 mM, adjusted to pH 7.5 $\pm$ 0.1 with 1% Triethylamine)-methanol (60:25:15, v/v/v) as mobile phase. The mobile phase was prepared freshly everyday. The mobile phase was filtered through a 0.2 µm membrane filter to

remove any particulate matter, mixed and degassed by sonication before use. The absorbance of Aconitine and Solanine were good at 223 nm and further it was free from any interference. Further, Piperine showed maximum absorbance at 343 nm. Hence, the eluted peaks were detected at 223 nm and 343 nm. The sensitivity of the detector was set at 0.01 AUFS. Prior to injecting solutions, the column was equilibrated for at least 60 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (RSD) was required to remain below 1.0% on peak area basis.

4.9.5.4) Optimization of Chromatographic conditions

Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers. Therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, mobile phase compositions, and flow rate to check the retention time, shape, resolution, and other system suitability parameters of all the peaks.

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase pH varied at 7, 7.5, and 8 keeping the composition of acetonitrile-KH_2PO_4 buffer (10 mM)-methanol (60:25:15 v/v/v) and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 65:15:15, 60:25:15 and 55:35:15 (v/v/v) with pH and flow rate kept constant at 7.5 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 60:25:15 (v/v/v) and 7.5, respectively. The effects of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the buffer system with the organic solvent in the desired proportions.

4.9.5.5) Validation

In order to verify that the proposed method is applicable to formulation analysis, validation was performed as per the ICH Guidelines.

a) Calibration curve (linearity)

Seven different concentrations of aconitine, solanine and piperine were analyzed and their calibration curve was constructed in the specified concentration range (10-100 μg/ml). The calibration plots were generated by replicate analysis (n = 3) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

b) Repeatability, precision and stability

The injection repeatability was determined by the analysis of six continuous injections using the same sample, while the analysis repeatability was examined by the injection of six different samples prepared by the same procedure. The mixture of standard solutions (40 μg/ml) was used for the test of injection repeatability and analysis repeatability.

The instrument precision was examined by performing the intra-day and inter-day assays of six replicate injections of the mixture of standard solutions at three concentration levels (10, 40, 100μg/ml). The intra-day assay precision was performed with the interval of 4 h in 1 day, while the inter-day assay precision was performed over 6 days.

c) Limit of detection and limit of quantification

LOD and LOQ were determined by kSD/s where k is a constant (3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

d) Specificity

Specificity is the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix. It was checked by determining the standards in laboratory prepared ternary mixtures. Moreover, the proposed method was also applied to the proprietary formulation.

e) Robustness

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 223 nm to 221 nm and 225 nm for Aconitine and solanine, while for piperine the wavelength was changed from 343 nm to 341 nm and 345 nm.

2) Column: Using another column (Hypersil ODS, particle size 5 µm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile and methanol supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

Standard solution was injected 6 times for each change. System suitability parameters like resolution, peak asymmetry, theoretical plates, capacity factor, and RSD were calculated for each peak. Recoveries and % RSDs were calculated for each component during each change.

f) Accuracy

The accuracy of the method was determined by calculating the recoveries of Aconitine, Solanine and Piperine by the method of standard additions. Known amounts of these standards (80%, 100% and 120 %) were added to the pre-analyzed sample solution, and the amounts of these standards were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

4.9.6) Development of HPLC method for the simultaneous estimation of Aconitine and solanine.

4.9.6.1) Preparation of Standard Solutions

Standard stock solutions of aconitine and solanine (1000 µg/ml) were prepared separately by dissolving 10 mg of pure drug in 10 ml methanol. Binary mixture was prepared by taking appropriate and accurate volume aliquots of the stock solutions in 10 ml calibrated flasks and diluting to the volume with methanol in the range of 10-100 µg/ml.

4.9.6.2) Preparation of sample solutions

Twenty tablets of all the three formulations were powdered and about 1 gm each of the three formulations was accurately weighed and basified using ammonia and extracted with chloroform (3 X 25 ml). The total extract was concentrated and evaporated. About 10 mg of the extract was dissolved in methanol in a 10 ml volumetric flask and sonicated. The volume was made up and further dilutions were made.

4.9.6.3) *Analytical conditions*

Analysis was isocratic at 1.0 ml/min flow rate with Phosphate buffer (10 mM, pH 7.5): acetonitrile: methanol (15:70:15, v/v/v) as mobile phase. The mobile phase was prepared freshly everyday. The mobile phase was filtered through a 0.2 µm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. The absorbance of aconitine and solanine were good at 223 nm and further it was free from any interference. Hence, the eluted peak was detected at 223 nm. The sensitivity of the detector was set at 0.01 AUFS. Prior to injecting solutions, the column was equilibrated for at least 60 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the %RSD was required to remain below 1.0% on peak area basis.

