4 Material and Methods

4.1 Material

4.1.1 Inducing and sensitizing agents:

Complete Freund's Adjuvant (CFA- each ml contains 1 mg heat killed and dried *Mycobacterium tuberculosis* in 0.85 ml paraffin oil and 0.15 ml mannide monooleate) and Lipopolysaccharide (cell wall components of gram negative bacteria *Escherichia coli* O111:B4) were purchased from Sigma-Aldrich, Bangalore. Bovine Collagen (Type II) was purchased from Chondrex Inc Redmond, WA supplied by Krishgen Biosystem Mumbai, India. (Annexure I)

4.1.2 Standard drug:

Methotrexate (Mtx) was obtained as gift sample from Alembic pharmaceuticals, Vadodara, Gujarat. (Annexure II)

4.1.3 Plant material for test drug:

Nigella sativa seed were purchased from a local grocery store from Siyagunj Indore, Madhya Pradesh and *Carica papaya* Seed were collected from papaya farm Japodad, Gujarat. Dried seeds of *Momordica charantia* were collected from a vegetable field situated at Khargone, Madhya Pradesh. The samples of the crude herbs were identified and authenticated by Dr. Padmanabhi S. Nagar, Assistant Professor, Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara. Reference no. for the authentication certificate is **Ref: Bot/30317/aut/1/ TD04.** The selected and prepared plant seed extracts were also compared and standardized by same extracts prepared by Ms. Swati and Mr. Vyankat Parihar from AMSAR Private limited 47, Laxmibai Nagar, Industrial Estate, Fort, Indore (Madhya Pradesh) for standardization of heavy metal content, microbial load and total extractive values of extracts (Annexure III).

4.1.4 Chemicals:

99% IPA (Isopropyl Alcohol/propane-2-ol) and Acetone was purchased from Thermo Fisher Scientific, Vadodara. Ethylenediamine tetra acetic acid (EDTA), Hydrochloric acid (HCL), Trisodium citrate was obtained from S.D. Fine Chemicals, Mumbai. Standard of

thymoquinone, gallic acid and chlorogenic acid were purchased from Sigma-Aldrich, Bangalore, India (Annexure IV).

4.1.5 Colorimetric and ELISA estimation kits:

Kits for estimation of C - reactive protein (C-RP) and Rheumatoid factor were obtained from ADI enterprises, Vadodara. ELISA kits for estimation of TNF-α, Interleukin-6, ACPA, TLR-4 and homocysteine were purchased from Krishgen biosystems Mumbai. Kits for estimation of total cholesterol, triglycerides, HDL-C were purchased from Span Diagnostics Pvt. Ltd., Surat, India (Annexure V).

4.1.6 Components of High Fat Diet:

Casein, corn starch and sucrose were obtained from Spectrochem Pvt. Ltd, Mumbai. Vitamin mix was purchased from Neelam enterprises for veterinary supplies, Vadodara. Lard was procured from licensed supplier from sadarbazar, Vadodara. Normal pellet diet for rats was obtained from VRK laboratory food supplies, Vadodara, India.

4.1.7 Pathological and clinical investigations:

Total WBC count, homocysteine and Anti-CCP estimation for initial phase were done by Dr. Kaushik A. Patel MD path& bact. Divine lab, Mangalkirti apartments, Fatehgunj, Vadodara. I am thankful to Dr. Kaushik Patel for all his help for these estimations. X-Rays of rats were done by Mr. Veer at Dr. Angela Lobo's Veterinary Clinic, Kirti mandir compound, Tilak Road, opp. SSG Hospital and Dr. Harsh Soni from Vadodara Welfare against Cruelty against Animals, centre, Chapad, Vadodara. Receptor studies for TLR-4 and NLRP-3 at initial study phase were performed by Mr. Amit Deshpandey from Deshpande Laboratories (DL) Pvt. Ltd. D, 25 Anushka Estate, Kalkheda-Neelbud, Bhopal, Madhya Pradesh. Histopathologies were done from Sakshi Histopathology, Makarpura, Vadodara, Gujarat, India.

4.1.8 Animals for *in-vivo* experimentation:

All experiments were carried out on male Wistar rats weighing 150 - 200gms, obtained from registered breeders of experimental animal. Animals were housed in well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12hrs/12hrs light-dark cycle maintained according to the guidelines. Animals were kept in polypropylene cages with corn cobs lining as bedding material. Three rats per cage were kept till start of experiment, after induction single animal was kept in each cage. The animals had free access to conventional laboratory diet in all groups and high fat diet in specific groups with purified water *ad libitum*.

Animals were reared, handled and treated in accordance with the guidance of the Committee for the Control and Supervision of Experiments on Animals (CCSEA) and The Prevention of Cruelty to Animals act (PCA), 1960, Department of Animal Husbandry and Dairying, Ministry of Fisheries Animal Husbandry and Dairying Government of India (DAHDMoFAH&D), formerly known as Department of Animal Welfare, Government of India. All the mentioned studies were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Dept. Faculty of Pharmacy, The M. S. University of Baroda vide the protocol number MSU/IAEC/2015-16/1661 dated 30/12/2016, MSU/IAEC/2018-19/1802 dated 29/12/2018 and MSU/IAEC/2019-20/1904 dated 21/08/2019. (Annexure VI)

4.2 Methods

As the whole work is outcome of different objectives in sequence and multiple methods were adopted in this study. On the basis of specific aim, methodology is divided in following sections -

Section I- Methodology for pharmacognostic, physicochemical and phytochemical evaluation of selected crude drugs, powders and their extracts

Section II- Methodology for *in-vivo* studies adopted for model development

a) Protocol for study design I

b) Protocol for study design II

Section III- Methodology adopted for model validation steps

Section IV- Methodologies adopted for pharmacological evaluation of NSAE, CPAE, and MCAE in optimized and validated model

Section V- Methodologies for formulation development and its standardization

4.2.1 Methodology for section I (Methodology for pharmacognostic, physicochemical and phytochemical evaluation of selected crude drugs, powders and their extracts)

The methodologies adopted for section I are comprised in following flow chart as summary of section I-

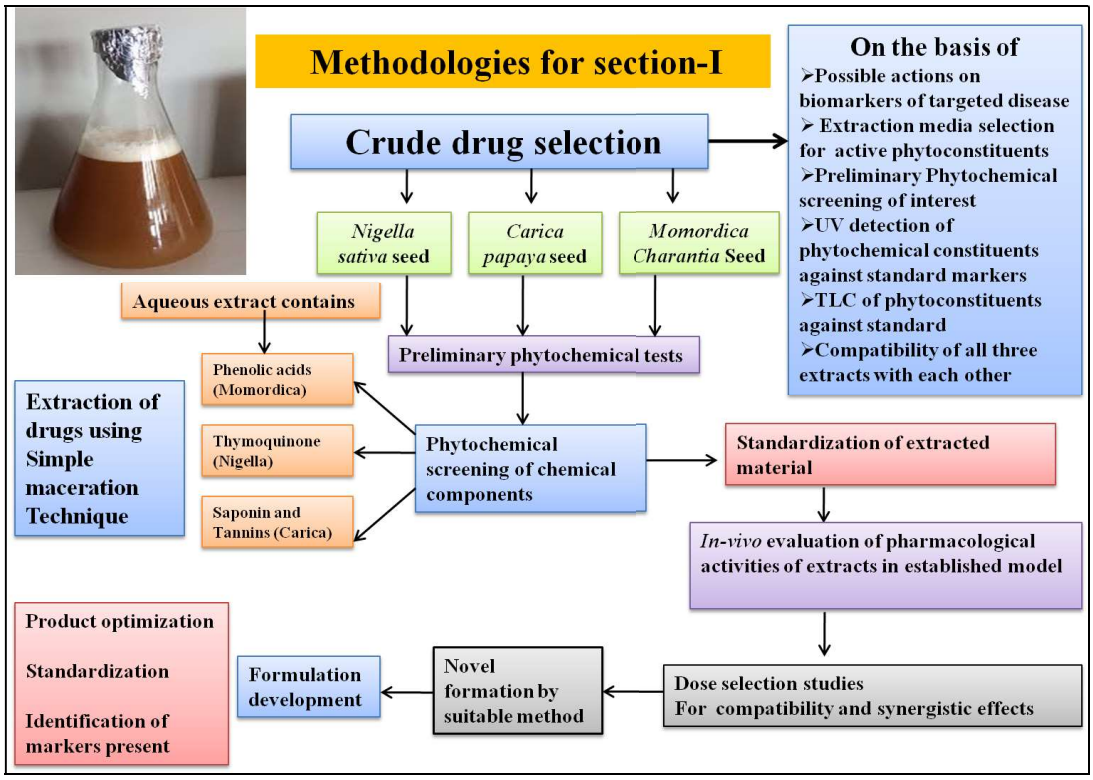

Fig. 4-1 Flow chart for summary of extraction and standardization procedure

4.2.1.1 Preparations for test drugs

I) Preparation of aqueous extract of *Nigella sativa* seed:

Simple maceration technique was used to extract out all the components of seed in aqueous extract of *Nigella sativa*. Dry seeds procured from local market Siyagunj Indore were powdered in grinder with breaks to prevent the oozing of oil from seed by heat generated by grinding. Coarsely powdered *Nigella sativa* seed was macerated in water 25%w/v (25gm powder in 100ml distilled water) for 10-12 hrs. at room temperature with occasional shaking. The filtrate was taken out by muslin cloth (cheese cloth) after 12 hrs. The filtrate was evaporated at controlled temperature. Practical yield of extract was calculated and was labeled as NSAE (*Nigella sativa* aqueous extract). It was stored at 2-8°c till further use. At

the time of dosing the extract was taken out from the storage and bought to the room temperature for preparing the fresh dose to be given.

II) Preparation of aqueous extract of *Carica papaya* seed:

Simple maceration technique was used to extract out all the components of seed in aqueous extract of *Carica papaya* Linn. Dry seeds procured from papaya farm Japod Gujarat, these seeds were completely dried and devoid of transparent mucilaginous outer membrane of seed. The seed were powdered in grinder with breaks to maintain the temperature. Powdered seeds were macerated in water 25%w/v (25gm powder in 100ml distilled water) for 10-12 hrs. at room temperature with occasional shaking. The filtrate was taken out by muslin cloth (Cheese cloth) after 12 hrs and the filtrate was evaporated on controlled temperature. The residue was observed for practical yield of extract and was labeled as CPAE (*Carica Papaya* Aqueous Extract). It was stored at 2-8°c till further use. At the time of dosing the extract was taken out from the storage and brought to the room temperature for preparing the fresh dose to be given.

III) Preparation of aqueous extract of *Momordica charantia* seed:

Simple maceration technique was used to extract out all the components of seed in aqueous extract of *Momordica charantia.* Dry seeds procured from vegetable field Khargone, M.P. were washed thoroughly and dried completely. The seed coat of seed was removed then the inner kernel of seed was powdered in grinder by controlling the temperature to prevent the oozing of oil. Powdered kernels were macerated in water 25%w/v (25gm powder in 100ml distilled water) for 10-12 hrs at room temperature with occasional shaking. The filtrate was taken out by muslin cloth (cheese cloth) after 12 hrs. and the filtrate evaporated and the practical yield of residue was calculated and was labeled as MCAE (*Momordica charantia* aqueous extract). It was stored at 2-8°c till further use. At the time of dosing the extract was taken out from the storage and brought to the room temperature for preparing the fresh dose to be given.

4.2.1.2 Standardization of the prepared aqueous extracts[85, 86]

All the procedures of extraction preparation were performed at Shri G. H. Patel Pharmacy building, The Maharaja Sayajirao University of Baroda, Vadodara and the extracts were also standardized by AMSAR Private Ltd. Indore (M.P.) for standardization of heavy metal content, microbial load and total extractive values of extracts.

4.2.1.3 Organoleptic examination:

The procured plant material was studied primarily on the basis of organoleptic and morphological characteristics. Seeds as a crude drug of all three plants were used in the study. Before extraction the crude drug was examined on the basis of appearance, shape, size, texture to identify the organoleptic properties.

4.2.1.4 Proximate analysis:

After confirmation of crude plant, material in powdered form was also analyzed for the determination of moisture, total ash, acid-insoluble, water-soluble ash and protein contents. Other analysis (loss on drying, heavy metals, microbial profile) was also done. After suitable confirmation, qualitative phytochemical screening of plant material was also performed where preliminary tests were performed for the qualitative measures of the phytochemical components in crude material as well as in prepared extracts. All the procedures were followed according to WHO guidelines (For Quality Standardized Herbs And Herbal Formulations).

4.2.1.5 Determination of moisture by Loss on drying (LOD):

Gravimetric method was used to estimate the moisture content in procured seeds. About 1.5 g powder of seeds was taken separately in flat porcelain dish and the content was heated in oven at 100^0 C to 105^0 C until two consecutive weighings do not differ by more than 0.5mg. Components were cooled in desiccators and weighed and LOD was recorded as moisture presented in crude samples of seed powder of *Nigella sativa, Carica papaya* and *Momordica charantia.*

4.2.1.6 Ash values:

Ash value is determination of quality and purity of crude drugs. Following procedures were performed for estimation of Ash values in crude powdered samples of selected seeds.

4.2.1.7 Determination of total Ash value:

A tared silica crucible was weighted and ignited. About 2 g of powdered drug was weighted in the crucible. This sample was heated using burner with a flame about 2cm high and supported the crucible about 7cm above the flame. The sample was heated till the vapours cease to be evolved and then the components were heated very strongly until the carbon is burnt off. The obtained material was cooled in a desiccator and weighted to calculate percentage of total ash.

4.2.1.8 Determination of Acid insoluble ash:

After getting total ash, the ash was washed using 25ml dilute hydrochloric acid and this content was boiled for 5min. the solid contents were collected by filtering and residue was washed with hot water. The content was collected in pre heated and weighted silica crucible. Heat the crucible as per method mentioned in total ash to remove carbon from the content. This ash was cooled into a desiccator and weighted to get acid insoluble ash value.

4.2.1.9 Determination of water soluble ash:

The above procedures mentioned in acid insoluble ash value were followed here but the dilute hydrochloric acid was replaced with 25 ml water.

4.2.1.10 Qualitative determination of preliminary phytochemical:

The extracts were suspended in water and kept aside for one hour. The supernatant was then collected to perform the basic chemical tests using standardized methods.

Table 4-1 Chemical tests for phytochemical identification

Sr. no	Test	Procedure	Observation
1.	Tests for Carbohydrates a) Molisch's test(General test)	Sample (2-3ml) + alpha-naphthol solution in alcohol Shake and add conc. H_2SO_4 from side of test tube.	Violet ring is formed at the junction of two liquids
b) Reducing sugar tests Fehling's test		Mix 1 ml of Fehling A and 1 ml of Fehling B solutions, boil for one minute add equal amount of sample solution. Heat in boiling water bath for 5-10min.	First yellow and then brick red ppt. is observed
2.	Tests for Proteins Biuret test	3ml sample + 4% NaOH + few drops of $CuSO_4$ solution	Violet Pink color appears.
3.	a) Filter Paper test Tests for Fats and oils	Take a piece of filter paper and add a drop of sample using glass rod	If oil of fat is present filter paper get permanent stain
b) Test for fat		Place a thick section of drug on glass slide, add few drops of Sudan Red III reagent After 2min. wash with 50% alcohol. Mount in glycerin	When observed in microscope oil globules appears red
4.	Tests for Steroids a)Salkowski reaction	2ml sample+ 2ml chloroform + 2ml conc. H_2SO_4. shake well	Chloroform layer appears red and acid layer shows greenish yellow fluorescence
5.	Saponin Glycosides a) foam test	Shake the drug extract or dry powder vigorously with water	Persistent foam observed
6.	Cardiac glycoside Legal's test	To aqueous or alcoholic extract, add 1ml pyridine and 1ml sodium nitroprusside	Pink to Red color appears
7.	Flavonoids a) Sulphuric acid test b) Lead acetate test	Sulphuric acid (66% or 80%) + sample Sample + Lead acetate solution	On addition of sulphuric acid flavones and flavones dissolve and give a deep yellow solution. Chalcones and aurones give red or red bluish color Flavanes give orange to red color. If Flavonoids is present yellow ppt occurs
8.	Alkaloids Dragendorff's test	Take residue of extracts and mix with dilute HCL shake well and filter Take 2- 3 ml filtrate add few drops of Dragendorff's reagent	Orange brown ppt. forms
9.	Tannins and	1) To 2-3 ml aqueous or alcoholic extract, add 5%	Deep blue- black color