4.9.6.4) *Optimization of Chromatographic conditions*

Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers. Therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, mobile phase compositions, and flow rate to check the retention time, shape, resolution, and other system suitability parameters of all the peaks.

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase pH varied at 7,7.5 and 8 keeping the composition of Phosphate buffer (10 mM, pH 7.5 was adjusted using 1% Triethylamine): acetonitrile: methanol (15:70:15 v/v/v) and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 20:65:15, 15:70:15 and 10:75:15 (v/v/v) with pH and flow rate kept constant at 7.5 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 15:70:15 (v/v/v) and 7.5, respectively. The effects of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the buffer system with the organic solvent in the desired proportions.

4.9.6.5) Method Validation:

In order to verify that the proposed method is applicable to formulation analysis, validation was performed as per the ICH Guidelines.

a) Calibration curve (linearity)

Seven different concentrations of solanine and aconitine were analyzed and their calibration curves were constructed in the specified concentration range (10-100 µg/ml). The calibration plots were generated by replicate analysis (n = 3) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

b) Repeatability, precision and stability

The injection repeatability was determined by the analysis of six continuous injections using the same samples, while the analysis repeatability was examined by the injection of six different samples prepared by the same procedure. The standard solution (40 µg/ml) was used for the test of injection repeatability and analysis repeatability.

The instrument precision was examined by performing the intra-day and inter-day assays of six replicate injections of the standard solutions at three concentration levels (10, 40, 100µg/ml). The intra-day precision was performed with the interval of 4 h in 1 day, while the inter-day precision was performed over 6 days.

c) Limit of detection and limit of quantification

LOD and LOQ were determined by kSD/s where k is a constant (3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

d) Specificity

Specificity is the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix. It was checked by determining the standards in laboratory prepared binary mixtures. Moreover, the proposed method was also applied to the proprietary formulations.

e) Robustness

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 227 nm to 225 nm and 229 nm

2) Column: Using another column (Hypersil ODS, particle size 5 µm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile and methanol supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

Standard solution was injected 6 times for each change. System suitability parameters like resolution, peak asymmetry, theoretical plates, capacity factor, and RSD were calculated for each peak. Recoveries and % RSDs were calculated for each component during each change.

f) Accuracy

The accuracy of the method was determined by calculating the recoveries of Aconitine and solanine by the method of standard additions. Known amounts of these standards (80%, 100% and 120 %) were added to the pre-analyzed sample solution, and the amounts of these standards were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

4.9.7) HPLC method development for simultaneous estimation of Solanine and Piperine.

4.9.7.1) Preparation of Standard solutions

Approximately 5 mg of standard Solanine and 5 mg of standard Piperine were weighed precisely and dissolved in 5 ml of acetonitrile obtaining stock concentrations of 1000 µg/ml. Aliquots of each standard were mixed to obtain solutions in the range of 1-100 µg/ml in acetonitrile. The stock solutions were refrigerated and were found to be stable for few weeks.

4.9.7.2) Preparation of sample solutions

About 1gm each of the three formulations were powdered and extracted in 25 ml of 0.1N HCl by sonication for 10 minutes at room temperature which was then fractionated with 10 ml ethyl acetate thrice to remove the non-alkaloid components. The acidic aqueous solutions were basified using ammonia to pH 11 and further extracted with 10 ml chloroform thrice. Chloroform was evaporated under reduced pressure. The residue was dissolved in methanol by sonication and further dilutions were made in acetonitrile.

4.9.7.3) Analytical conditions

Analysis was isocratic at 1.0 ml/min flow rate with acetonitrile-KH_2PO_4 buffer solution (10 mM, adjusted to pH 7.5 ± 0.1 with 1% Triethylamine)-methanol (55:20:15, v/v/v) as mobile phase. The mobile phase was prepared freshly everyday. The mobile phase was filtered through a 0.2 µm membrane filter to remove any particulate matter, mixed and degassed by sonication before use. The absorbance of Solanine was good at 223 nm and was free from any interference. Further, Piperine showed maximum absorbance at 343 nm. Hence, the eluted peaks were detected at 223 nm and 343 nm. The sensitivity of the detector was set at 0.01 AUFS. Prior to injecting solutions, the column was equilibrated for at least 60 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (RSD) was required to remain below 1.0% on peak area basis.