	Phenolic Acids	$FeCl_3$ solution 2)Sample + 1 drop of NH_4OH + excess amount of 10% $AgNO_3$ solution, heat for 20 min in boiling water bath	appears White ppt. observed then dark silver mirror deposits

4.2.1.11 Qualitative and qualitative estimation of phytochemicals of interest for anti RA activity:

After estimation of primary data for presence of metabolites, Thin Layer Chromatography (TLC) was also performed and further serial dilution of extracted materials were prepared to confirm and quantify the major metabolites against markers using UV visible photo spectrometer

I) Thin Layer Chromatography for qualitative determination of markers[(87)]:

Thin Layer Chromatography was also performed for thymoquinone, chlorogenic acid and gallic acid as selected markers for aqueous extracts. TLC was performed on pre-coated plates (silica gel $60F_{254}$). The plates were cut with scissors and marked about 1cm from bottom of plate using pencil. One ascending technique was used for detection, each sample was dissolved in suitable solvents and capillary tubes were used to apply on TLC plates and allowed to dry. These plates were developed in TLC chamber (chromatographic tank) using different solvent systems known as mobile phase. The following mobile phases were evaluated for all three test compounds (table 4-2)-

Table 4-2 Mobile Phases for TLC for different marker identification

Markers	**Mobile phases**
Thymoquinone	
1	n-Hexane: Ethyl acetate: Methanol (7:2:1)
2	Hexane: 2propranol (99:1)
3	Acetonitrile (2mM): Ammonium Formate (50:50)
4	Benzene: glacial acetic acid (1:1)
5	Carbon tetra chloride : acetone: glacial acetic acid (15.2: 3:1)
6	**Chloroform: Benzene (50:50)**
Chlorogenic Acid	
1	**Ethyl acetate: Water: Formic Acid (7.7:1.3:0.9)**
2	Ethyl acetate: Acetic acid: Formic Acid: Water (10:11:1.1:2.3)
3	Ethyl acetate: Methanol: Water (77:13:10)
4	Toluene: Ethyl acetate: Formic Acid: Water (15: 90: 5:5)
Gallic Acid	
1	**Toluene: Ethyl acetate: Formic Acid: Methanol (3.3 :0.8:0.2)**
2	Toluene: Ethyl acetate: Methanol: Formic Acid (6:6: 0.4:1.6)
3	Toluene: Ethyl acetate: Formic Acid (6:6:1)

4	Ethyl acetate: Methanol: Formic Acid (8:2:1)

The plates were prepared and developed in mobile phase and R_f value was calculated using following equation and photography was done in under UV visible mode to identify and mark the solute retention spot.

$$Rf = \frac{\text{Distance moved by the solute/compound}}{\text{Distance moved by the solvent (solvent front)}}$$

II) UV visible Method:[88-90]

Analytical method was developed for quantification for detection of Thymoquinone (TQ) in *Nigella sativa* seed aqueous extract, Chlorogenic acid (CGA) and Gallic acid (GA) in *Carica papaya* and *Momordica charantia* seed aqueous extracts respectively.

Preparation of Phosphate buffer solution:

Phosphate buffer solution (pH 7.4) was prepared by dissolving 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride in water to make 1000ml solution.

Method development for Thymoquinone:

All chemicals and solvents of analytical grade were used for preparations of reagent required for method development for thymoquinone, chlorogenic acid and gallic acid.

Preparation of standard solution and sample:

All standard reagents were prepared in aqueous medium. Dimethyl sulfoxide (DMSO), dimethyl formamide (DMF) and ethanol were also prepared in different concentration to check solubility of thymoquinone. Here the sample standard of thymoquinone used in this study was found to be freely dissolved in methanol.

Determination of λmax of thymoquinone:

The reported λmax of thymoquinone (254nm) was confirmed by preparing methanol standard solution in different concentrations ranging 1µg to 16 µg for UV-spectroscopy analysis and absorbance was taken in UV-Visible spectrophotometer (1800 Shimadzu, Japan). Calibration curve of these readings of absorbance were plotted against concentration for detection.

Thymoquinone presence in sample was confirmed by preparing a standard solution of 10 µg/ml of NSAE in methanol for which 1 ml of the stock solution of NSAE was transferred to a 10 ml volumetric flask and diluted with methanol to make up the volume. The solution thus prepared (10 µg/ml) of thymoquinone was scanned in the range of 200 to 400 nm using methanol as blank.

Determination of λmax of chlorogenic acid:

Standard chlorogenic acid was dissolved in methanol to make standard solution of different concentrations. The λmax for chlorogenic acid is reported as 270nm and it was confirmed with different concentrations ranging from 10µg/ml to 100µg/ml. the calibration curve was plotted for absorbance vs. concentration.

Chlorogenic acid presence in NSAE, CPAE and MCAE sample was confirmed by preparing a standard solution of 10 µg/ml each sample separately in methanol for which 1 ml of the stock solution was prepared and further diluted for UV scan in the range of 200 to 400 nm using methanol as blank.

Determination of λmax of gallic acid:

The known λmax for gallic acid ranges from 268-270nm which was confirmed by preparing standard solution of gallic acid standard in methanol in different concentrations (10µg/ml to 100µg/ml) and calibration curve was plotted between absorbance and concentration.

Detection of gallic acid in all three selected samples NSAE, CPAE and MCAE was performed as mentioned above for chlorogenic acid.

Method Validation:

To validate the applied method, calibration curve was plotted where linearity indicates the validation of method. Limit of detection (LOD) and Limit of quantification (LOQ) were also analyzed for the same as mentioned below-

The Limit of detection (LOD) may be expressed as:

$$DL = 3.3\ \sigma/S$$

The Limit of quantification (LOQ) may be expressed as:

$$QL = 10\ \sigma/S$$

Where,

DL- Detection Limit

QL- Quantification Limit

σ = the standard deviation of the response

S = the slope of the calibration curve.

4.3 Methodology for section II (Methods for *In-vivo* experimental design)

In- vivo model development was performed on the basis of diverse actions of inducing agents for disease generation (RA). In this line of study different secondary inducers were also added at different time to generate further extra organ manifestations in RA (cardiovascular complications). The *in- vivo* methods were further divided in the sections in later part as study designs to link the two different problem statement taken for model development.

4.3.1 Preparation, development and standardization of drugs and inducing agents for *in- vivo* experiments

4.3.1.1 Preparation for standard drug Methotrexate:

Animals were treated with Methotrexate (Mtx.) with 0.6mg/kg dose weekly. Each time Mtx solution was prepared freshly by dissolving powdered drug needed according to animal body weight in Phosphate buffer solution having pH 8.00. The doses to be injected by i.p. route were calculated according to body weights of animals.

4.3.1.2 Dose selection for extracts as test drug:

All the selected herbs were taken in similar concentration in first stage (100, 200, 400mg/kg) due to wide range of effective concentrations reported in different conditions and to maintain dose variation to reduce noise. These selected doses were based on either similar marker blockage action or on the basis of connecting disease pathophysiology like inflammation, pain, immune responses and metabolic dysbiosis associated with RA.

Nigella sativa has highest tolerated dose 800mg/kg in animals. Dose of *Nigella sativa* for this study was selected from literature which has treatment indications for hypolipidemia and Anti- inflammatory activity, due to this said reason 100, 200 and 400 mg/kg for rats were selected for evaluation of anti arthritic and anti atherosclerotic properties. *Carica papya* seed are having dose specific activities in different diseases and the dose range is started from 50 to 1000mg/kg dose. The dose of *Carica papaya* seed was taken from the line of treatment with the connected diseases like diabetes and inflammatory diseases which was 100, 200 and 400mg/kg for rats. *Momordica charantia* seeds can be used for wound healing to the cancers which has specific activity on markers the dose range was from 200 to 800mg/kg. As *Momordica charantia* shows cell proliferation in synovial cells in primary cell line studies in higher doses the range was set as 100, 200, 400mg/kg to check the dose dependent variation.

4.3.1.3 Preparation of test drugs (Selected herbal extracts):

All the aqueous extracts were prepared and stored at 2-8 ^{0}C with labels NSAE (*Nigella sativa* aqueous extract) for *Nigella sativa* seed CPAE (*Carica papaya* aqueous extract) for *Carica papaya* seed and MCAE (*Momordica charantia* aqueous extract) for *Momordica charantia* seed till further use. At the time of dosing 1mg/ml solution as per the animal weight were prepared for each extract in distilled water and administered in the rats by oral route.

4.3.1.4 Lipopolysachcharide:

In this study, Lipopolysachcharide (LPS) of gram negative bacteria *Escherichia coli* was used as secondary inducing agent. To prepare a desired injectable solution LPS was dissolved to make stock solution of 100µl/ml in 0.9% saline and to get different concentrations (0.5, 0.1 and 10µg/ml) this stock solution was further diluted.

4.3.1.5 High Fat diet:

High Fat Diet (HFD) was selected in this study to develop cardiovascular complications in Rheumatoid Arthritis which served as another sensitizing agent with LPS induction as a secondary inducer. This diet was provided in selective group of animals for initiation of atherogenic events with RA.

Table 4-3 Composition and calories of HFD[91]

Ingredient	**Weight(gm)**	**Appox. Calories (KCAL)**	**Significance Of The Components**
Normal Pellet Diet (NPD)	200	500	Normal Food Diet
Casein	125	500	Source of Protein and for elevation of lipid profile specially TG Level
Lard	300	2700	Main source of fat
Sucrose	213	861.37	For sweetness
Corn Starch	150	600	Elevate Insulin resistance
Vitamin	6	---	For normal health conditions
Salt	1	---	Taste and for homeostasis
Soya Bean Oil	5	45	Fat content
Total	1000	**5206.37**	
			TOTAL FAT=48.93%

To prepare this diet, desirable quantity of dry ingredients; powdered NPD, casein, sucrose and corn starch were weighted and mixed primarily according to preparation (above measure is for 1kg diet) in a large clean and dry container. After mixing this thoroughly, desirable amount of lard, Soyabean oil and salt were added and mixed until semisolid dough like consistency was achieved. The mixture was shaped in small balls to make it compatible with

food sack of cages. At a time four days diet was prepared and per 6 animal total 150g diet was weighted and provided to the animals in gap of 24 hrs. to measure accurate amount of food consumption. The percentage of lard in this diet is 30% and the amount of saturated fats in lard is higher (approximately 25gm/100gm) which can lead cardiovascular complications.

4.3.2 Methods and protocols adopted for model development

Models can act as contributors or restrictors of the drug developing process. An invalid disease model can lead in a wrong direction wasting time and significant investment. The whole hypothesis or the idea of construction of a preclinical model is to have a comparative hierarchy of clinical situation with preclinical condition. As preclinical trial design is different in many aspects with clinical trial, the hierarchy of both the situations is different in many aspects but the major connecting link is still somewhere between evaluation and assessment of data at particular points which are denoted as checkpoints of preclinical protocol design for two problem identified for model development for Rheumatoid Arthritis and its associated cardiovascular complications. Moreover these preclinical loops were tried to rectify in development of model as well as via validation tools to give a new and validated model for cardiovascular complications in RA.

In the following table 4-4 the justification for selecting the different inducers (primary and secondary) as well as the impact of each selected criteria is summarized. On the basis of these criteria models were developed for both the conditions (RA and CVD in RA) which are further validated on different parameters which support and link the present justification (table 4-5).

Table 4-4 Criteria adopted for model design hypothesis

Selection criteria for model designs for RA and RA with CVD in current study			
Sr. no.	**Selection criteria**	**Applicability of criteria**	**Model specific Reason for selection criteria**
1	**Model selection (Species/strain)** (Constrict validity criteria) External validity	**Wistar male rats** were selected as model simulator due to higher resemblance of anatomical and physiological responses involved in RA and associated complications	High susceptibility with inducing agents (CFA and CIA) as compare to other strains of rats. The targeted biomarkers respond similar to human in developed conditions [92, 93].
2	**Degree of complexity** (Face validity criteria) External validity	Degree of complexity in this study was created by adding primary inducing agents **(CFA and Collagen)** with secondary inducing agents **(LPS and HFD)** on molecular level	Complexity level is near to human in terms of anatomy of targeted area (synovium, cartilage, muscles and bones) and similar pathophysiological responses (transduction pathways) of inducing agents and selected drugs[70, 94].
3	**Duration of model development** (Predictive Validity) External validity	CFA induced model is **21 day** model for Rheumatoid arthritis which prominently involves inflammatory responses. The model with Collagen was kept for **42 days** to check the immune responses with inflammatory responses[69]. LPS and HFD were used to involve extra organ manifestations in RA as atherosclerosis.	There is a difference between life span of Rat and human 1 human day is equal to approximately 27 days of rat life span([95]).Thus is study is designed to get results for long term complications arises in human in rat nearly for 3years on the basis of adopted literature for time calculation between rat and human
4	**Treatment design** (Target Validity) internal validity	Preventive/Prophylactic therapy was designed using standard drug Methotrexate (Mtx0.6mg/kg)[96] and test drugs (Herbal aqueous extracts). Induction was done on day 0 and treatment therapy was initiated from day 1 as preventive protocol till end of study **(28 and 42 day in respective model)**	The test compounds selected in this study were taken from herbal origin and all the drugs were given as preventive treatment to check the effects on inflammatory and immune responses on different time points and evaluation was done on the basis of perceptive, physical and biochemical estimation similar to human parameters.
5	**Disease specificity criteria** (Predictive and Target Validity) Internal validity	**Wistar rats of 8-12 weeks** were preferred here as they have well developed skeleton system, synovium and cartilages with joint muscles responsible for movement and strength and major target of RA.	Cell infiltration, Pannus formation, Chemotaxis and cartilage damage was seen in the selected models which resembles with the human like RA conditions[70]. And metabolic dysbiosis, lipid profile, steatosis and atherogenic index showed CV complications[65].

6	**Subjectivity** (Face Validity) Internal validation	The selected healthy wistar rats after suitable screening resembles the human like symptoms, Involvement of metapharyngeal joints and edema in specific area of ankle joint resembles with human knee are significant in development of disease and assessment of walking disability, extra organ manifestation[97] and metabolic dysbiosis[74]	Primary and secondary lesions were clearly visible in rats. Symmetric patterns of disease and digestion of digits were also seen as human like RA disability and atherosclerosis was marked as extra organ manifestation. The treatment groups showed clear indication of lesion recovery and changes in radiography as well as Histopathological changes[98] were significant as human conditions
7	**Reproducibility and Robustness** (Face, Predictive and Target Validity) Internal validity	Different inducing agents (CFA and CIA) were used in the past by different researchers and the similar procedure was used to reproduce Rheumatoid Arthritis. For reproducibility of RA with different situation diet manipulation was done to see the effect of inducing agent and their targets.	The inducing agents were compared with the literature and also in the lab with different strain (SD and Wistar rats) and with the male and female rats to see the reproducibility and robustness to optimize the inducing agents and to validate the model from data collected with different personnel at different time points. As well as with the diet manipulation inducing agents for RA done their job similarly this showed the reproducibility of model in different conditions.
8	**Ethical confirmations**	CPCSEA guidelines were followed for experimentation and PREPARE and ECVAM were taken as reference to check the points for designing the protocol and ARRIVE guidelines were followed for reporting and data collection specifically for animal studies and SMASH guideline was used for evaluating Rheumatoid Arthritis in developed model	Animal experiments were performed under guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Dept, Faculty of Pharmacy, The M. S. University of Baroda. Sanctioned protocol numbers- MSU/IAEC/2015-16/1661, MSU/IAEC/2018-19/1802, MSU/IAEC/2019-20/1904

On the basis of all the above discussed points this experimental research was framed with following studies-

Study design I- Development of animal models for replication of Rheumatoid arthritis

Study design II- Development of animal model for replication of RA and CV complication

4.3.3 Role of specific antigen in disease progression

The first part of the study was designed to develop an animal model only for Rheumatoid Arthritis which can resemble the pathogenesis and etiology of human disease.

Inflammatory responses are key components for generation of RA in animals which can be activated using well established Adjuvant models. Here in the study path, Complete Freund's Adjuvant (CFA 0.1ml sub planter) was considered as primary inducing agent principally for activation of inflammasomes and cytokine release.[68] As the aim of study was to evaluate the best model for RA, Bovine collagen type II (Collagen 0.1ml sub planter) was selected for Collagen Induced Arthritis (CIA) as individual inducing agent to incorporate the complexities in form of immunological components.[13] as well as to compare disease severity in different study time lines (induction of RA with CFA is 28 days and collagen model is 42 days).