4.9.7.4) Optimization of Chromatographic conditions

Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers. Therefore, before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, mobile phase compositions, and flow rate to check the retention time, shape, resolution, and other system suitability parameters of all the peaks.

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase pH varied at 7, 7.5, and 8 keeping the composition of acetonitrile-KH_2PO_4 buffer (10 mM)- methanol (55:20:15 v/v/v) and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 60:15:15, 55:20:15 and 50:25:15 (v/v/v) with pH and flow rate kept constant at 7.5 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 55:20:15(v/v/v) and 7.5, respectively. The effects of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the buffer system with the organic solvent in the desired proportions.

4.9.7.6) Method Validation

In order to verify that the proposed method is applicable to formulation analysis, validation was performed as per the ICH Guidelines.

a) Calibration curve (linearity)

Seven different concentrations of Solanine and Piperine were analyzed and their calibration curve was constructed in the specified concentration range (10-100 µg/ml). The calibration plots were generated by replicate analysis (n = 3) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

b) Repeatability, precision and stability

The injection repeatability was determined by the analysis of six continuous injections using the same sample, while the analysis repeatability was examined by the injection of six different samples prepared by the same procedure. The mixture of standard solutions (40 µg/ml) was used for the test of injection repeatability and analysis repeatability.

The instrument precision was examined by performing the intra-day and inter-day assays of six replicate injections of the mixture of standard solutions at three concentration levels (10, 40, 100µg/ml). The intra-day assay precision was performed with the interval of 4 h in 1 day, while the inter-day assay precision was performed over 6 days.

c) Limit of detection and limit of quantification

LOD and LOQ were determined by kSD/s where k is a constant (3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal, and s is the slope of the concentration/response graph.

d) Specificity

Specificity is the ability of the analytical method to measure analyte response in the presence of interferences present in the sample matrix. It was checked by determining the standards in laboratory prepared binary mixtures. Moreover, the proposed method was also applied to the proprietary formulations.

e) Robustness

Robustness of the proposed method was evaluated by keeping the chromatographic conditions constant, except for the followings changes:

1) Detection wavelength: Changed from 223 nm to 221 nm and 225 nm for Solanine, while for Piperine the wavelength was changed from 343 nm to 341 nm and 345 nm.

2) Column: Using another column (Hypersil ODS, particle size 5 µm; 250 mm X 4.6 mm ID)

3) Solvent Brand: Acetonitrile and methanol supplied by Spectrochem Pvt Ltd. (Mumbai, India) and Rankem (Mumbai, India).

Standard solution was injected 6 times for each change. System suitability parameters like resolution, peak asymmetry, theoretical plates, capacity factor, and RSD were calculated for each peak. Recoveries and % RSDs were calculated for each component during each change.

f) Accuracy

The accuracy of the method was determined by calculating the recoveries of Solanine and Piperine by the method of standard additions. Known amounts of these standards (80%, 100% and 120 %) were added to the pre-analyzed sample solution, and the amounts of these standards were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

4.9.8) Development of analytical methods for the estimation of Sulphur in Mahamrutyunjaya rasa.

4.9.8.1) Chemicals and Reagents

Pure Sulphur was purchased from Qualigens, (Mumbai, India). Sodium hydroxide, Toluene and Hydrazine hydrate of analytical grade was purchased from Rankem (Mumbai, India). Double distilled water was used to prepare all the solutions.

4.9.8.2) Preparation of Reagents.

0.1 N NaOH was prepared using double distilled water. An aliquot of 25 ml of Hydrazine hydrate was taken in a volumetric flask and volume was made up using 0.1 N NaOH.

4.9.8.3)Preparation of standard sulphur solution.

10 mg of standard sulphur was accurately weighed and transferred to a 10 ml volumetric flask. The Hydrazine hydrate solution was added and sonicated for

10 minutes. The volume was made up using the hydrazine hydrate solution. From this solution, further dilutions were made in 0.1N NaOH to obtain solutions in the range of 20-70 µg/ml of sulphur.

4.9.8.4) Preparation of sample solution.

Twenty tablets were powdered and about 500 mg was weighed accurately and transferred to a conical flask. 50 ml of toluene was added to the powdered material and the mixture was sonicated for 20 minutes. The mixture was filtered using Whatmann No.42 filter paper. The procedure was repeated using the same residue. The extract was collected and evaporated to dryness. The extractive value was calculated. About 10 mg of the residue was weighed and transferred to a 10 ml volumetric flask and volume was made up using Hydrazine hydrate solution. Further dilutions were made to set the concentration in the linearity range.