Additionally lipopolysaccharide (LPS) sensitization suggests the activation of macrophages and TLR-4 receptors.[16, 99] On the basis of pilot studies LPS 0.1, 0.5 and 10µg/ml doses were selected for receptor activation with both the inducing agents in animal experiments.

I) Sensitization of animals with CFA

There are many models available for the induction of RA in rat and mice. Complete Freund's Adjuvant induced RA (adjuvant induced RA) is one of the oldest and well established models, which can induce RA in rats within 10 to 15 days and severity increases in 28 days.[93, 100] The mechanism followed by CFA is activation of inflammatory components due to presence of M. tuberculosis. The prominent features of CFA induced RA are; edema, Pannus formation, infiltration into joints of mononuclear or polynuclear cells which causes the erosion and destruction of cartilages.[68] With the above accounts, in this study CFA was utilized as a single inducing dose of 0.1ml/ animal by sub planter route on day 0 for induction of RA.

II) Sensitization of animals with collagen

In different studies, some limitations were discussed for CFA model as the severity was less in those models in comparison to human and it obeys only inflammatory paths which have chances to recover in or after 21 days. Immunological link is still unclear as RF is stimulated in some models due to role of *HSP- 65* and it was not generated in some models. The role of HLA-DRB in CFA model is also unclear.[21, 68]

Due to this, the higher grade model was recreated using bovine collagen type II as another inducing agent. Collagen Induced Arthritis (CIA) model is established successfully in mice, rats and primates and the pathogenesis includes activation of immunological components followed by inflammatory responses which gives the better insight of disease progression and the studies of bovine collagen type II in primates support strong evidences and high severity of onset of RA with genetic component involvement similar to human conditions.[92] In this study, bovine collagen type II was used in 0.1ml quantity after forming emulsion of CFA and collagen in 1:1 ratio. Animals were sensitized with this emulsion by sub planter route for induction of RA once using 0.1ml dose on day 0.

III) Challenge with high fat diet

High fat diet[91] was used here for creating atherogenic environment in animals. Lard rich diet (30%) was used in this study to activate the metabolic dysbiosis with the other expects of inflammation to link it with atherogenic episodes. Obesity is one of the major factors to initiate inflammatory responses and it will provide a leaky GUT via endotoxemia which is hypothesized to provide a ground for production of LPS and to increase this process, LPS was also added after 28 days of diet modification.

IV) Sensitization of animals with LPS

Lipopolysachcharide (LPS) is an endotoxine which is known for activation of cytokine release in *in-vitro* and *in-vivo* conditions. The association of these LPS molecules with pattern recognition receptors (TLR and NLRP) is proven in previous studies.[16] LPS promotes the TLR-4 binding with its co receptor MD-2 on epitopes present on defense cells (especially on macrophages) and initiates two major intracellular signaling pathways, MyD88-dependent and TRIF-dependent (MyD88-independent). The main role of the MyD88-dependent pathway (downstream of TLR4 is to induce the expression of inflammatory cytokines such as IL-6, IL-12, and TNFα, whereas the main role of the TRIF-dependent pathway is to induce up

regulation of co stimulatory molecules and the expression of type I IFNs.[101] These two pathways seem to cooperate to maximize the expression of inflammatory cytokines. In this study, LPS of gram negative bacteria *Escherichia coli* was dissolve in saline to get the desired concentrations (0.5, 0.1 and 10µg/ml via subcutaneous route were selected in this study to prevent sepsis like conditions and to only boost the LPS production in association with HFD.

The LPS sensitization was done from day 14 to 28 in 0.1ml dose by subcutaneous route in rats pre challenged with CFA as an inducing agent. The LPS (LPS 0.1, 0.5 and 10µg/ml) sensitization was started after first sign of immunological intervention as increased neutrophil and lymphocytes in hematological investigations on day 12 of study. In CIA groups also, the rats were sensitized with LPS (0.5, 0.1 and 10 µg/ml) doses from day 14 to 42 by subcutaneous route.

4.3.4 Protocol followed for study design I

Objective one fulfilled by using different inducing agents (CFA, collagen and LPS) followed by evaluating their resemblance to humans in terms of etiology, mode of actions, generated symptoms and progression of disease through degree of complexity, pathogenesis, robustness and repetition of symptoms with similar conditions on recreation of model where-

Models only for **Rheumatoid Arthritis** were developed to evaluate the disease progression, severity and duration of induction of disease in different study time period (28 days for CFA and 42 days for collagen) using two separate inducers (as shown in Fig. 4-1) considered as primary stimulants in different groups of animals with LPS as secondary sensitizer and groups were divided as-

Model developed for RA with CFA
Model developed for RA with CFA + LPS in different concentrations
Model developed for RA with collagen
Model developed for RA with collagen + LPS in different concentrations

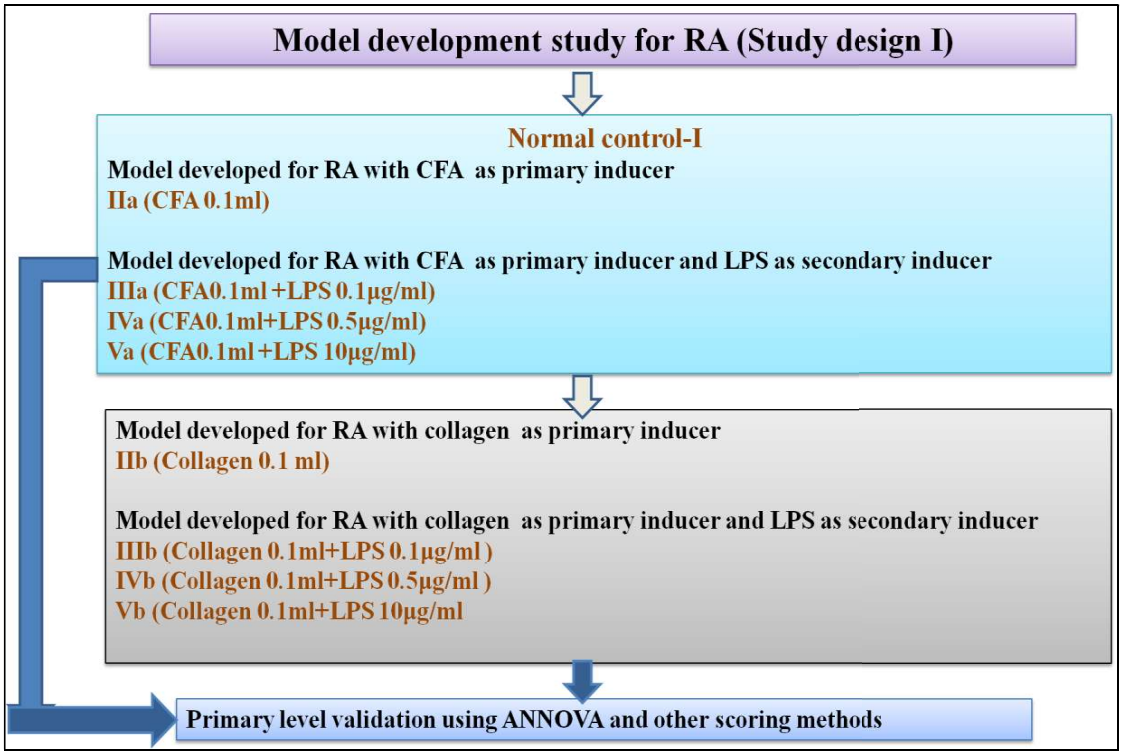

Fig. 4-1Flow chart for model development study for RA (study design I)

CFA induced RA

After suitable acclimatization, animals were divided into five groups where, Group I was considered as normal control group and animals of this group were not sensitized with any agent. CFA induced RA study was designed for 28 days and group IIa, considered as CFA model which received CFA 0.1ml by sub planter route on left hind paw on day 0 for induction of RA. Group IIIa, IVa and Va also received CFA 0.1ml on left hind paw by sub planter route on day 0 of study. On day 14 to 28 animals of these groups (IIIa, IVa, and Va) were further sensitized with LPS in different doses by s.c. route. Group IIIa received, CFA0.1ml+ LPS 0.1µg/ml. Group IVa received CFA0.1ml+ LPS 0.5µg/ml and Group Va received CFA0.1ml+ LPS 10µg/ml. All the evaluation parameters were performed for assessment of progression of RA on different time points. Paw volume was measured on day 1,3,5,7,9,11,14,21,28 by digital plathysmometer. Photography was done on alternate days and arthritic index was calculated on the basis of paw volume and arthritic score taken on day 5 and 21, Rheumatoid Factor (RF) was measured on day 5 and 21. ESR was measured on day 1, 7, 14, 21 and 28. C-RP was performed on day1, 7, 14, 21 and 28. IL-6 and TNF-α were estimated on day 28. CBC, homocysteine and Anti-CCP were performed on day 12 and 28 and at the end of the study X-Ray and histopathology were done for evaluating the

development of model.