4.9.8.5) General procedure

The absorbances of yellow colored standard and sample solutions were measured at 368 nm against the corresponding blank solution. The reagent blank solution was prepared in the same manner without Sulphur. Calibration plots were drawn to calculate the amount of drugs in analyte sample solution. All measurements were made at room temperature.

4.9.8.6) Optimization of conditions

4.9.8.6.1) Spectral characteristics

The absorption spectra of hydrazine sulphate, was measured in the range 350–550 nm against the blank solution. The colorless reagent blank has practically no absorption at these wavelengths in all cases. The variables like concentration of NaOH and Hydrazine hydrate were studied to determine the optimum concentrations and their effect on the position of their λ_{max} and the shape of these absorption spectra.

4.9.8.6.2) Effects of Hydrazine hydrate concentration

The effect of the concentration of hydrazine hydrate was tested by adding different volumes of hydrazine hydrate in the range 0.5-5.0 mL, to a constant amount of sulphur.

4.9.8.6.3) Effects of pH on the ion-pair formation

The effect of concentration of NaOH on the hydrazine sulphate was studied over the concentration range of 0.01 N- 0.5N NaOH.

4.9.8.6.4) Effect of Diluting solvent

The most favourable solvent as dilution medium was studied using Sodium hydroxide, Hydrochloric acid and water. The final dilutions were made using the solvents and scanned in the range of 350-500 nm. The stability of the samples in the solvents was also checked by scanning the same samples at equal interval of time.

4.9.8.6.5) Composition of ion-pair extraction

The composition of the compound was established by Job's method[218] of continuous variation.

4.9.8.6.6) Optimisation of extraction conditions

The concentration of sulphur extracted in different solvents like hexane, toluene and benzene was studied. The volume of toluene required for extraction of sulphur was also optimized.

4.9.8.7) Method Validation

a) Linearity and range

The linearity of the hydrazine sulphate was evaluated by analysing a series of different concentrations of sulphur. In this study, seven different concentrations of sulphur were chosen within the linearity range, and each was repeated three times. A linear relationship was found between the absorbance and the concentration of sulphur in the range 20-70 µg/ml. The optical characteristic such as Beer's law limits, molar absorptivity and regression equation (slope, intercept and correlation coefficient) were determined for the method. The high molar absorptivities of the resulting colored solution indicate the high sensitivity of the proposed method.

b) Detection and Quantitation Limits

The LOD and LOQ values for hydrazine sulphate were calculated from the calibration curves as kSD/b where k=3 for LOD and 10 for LOQ, SD is the standard deviation of the intercept and b is the slope of the calibration curve.

c) Precision

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels 20, 40 and 70 µg/ml for sulphur. The experiments were repeated six times a day for intra-day precision and on three different days for inter-day precision.

d) Accuracy

To confirm the accuracy of proposed method, recovery study was performed by the standard addition technique. Three different levels (80,100 and 120%) of standards were added to pre-analyzed tablet samples, and each level was repeated three times.

4.9.9) Development of analytical methods for the estimation of Boron in Mahamrutyunjaya rasa.

4.9.9.1) Spectrofluorimetric method.

4.9.9.1.1) Chemicals and Reagents

Sodium metaborate was purchased from Qualigens. Alizarin red S was purchased from Fluka. The working solutions were prepared daily. The other chemicals used throughout the study were of analytical grade. All the solutions were prepared with triple distilled water.

4.9.9.1.2) Preparation of standard solution

Stock solution was prepared by weighing sodium metaborate equivalent to 10mg of boron and diluting to 100ml with triple distilled water. The solution was stored in a polyethylene bottle.

4.9.9.1.3) Preparation of Reagents

Alizarin Red S *solution* (1mM) was prepared by weighing exactly 0.171 g of the reagent and diluting to 500 ml with doubly distilled water.

Buffer solution (pH 7.4) was prepared by mixing 0.1M disodium hydrogen phosphate with 0.1M potassium dihydrogen phosphate.

4.9.9.1.4) Preparation of sample solution

About 1 g of powdered formulation was weighed accurately in a tarred silica crucible. The sample was incinerated in a muffle furnace at 500 °C for 2 h and then left to cool inside the furnace. The ash was humidified using triple distilled water, and 1 ml of concentrated hydrochloric acid was added. The mixture was

heated at 70 °C on a heating mantle. The solution was filtered and washed with hot water. 5 ml of 0.1M EDTA solution was added. The solution was neutralized using sodium hydroxide solution and transferred into a 25 ml calibrated flask and diluted to the mark with doubly distilled water. Different aliquots of not more than 5 ml were taken for the determination of boron with Alizarin Red S by the procedure described above for the samples.