Collagen induced RA

After suitable acclimatization, animals were divided in to five groups where group I was considered as normal control group here also for comparison.

Collagen induced RA protocol was designed for 42 days and group IIb, IIIb, IVb and Vb were sensitized with collagen 0.1ml (CFA1: collagen 1). Bovine collagen type II was premixed with CFA before injecting in animals in 1:1 ratio to get a stable emulsion (tested by uniform droplet formation in cold water) and 0.1ml of this prepared emulsion was injected by sub planter route on day 0 for induction of RA[21]. Group IIb received CIA 0.1ml only on day 0. Group IIIb, IVb and Vb received CIA 0.1ml on day 0 and these groups were further sensitized with LPS from day 14 to day 42 with 0.1, 0.5 and 10µg/ml of LPS doses respectively by s.c. route. Different parameters; biological ESR, CRP were preformed on day 7, 14, 21, 28, 35, 42.

CBC, Anti-CCP and homocysteine were performed on day 14 and 35. Paw volume of left hind paw was measured on day 1, 3, 5, 7, 11, 14, 17, 21, 28, 35, 42 and arthritic score was assessed on day 5, 21 and 35. On the basis of paw volume, secondary lesions and severity arthritic index was calculated. Photographic assessment was done on alternative days, radiographic (X-ray) were performed on day 14, 28 and 42 and at the end of the study histopathological assessment of required organ was done.

Among all the experiments CFA sensitized groups; group Va (CFA0.1ml+ LPS 10µg/ml) represented higher severity among all the groups and gives statistically significant results which were further carried out for comparison with CIA groups to get a best model. Among the CIA sensitized groups, group Vb (CIA0.1ml+ LPS 10µg/ml) represent the severity index higher among all other groups.

The initial results for all the groups compared using Primary level validation with ANOVA as statistical tool by using GraphPad Prism software and the external validation criteria given by Frank Sams-Dodd and Denayer Tinneke (attached as supplement Annexure VII). The best fit four models were further compared on higher level validation methods for external as well as internal validity criteria using FIMD method.

4.3.5 Protocol followed for study design II

4.3.5.1 Role of secondary inducers in development of CVD in RA model

There are two main reasons of occurrence of CVD in RA: The first being inflammation driven by TLR receptor activation, works as underpinning factor in both the diseases and second one is the side effects and drawbacks of existing therapies.[102] The proposed hypothesis is designed to develop an animal model replicating CVD in RA using Lipopolysachcharide (LPS) and High Fat Diet (HFD) with CFA and Collagen. This model is designed by-

i) Activation of inflammatory responses via adjuvant/immunological component.

ii) Activation of macrophage as immune responses via TLR-4 (Toll like Receptor Proteins) receptors.

These receptors link inflammation with Immunogenicity via metabolic dysbiosis generated through HFD and LPS sensitization which trigger TLRs; the prime factors and highly expressed proteins in the synovial lining and sub lining layer of RA synovium and cardiac muscles.[66] TLRs process endotoxemia, infiltrate the cells with leaky gut and stimulates cytokines like TNF-α, IL-6, IL-8, and NF-ƘB responsible for CVD in RA.[13] With above pathological events in signal transduction chain here in this investigation, Rheumatoid Arthritis model along with co morbid conditions (cardiovascular complications in this study) were developed via diet modification for which, high fat diet with LPS were used as secondary sensitizing agent to activate the crosslinking biomarkers for generation of metabolic dysbiosis and leaky GUT for cell infiltration in existing RA conditions to develop the human like pathogenic condition in the developed rat model of RA.

4.3.5.2 Model development for CVD in RA

In study design II models for **Rheumatoid Arthritis with its associated co-morbid CV complications**, resembling human with translational competence between preclinical and clinical studies were developed as depicted in fig.4-3. Here high fat diet and LPS were added as additional sensitizers or secondary inducers separately and in combination with primary inducers (CFA and collagen) and groups were denoted as-

Model developed for RA with CV complications using CFA + LPS
Model developed for RA with CV complications using CFA + LPS + HFD
Model developed for RA with CV complications using collagen + LPS
Model developed for RA with CV complications using collagen + LPS + HFD

*LPS dose in these models was taken from best dose proved from RA models

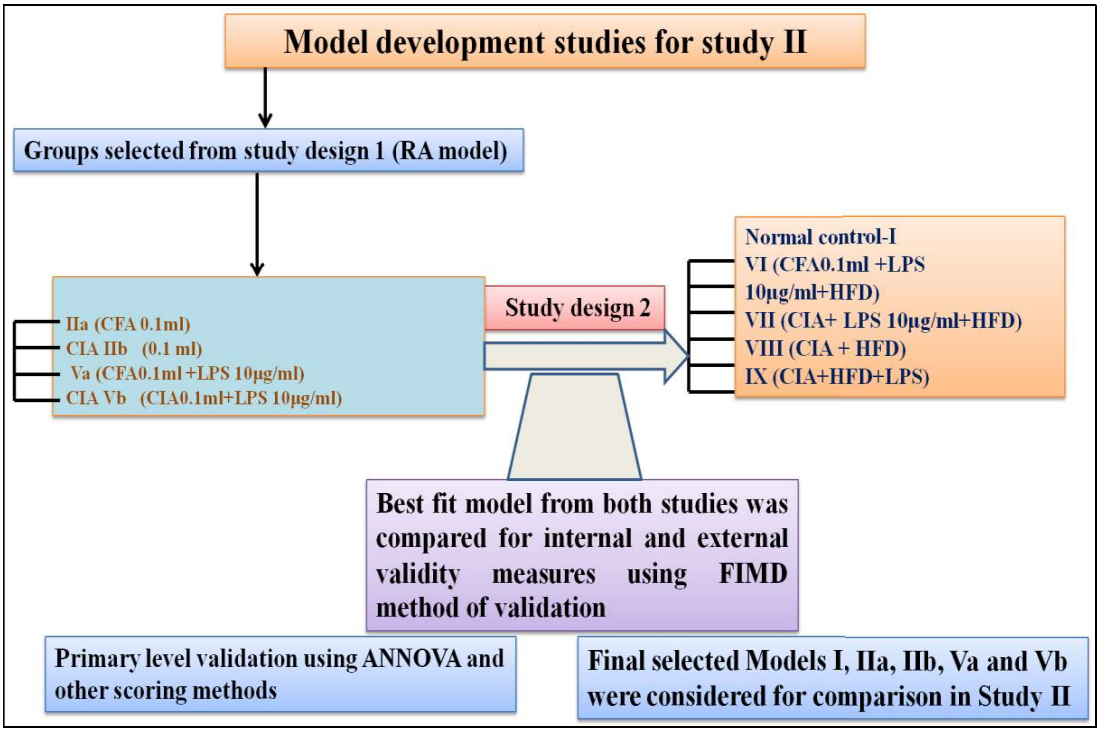

Fig. 4-2 Flow chart for model development study for RA (study design II)

The final selected groups from study 1- group Va (CFA+ LPS10µg/ml) and group Vb (CIA0.1ml+ LPS 10µg/ml) were carried out for further assessment here.

All the animals carried out for this protocol were fed with modified diet (HFD) throughout the study period. CFA and CIA were used here also for primary induction and for secondary sensitization LPS and HFD were used. Two groups mentioned above were compared with the following group of animals; group VI (CFA0.1ml+ HFD) which received CFA 0.1ml on day 0 along with HFD for 28 days. Group VII (CFA0.1ml+ HFD+ LPS 10µg/ml) was sensitized with CFA 0.1ml on day 0 and further sensitized with LPS 10µg/ml and HFD was given to the animals for 28 days. Group VIII (CIA0.1ml+ HFD) received CIA 0.1ml on day 0 with HFD for 42 days and group IX (CIA0.1ml+ HFD+ LPS 10µg/ml) received CIA0.1ml on day 0 and further sensitized with LPS 10µg/ml on day 14 till day 42 and animals were fed with HFD throughout the study period (42 days). All the parameters for assessment of RA were performed on different time points for 28 days in CFA groups and 42 days in CIA groups respectively. These groups were further analyzed for the progression of Atherosclerosis on different time points using Biological parameters (Triglyceride levels, Cholesterol, HDL,

LDL levels, atherogenic index and TLR-4 activation), Perceptive indicators (fibre length of Vastus Medialis and Biceps Femoris muscle)[103] were performed to check the development of Atherosclerosis. At the end of the study X-Ray and histopathology [104] of paw, aorta and Vastus Medialis and Biceps Femoris muscle were done for confirmation.

As these statistical data are predictive values and there outcome is insufficient to give a model which can be called as validated, so among all nine groups, four groups; group Va (CFA 0.1ml + LPS 10µg/ml), group Vb (CIA0.1ml+ LPS 10µg/ml), VII (CFA0.1ml+ HFD+ LPS 10µg/ml) and group IX (CIA0.1ml+ HFD+ LPS 10µg/ml) were carried forward for external validity measures, using FIMD questionnaire based model validation method.

Notation: For better understanding in succeeding sections the final compared groups will be denoted as-

Va (CFA 0.1ml + LPS 10µg/ml) as Model I

Vb (CIA0.1ml+ LPS 10µg/ml) as Model II

VII (CFA0.1ml+ HFD+ LPS 10µg/ml) as Model III

IX (CIA0.1ml+ HFD+ LPS 10µg/ml) as Model IV

4.4 Methodology for section III (Methodology adopted for model validation steps)

An animal model is valid, if it resembles the human condition in etiology, pathophysiology, symptomatology and response to therapeutic intervention[22, 105]. Hindrances in model resemblance to human etiology and symptomatology are affected with the common laboratory limitation and the improper selection of hypothesis which is based only on **predictive data**. If a model selected is unable to prove **modality** and **reproducibility** it can fail to impart the gap between preclinical and clinical trials which is the most prominent reason of this defeat. The flawed preclinical data without validation tool are inadequate to generate the sufficient data for further proceeding.

Validity indicates how refined your research is, in terms of proving the correctness of your hypothesis. In any research the concept, findings and conclusions should be able to generate the same results on recreating the model fundamentals with all variables.

These concepts of model validation are based on-

Predictive validity-Basic target of predictive validity is to check at how much extent the demonstrated model in particular species replicates the human disease condition. The predictive validity is based on the evaluation of the end points (parameters) based on statistical tools for terms of reliability and relevance. Reliability is assessed by calculating the inter-laboratory reproducibility and intra laboratory repeatability.

Face validity - Face validity is logical validity this is primarily the theoretical consideration of the procedure at which extent it is similar to the set hypothesis.

Constrict validity and target validity- The target under investigation at the time of recreation should have a similar role in the disease model as in the clinical situation.

In context to these basic validation strategies given by Wilner and McKinney and Bunney, Frank Sams- Dodd and Tinneke Denayer *et. al.* also given the points for optimization of **External validation** for minimizing the translational gaps between preclinical and clinical relevancies at the time of model selection on the basis of **species, strain, complexicity level and duration of treatment.**

These basics can be used to avoid the lower level faults in conceptualization of a model designing which can further be optimized on a detailed validation parameters. Furthermore animal model should also be replicating the **internal validity (symptomatology, disease specific criteria, subjectivity and reproducibility)** which is a prime requisite for resemblance of clinical conditions. On the other side if they are adequately designed and conducted, animal models can contribute valuable information to our knowledge of biology and medicine, including the discovery and development of new drugs.

On the basis of all the above discussed points the sequences in this study were conceptualized on two problem statement mentioned in relevance to RA model development.

1. Model development (as per section-II)

The first and foremost challenge was to develop an animal model which similitude clinical (human like) complications arisen in **RA which was done by -**

a) Comparing primary inducing agents CFA and Collagen

b) Comparing secondary inducing agent LPS with primary inducing agent (CFA and Collagen) in model developed for RA.

The another challenge in model development was to take one more step in relation with complexity of diseases as incorporation of clinical **co morbid conditions (RA along with CVD) in preclinical models via incorporation of one more secondary inducing agent HFD** with- CFA, collagen and LPS model.

2. Model validation

The second and the most important chain of event was to validate these models, which was performed primarily with core pharmacological background using statistical approaches for comparison in between the developed models and strategies to optimize external and predictive validation methods. After getting the results of this ground level the data were further validated using questionnaire based FIMD validation tool to get better perception of study on external as well as internal validity measures.

4.4.1 Selection of validation criteria for model development for RA and CVD in RA

The current study focused to develop a new model with multiple newer inducing agents in combination. In this attempt various methods for validation were considered previously among which two different validation methods were adopted-

1. Primary validation on the basis of method adopted by general criteria and scoring system (Pre validation)

2. Final validation by FIMD method

4.4.1.1 Methods adopted for pre validation:

The basic external and internal validity criteria given by the different researchers adopted in designing the model for both the situations to rectify the basic disbalances between preclinical and clinical situations. The points in table 4-5 were taken from the deep literature survey available from different authorities and agencies working to reduce the inaccuracy or organize the work for animal ethics. According to these guidelines there are some points to be remembered from basic system to the higher ethical considerations for prevalidation and validation. Experimental protocol which includes utilization of animals when designed according to this criterion can reduce the burden of insufficient data of preclinical studies to refine clinical relevance via statistically significant test results to reproduce and reliable model development via using ECVAM, ARRIVE, PREPARE, OECD, ICH S5 CR3 and SMASH guideline.

Table 4-5Generalized experimental model design based on standard validity criteria for *in-vivo* study on model validation ground for RA model and model for RA with co-morb conditions

Sr. no.	Selection criteria	Selection criteria given for the study design for model validation as per ARRIVE, EVACM PREPARE, OECD, ICH S5 CR3 ,CPCSEA, SMASH guidelines	
		Applicability of criteria	**Reason based on generalized examples**
1	**Model selection (Species/strain)** (Constrict validity criteria) External validity	Model is counted as disease simulator in pharmacology so the species should be closer to human in phylogenic proximity to increase the chances of mimicking the clinical situations.	Species should be selected cautiously for successful conduct Example - Rat, is not suitable for asthma model due to absence of histaminic receptors but guinea pig can be used for this purpose.
2	**Degree of complexity** (Face validity criteria) External validity	A higher similarity in complexity of test systems between animals and humans enhances replication of correlated mechanisms and biochemical involvement.	Equal state of complexity can provide the better results. Examples - Comparison of *in-vitro* and *in-vivo* results of drugs, where *in-vivo* system can give more precise results on the basis of organ level complexity.
3	**Duration of model development** (Predictive Validity) External validity	The principles and pathway of disease progression in human (Clinical) and in animals (Preclinical) have different mechanistic approaches and distinct time periods which may or may not follow all the components due to species and lifespan differences.	Chronic inflammation is one such example which progressed slowly in human being with environmental conditions, genetic constituents and immune responses over the period of time which takes some days to years. On the other hand, inflammation in animal models can be dependent on inducing agents which can be slow or fast depending upon the responses of particular species and strain.
4	**Treatment design** (Target Validity) internal validity	After deciding the basic conceptual design of model, treatment schedule is equally important to reduce the clinical complexity in terms of safety and efficacy. Preventive/Curative/Prophylactic treatment should be followed strictly to get the authentic data for clinical investigation.	The treatment schedule is shorter in the animals as compared to humans. The potential pharmacological effect could be overestimated as therapeutic intervention occurs earlier in the form of preventive treatment before disease pathology initiation. However in a clinical situation treatment begins on onset of symptoms.
5	**Disease specificity criteria** (Predictive and Target Validity) Internal validity	Every species and strain is different in their environmental and genetic background. When we choose any disease to induce in the models, particular species, strain, age, gender and physical condition of the animal are basic things which interfere in induction of disease and optimization of drugs.	For example the nulliporus females of age 50 days are chosen for the development of breast cancer in rats using MNU but in human condition Breast cancer is noticed in females most commonly in the age group of late 40s (menopausal state).
6	**Subjectivity** (Face Validity) Internal validation	Subjectivity is one of the strongest reasons in failed compilation between the clinical and preclinical comparisons. In the animal model the researcher takes the biochemical and physical parameters according to a set protocol. Sometimes the animal may suffer with some other complications which can be misinterpreted. Manual errors are also responsible for biasness in some cases.	For example if we take a disease which has a scoring for disease severity (IBD, Inflammation, Diarrhea) the scores given, differs in observer who is unaware and the observer who is aware about the treatment. These subjective errors may occur which are not suitable for the optimization. Again the species selection also give different responses