4.9.9.1.5) Instrumental Parameters:

Scan speed: Medium, Abscissa scale: 2, Ordinate scale: 6, Sensitivity:1, coarse and 1 fine; Slit-widths: 5 nm for excitation and 3 nm for emission; Excitation wavelength:470 nm; and Emission wavelength: 570 nm.

4.9.9.1.6) General Procedure for the Determination of Boron

A suitable aliquot containing 0.25-10 µg/ml of Boron was transferred in a 10ml calibrated flask. 1 ml of buffer solution and 1.5 ml Alizarin Red S were added and diluted to the mark with triple distilled water. Standard solution of sodium metaborate was scanned in the range of 300-600 nm using spectrofluorometer and the excitation wavelength was determined to be 470 nm. Same solution was scanned for emission wavelength using the spectrofluorimeter in the range of 471-600 nm keeping 470 nm as the excitation wavelength and it was found to be 570 nm. Each time the solvent blank was also scanned.

The variables like pH, reagent concentration, order of addition of reagents and temperature were studied to determine the optimum conditions and the effect of the variables on the position of their excitation and emission wavelength and the intensity of these spectra.

4.9.9.1.7) Effect of pH

The effect of the pH of the medium on the fluorescence intensity was studied in the range of 3.0-9.0. The pH was maintained using HCl and NaOH solution.

4.9.9.1.8) Effect of Reagent concentration

The effect of reagent concentration on the fluorescence intensity for solutions containing 10 µg/ml of boron was studied. The fluorescence intensity was studied with respect to the the reagent concentration in the range of 0.05 mM to 0.2 mM.

4.9.9.1.9) Effect of Order of Addition of Reagents

The sequence of addition of analyte-reagent-buffer was studied by one variation at a time. The effect on the fluorescence intensity of 5 µg/ml of boron was used for the study.

4.9.9.1.10) Effect of Temperature

The effect of temperature on the measurements was studied by heating a series of samples to between 5 and 50°C in a water bath. An increase in temperature from 5 to 50°C reduced the fluorescence intensity, it was found necessary to maintain the temperature at 25 °C.

4.9.9.1.11) Stability of the samples

The fluorescence intensity of the boron-Alizarin Red S complex was measured over a period of 24 h at equal intervals after sample preparation.

4.9.9.1.12) Influence of Foreign Ions

In order to assess the possible analytical applications of this fluorescence reaction, the effect of a number of foreign ions was studied by carrying out determinations of 15 µg/ml of boron in the presence of each of these ions. The criterion for interference was a fluorescence intensity value varying by more than 5% from the expected value for boron alone.

4.9.9.1.13) Method Validation

a) Linearity and range

The linearity of the Boron-Alizarin red S complex was evaluated by analyzing a series of different concentrations of Boron. In this study, seven different concentrations of Boron were chosen within the linearity range, and each was repeated three times. A linear relationship was found between the absorbance and the concentration of the Boron in the range 7.5-25 µg/ml. The optical characteristics such as linearity range and regression equation (slope, intercept, correlation coefficient and standard error of estimation) were determined for the method.

b) Detection and Quantitation Limits

The LOD and LOQ values for Boron were calculated from the calibration curves as kSD/b where k=3 for LOD and 10 for LOQ, SD is the standard deviation of the intercept and b is the slope of the calibration curve.

c) Precision

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels (7.5, 15 and 25 µg/ml) for Boron. The experiments were repeated six times a day for intra-day precision and on three different days for inter-day precision.

d) Accuracy

To confirm the accuracy of proposed method, recovery study was performed by the standard addition technique. Three different levels (80,100 and 120%) of standards were added to pre-analyzed tablet samples, and each level was repeated three times.

e)Robustness

The robustness of the method was checked by examining the reflection of the slight changes to the results in methodological parameters. The brand of reagents used in the analysis was purposely altered in order to determine the robustness of the method.

4.9.9.2) Voltammetric method

4.9.9.2.1) Chemicals and reagents

Sodium metaborate was purchased from Qualigens. Alizarin red S was purchased from Fluka. The working solutions were prepared daily. The other chemicals used throughout the study were of analytical grade. All the solutions were prepared with triple distilled water.

4.9.9.2.2) Preparation of standard solution

Stock solution was prepared by weighing sodium borate equivalent to 10 mg of boron and diluting to 100 ml with triple distilled water. The solution was stored in a polyethylene bottle.

4.9.9.2.3) Preparation of Reagents

Alizarin Red S *solution,* (1mM) was prepared by weighing exactly 0.171 g of the reagent and diluting to 500 ml with doubly distilled water.

Buffer solution (pH 7) was prepared by mixing 1M ammonium acetate and 1M potassium dihydrogen phosphate.

4.9.9.2.4) Preparation of sample solution

About 1 g of powdered formulation was weighed accurately in a tarred silica crucible. The sample was incinerated in a muffle furnace at 500 °C for 2 h and then left to cool inside the furnace. The ash was humidified using triple distilled water, and 1 ml of concentrated hydrochloric acid was added. The mixture was heated at 70 °C on a heating mantle. The solution was filtered and washed with hot water. 5ml of 0.1M EDTA solution was added. The solution was neutralized using sodium hydroxide solution and transferred into a 25 ml calibrated flask and diluted to the mark with doubly distilled water. Different aliquots of not more than 5 ml were taken for the determination of boron with Alizarin Red S by the procedure described above for the samples.

4.9.9.2.5) General procedure

100 µl of 0.01 M Alizarin S Red, 1ml of 1M ammonium acetate-phosphate supporting electrolyte (pH 7) and the required amount of Boron was pippetted into a 10 ml of volumetric flask and completed to 10 ml with distilled water. The solution was transferred to the quartz voltammetric cell and nitrogen was purged for 5 minutes. A new drop of mercury was extruded and the stripping was initiated immediately in the anodic direction starting from -700 mV by using differential pulse modulation without being stirred. The instrumental conditions were potential scan rate of 5 mV/s, pulse duration 0.02s and pulse amplitude 50 mV. All the measurements were performed at room temperature. The sample analysis was performed in the presence of 1 mM EDTA for the elimination of interference from metal ions.

4.9.9.2.6) Effect of pH and supporting electrolyte.

The peak current was measured by varying the pH in the range of 5.0-8.0. Additionally, different electrolytes at pH 7.0 were tested as supporting electrolyte (ammonium acetate, phosphate, ammonium actate-phosphate mixture, sodium nitrate, ammonium chloride, potassium perchlorate, sodium bromate). Among these, 0.1 M ammonium acetate and 0.1 M phosphate mixture (pH 7.0) has given the maximum peak current.

4.9.9.2.7) Effect of Alizarin Red S concentration

The effect of the concentration of Alizarin red S on peak current of the Boron-ARS complex was tested. Various concentrations of the Alizarin red S ranged from 0.1-10 µM in the presence of 5 µg/ml boron and supporting electrolyte at pH 7.0 were studied. The results have shown that the peak height of boron-Alizarin red S complex increases with the ligand concentration.

4.9.9.2.8) Method Validation

a) Linearity and range

The linearity of the Boron was evaluated by analysing a series of different concentrations of Boron. In this study, five different concentrations of Boron-Alizarin red S complex were chosen within the linearity range, and each was repeated three times. A linear relationship was found between the current and the concentration of the complex in the range 1-10 µg/ml. The linearity range and regression equation (slope, intercept, correlation coefficient and standard error of estimation) were determined.

b) Detection and Quantitation Limits

The LOD and LOQ values for Boron were calculated from the calibration curves as kSD/b where k= 3 for LOD and 10 for LOQ, SD is the standard deviation of the intercept and b is the slope of the calibration curve.

c) Precision

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels 1, 5 and 10 µg/ml for Boron. The experiments were repeated six times a day for intra-day precision and on three different days for inter-day precision.

d) Accuracy

To confirm the accuracy of proposed method, recovery study was performed by the standard addition technique. Three different levels (80, 100 and 120%) of standards were added to pre-analyzed tablet samples, and each level was repeated three times.

e) Robustness

The robustness of the method was checked by examining the reflection of the slight changes to the results in methodological parameters. The brand of the

reagents used in the analysis was purposely altered in order to determine the robustness of the method.

4.9.9.3) Estimation of Boron using Inductively coupled plasma.

4.9.9.3.1) Preparation of standard solution

Standard stock solution of Boron in the concentration range of 100 µg/ml was prepared in deionized water.

4.9.9.3.2) Preparation of sample solution

Twenty tablets were triturated to fine powder and about 1 gm of each was incinerated in muffle furnace at 500° C until a white color was obtained. Solutions of the ash were dissolved in 0.1N HCl by sonication and the volume was made up in the concentration range of 5 PPM.

4.9.9.3.3) General Procedure

Boron was estimated in the formulations using inductively coupled plasma-AES (Perkin Elmer, Optical Emission Spectrometer, Optima 2100 DV attached to Winlab 32 software). A calibration plot was prepared using standard Boron solution in the range (1-6 µg/ml). The samples were analyzed at 249.43 nm. The estimation was repeated three times and the %RSDs were calculated.

4.9.10) Estimation of mercury using Inductively coupled plasma

4.9.10.1) Preparation of standard solution

Standard stock solution of Mercury in the concentration range of 100 µg/ml was prepared in aqueous aqua regia.

4.9.10.2) Preparation sample solution

Twenty tablets were triturated to fine powder and about 1 gm of each was digested with acid. The cinnabar was extracted in aqua regia and diluted in deionized water.

4.9.10.3) General Procedure

Mercury was estimated in the formulations using inductively coupled plasma-AES (Perkin Elmer, Optical Emission Spectrometer, Optima 2100 DV attached to Winlab 32 software). A calibration plot was prepared using standard mercury solution in the range (1-6 µg/ml). The samples were analyzed at 253.56 nm. The estimation was repeated three times and the RSDs were calculated.

4.10) Biological Standardization of the three formulations.

4.10.1) Materials and Methods

4.10.1.1) Animals

Balb/c female mice weighing between 20-25g and male albino rats of Wistar strain weighing between 200-270 g were used for the study. They were housed under standard environmental conditions and fed with commercial diet and water *ad libitum.* All experiments were carried out as per the guidelines of Institutional ethics committee, Pharmacy department/ M. S. University, Baroda/404/01/1/CPCSEA.

4.10.2) Acute toxicity study[220-221]

Toxicity study was carried out on formulations as per the OECD guidelines in female albino mice. Animals were dosed with single oral dose of 2000 mg/kg body weight and observed for mortality. If the animals remained alive additional animals were dosed with the same bolus. In case of mortality fresh mice were administered with lower dose in the range of 1.75, 5.5, 17.5, 55, 175, 550, 2000 mg/kg body weight. Histopathological examination of the visceral organs like heart, kidney and liver was done in order to observe the toxic effects of formulations. Mice were observed for any reactions like tremors, convulsions, salivation, and diarrhea.

4.10.3) Isoproterenol-induced myocardial infarction in rats (MI)[219, 222-224]

Serum levels of creatine kinase, lactate dehydrogenase and transaminases are the diagnostic indicators of myocardial infarction. An increase in the activity of these enzymes in serum is due to the leakage of enzymes from the heart as a result of necrosis induced by ISO. Increase in serum uric acid could be due to excessive degradation of purine nucleotides and proteolysis.

4.10.3.1) Experimental design

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

Group-I: Served as a control, received 1% aqueous solution of tween 80 p.o. for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs);

Group-II: Received 1% tween 80 (p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs);

Group-III: Treated with FORM1 (50mg/kg.b.w.p.o.) for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs);

Group-IV: Treated with FORM1 (50mg/kg.b.w.p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs);

Group-V: Treated with FORM2 (50mg/kg.b.w.p.o.) for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs);

Group-VI: Treated with FORM2 (50mg/kg.b.w.p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs);

Group-VII: Treated with FORM3 (50mg/kg.b.w.p.o.) for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs);

Group-VIII: Treated with FORM3 (50mg/kg.b.w.p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs).

Group-IX: Treated with FORM1 (100mg/kg.b.w.p.o.) for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs);

Group-X: Treated with FORM1 (100mg/kg.b.w.p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs);

Group-XI: Treated with FORM2 (100mg/kg.b.w.p.o.) for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs);

Group-XII: Treated with FORM2 (100mg/kg.b.w.p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs);

Group-XIII: Treated with FORM3 (100mg/kg.b.w.p.o.) for 15 days followed by normal saline (2 ml/kg, s.c. twice at an interval of 24 hrs);

Group-XIV: Treated with FORM3 (100mg/kg.b.w.p.o.) for 15 days followed by ISO (25 mg/kg, s.c. twice at an interval of 24 hrs).

The body weights of the animals were recorded through out the experimental period. At the termination point of the experiment the rats were sacrificed under light ether anesthesia and blood was collected by cutting the carotid artery and the serum was separated and used for the assay of marker enzymes like lactate dehydrogenase (LDH), creatine phosphokinase isoenzymes (CK-MB), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALKP) and uric acid. Heart was dissected out, washed in ice-cold saline and weighed accurately to calculate the ratio of heart weight/ body weight (HW/BW)

for each group. Apart from these histopathological studies of heart was also carried out.

4.10.3.2) Estimation of serum marker enzymes

a) Creatine kinase Isoenzyme (CKMB)

Quantitative estimation of CKMB was done using ENZOPAK-CK-NAC kit [Reckon Diagnostics Pvt. Ltd., Baroda]. In this reaction creatine kinase catalyses the formation of ATP from creatine phosphate and ADP. Glucose is converted to glucose-6-phosphate by hexokinase using ATP as a source for phosphate moiety. Glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to 6-phosphogluconate reducing NADP to NADPH. Their action after the lag phase is monitored by the increase in absorbance at 340 nm and is directly proportional to the creatine kinase activity (i.e. the formation of NADPH is in equimolar amount as that of formation of creatine.

b) Lactate dehydrogenase (LDH)

Quantitative estimation of lactate dehydrogenase (LDH) was done using ENZOPAK LDH L→P kit [Reckon Diagnostics Pvt. Ltd., Baroda]. LDH catalyses the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD. Units were expressed as U/L.

c) Uric acid

Quantitative estimation of uric acid was done using diagnostic kit [Span Diagnostics Ltd., Surat, India]. Uric acid in alkaline medium reduces phosphotungstic acid to "tungsten blue" a blue colored complex, which is measured colorimetrically. Units were expressed as mg/dL.

d) Aspartate aminotransferase (GOT)

Quantitative estimation of serum glutamate oxaloacetate transaminase (GOT) was done by using span diagnostic reagent kit [Span Diagnostics Ltd., Surat, India]. Oxaloacetate so formed is coupled with 2, 4-Dinitrophenyl hydrazine (2, 4-DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium and this can be measured colorimetrically. Units were expressed as U/L.

e) Alkaline Phosphatase (ALKP)

Quantitative estimation of alkaline phosphatase was done by using span diagnostic reagent kit [Span Diagnostics Ltd., Surat, India]. At pH 10.3 alkaline phosphatase catalyses the hydrolysis of p-nitrophenyl phosphate and the change in absorbance measured at 405 nm is directly proportional to enzyme activity. Units were expressed as IU/L.

4.10.3.3) Histopathological Studies

After the treatment period, the animals were sacrificed and heart was excised, made free of blood and tissue fluids and preserved in 4% v/v buffered normal saline solution. Tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5 μm thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The hematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were examined under Olympus BX40 photomicroscope and photographed.

4.10.4) In vitro studies using H9c2 cell lines

4.10.4.1) Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCO, Invitrogen Corporation, Carlsbad, California. 3-(4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide (MTT), streptomycin, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of the highest grade available.

4.10.4.2) Cell culture

The H9c2 cell line was originally derived from embryonic rat heart tissue using selective serial passages and was purchased from America Tissue Type Collection (Manassas, VA; catalog # CRL – 1446). Cells were cultured in DMEM medium supplemented with 10% 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. Cells were fed every 2–3 days, and sub-cultured once they reached 70 – 80% confluency in order to prevent the loss of differentiation potential.

Formulations were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was not more than 0.2 % during the experiments.

4.10.4.3) Analysis of cell viability

The viability of cells after treatment with formulations was assayed by the reduction of 3-(4, 5-dimethylthiazole-2-yl)-2, 5- diphenyl-tetrazoliumbromide (MTT) to formazan as described previously (Plumb, 1989). Briefly, cells were seeded in 96-well microtiter plates (5×10^3 cells per well), and left to adhere to the plates overnight before being exposed to different concentrations of test material. In each experiment, different concentrations of formulation (1, 2, 5, 10, 20, 50 and 100 µg/ml) were tested in three separate wells and the cytotoxicity curve was constructed from three different experiments. After exposure to formulation, 10 µl of 5 mM MTT solution was added to each well, and the cells were incubated in the dark at 37°C for an additional 4hrs. Thereafter, the medium was removed, the formazan crystals were dissolved in 100 µl of DMSO and the absorbance was measured at 570 nm in a microplate reader (Molecular Devices, Spectra MAX 250). The data of the survival curves were expressed as the percentage of untreated controls.

4.10.5) Statistical Analysis of Data

Results of all the in vivo experiments have been expressed as mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons test and Dunnett multiple comparisons tests. For i*n vitro* experiments values are expressed as mean± SEM.