7	**Reproducibility and Robustness** (Face, Predictive and Target Validity) Internal validity	The ability to recreate the same impact every time by different researchers (Reproducibility) is one of the prime requisite for the standardization of the model and to evaluate the new molecules with the high impact on the treatment of the condition to help clinical interventions (Robustness) which depend upon the environmental factors, lab conditions and different time points and parameters for the same model with different conditions are two major concerning things in the preclinical trials.	Reproducibility is important criteria because model is the only tool to check the therapeutic effectiveness of the drugs and if it cannot be replicated or recreated due to certain restrictions the resources used for the drug development can be wasted. This can be achieved by inter species and inter/intra lab data collection for same model developed in different situations by one or more researchers.
8	**Ethical confirmations**	The ethical confirmations regarding the preclinical trials are equally important as the inform consent in the clinical trials. The number of animals used for any study should have a justification to sacrifice the lives of innocent animals after experimenting on them.	European and U.S. authorities have published some guidelines which are listed on the basis of cross species, comparison, target, homology distribution, signal transduction pathways and pharmacological effects. These guidelines (ECVAM, ARRIVE, PREPARE, OECD, ICH S5 CR3) can help the industries to use the biological products for approval before conducting an *in-vivo* experiment.

After designing models on adopted standardized methods the points of preclinical and clinical gaps were analyzed and rectified as effectively as possible-

- First step of proper conduct of model design is randomization of animals, which was done by creating different groups on the basis of approximate average weight (150-200gm as in some groups diet modification was needed) and age (8-12 weeks as the rats of this age achieved adult human like anatomical development of skeleton system) and primary screening in the form of biochemical tests to avoid the noise in primary data.
- All groups were allotted similar number of animals (n=6) and the experimentations were conducted on single strain (Wistar male rat) and similar lot of animals obtained from registered breeders.
- Animals from each group were marked for identification and the collected samples were also marked for estimation to avoid the errors.
- Samples (plasma/ serum/ organs) for different estimations were collected by standard procedures and processed according to need of evaluation parameter.
- On laboratory scale the basic tool for pharmacological comparison of models and evaluation of treatment groups against control group is software based statistical methods or mathematical equations. Different parametric and non parametric tests depending upon population size and the variable were analyzed.
- Before processing these data, the primary requisite is to obtain these values with minimum error to create same results every time which is considered as method validation. Here to maintain accuracy and robustness comparison of experimental models with four different inducing agents alone and in combination followed by ELUAR and Rheumatology guidelines of validation scoring systems containing three criteria-
- Predictive validity (graded for pharmacological parameters, CRP, ESR, Anti-CCP, IL-6, TNF-α, RF Factor, TLR-4, TG, TC, HDL, LDL levels
- Face validity (graded for core symptoms; Paw volume, Arthritic score, Arthritic index, X Ray, histopathology) and
- Constrict validity (graded for disease similarities and human resemblance; pain, symmetrical secondary lesions, digestion of digits, steatosis, Homocysteine levels and co morbidities).were performed with basic statistical methods of comparison between control group and model groups to check the disease progression.
- These data were further validated to get the better comprehension between models for Rheumatoid Arthritis and Models for Rheumatoid Arthritis with co morbid conditions by adopting scoring system again based on system biology as given in following table-

Table 4-6 Primary validation criteria for model development based on external validity criteria

Criteria	Value	Score
1. Species	Human Non-human primate Non-human mammal Non-mammal	4 3 2 1
2. Disease simulation	True Complex Pharmacological No	4 3 2 1
3. Face validity	>1 core symptom	4

	1 core symptom 1 symptom No	3 2 1
4. Complexity	*in-vivo* Tissue Cellular Sub-cellular/molecular	4 3 2 1
5. Predictivity	Graded for all pharmacology principles Graded for certain pharmacology principles All or none for certain pharmacology principles No or not shown	4 3 2 1

However the disease targeted in this study (RA) is one of the diseases facing a dialectical integrity between disease symptoms and the treatment applicability in humans coupled with the involvement of auto immune responses. Suggestions and recommendations generated through these logical scientific discussions are categorically summarized on the basis of **standard validation criteria (face, constrict and predictive validity)** and were used in preclinical *in-vivo* model designing in the current study replicates **external and internal validity parameters** were selected after a deep literature search for validation of prepared models for identified problems; RA model of rat with human resemblance and RA with CVD model of rat with human resemblance as discussed in table 4-6.

Finally all the marked gaps were carried to develop models for different situations and a final compiled model was evaluated which lay down in the format to closely fit for imparting the gap between preclinical and clinical situations using optimization at higher level of validation approaches to justify statistical data on external and internal validity domain indicators (FIMD) based on same weight.

4.4.2 Evaluation parameters for Pre validation:

4.4.3 Physical Indicators

4.4.3.1 Paw volume measurement[(104)]:

Inflammation is first and foremost sign of RA and in animals, inflammation and edema (fluid accumulation in body parts) developed after sensitization of inducers can be measured using paw volume. This estimation was done by Digital Plethysmometer (Aud/2510/dt.27-03-17) acquired by Laboratory enterprises a division of Milton enterprises, Nashik, Maharashtra, India.

Paw of animal were marked with a permanent marker at similar height using a scale to minimize the error before measuring paw volume.

This instrument has a cavity to fill water in which paw of animal was inserted and it should be stable for 03 second and value in ml of water displacement appeared on screen of the instrument after 03 sec. Three consecutive readings for each animal were taken to rectify the manual error. The comparison of inflammation can be done by taking readings of control group vs. other groups as well as non injected paw against injected paw. In this study paw volume was measured on day 1, 3, 5, 7, 11, 14, 17, 21, 28 in CFA induced models and on day 1, 3, 5, 7, 11, 14, 17, 21, 28, 35, 42 in collagen induced models.

4.4.3.2 Walking ability (Gait score)[106, 107]:

Walking ability was assessed on day 0, 7, 14, 28 in CFA induced groups and 0, 7, 14, 28, 42 in collagen induced groups by giving scores to the animals on the scale of 0 to 6. Here individual animal was kept on the flat surface to walk freely for a set time (2 min.) and the scores were given by the blind observer who is unaware about the identity of groups. Following scoring pattern was used to assess the mobility of animals.

Table 4-7 Gait Score for animals to assess walking ability

Walking Pattern	**Score**
Animal unable to move	0
Animal crawls only using the fore paws	1
Animal is unable to touch floor by paws only toes	2
fully touches the contralateral hind paw and fully touches the floor	3
Only toe of the ipsilateral hind paw of the animal touches the floor	4
Walk is protective towards the induced paw (touches the hind paw to the floor)	5
Animals walks normally	6

4.4.3.3 Body weight:

Body weight of the animals in this study was important in two aspects; in first part of the study the decrease in body weight is indicator for disease severity in terms of RA. But in later part of study where high fat diet was incorporated as animal food the diet became primary criteria to check the obesity in terms of metabolic dysbiosis. Here percentage change in body weight and growth index was measured against the food intake of animals on daily basis.

4.4.4 Perceptible Indicators[104]

4.4.4.1 Arthritic Score[108]:

Arthritic scoring is a measure of disease progression and severity appears on the non injected sites of animals. This score is given on the basis of inflammation, primary and secondary lesions, nodule formation redness, symmetric pattern involvement and inflammation of metapharyngeal joints of non injected paws (hind paw and fore paws). These scores were given by blind observers on day 5 on the basis of primary lesions generated in response to inflammation and on day 21 on the basis of secondary lesions generated in response to immunological intervention. In collagen induced groups the severity of the disease was measured on day 35 also and scores were provided to the animals. These scores were given by a blind observer who was unaware about the group's identity.

Table 4-8 scoring for evaluation of arthritic score

Body site	Observation	Score
Ears	Absence of nodules and redness Presence of nodules and redness	0 1
Nose	No swelling of connective tissue Intensive swelling of connective	0 1
Tail	Absence of nodules Presence of nodules	0 1
Fore paws	Absence of inflammation Inflammation of at least 1 joint	0 1
Hind paws	Absence of inflammation Slight inflammation Moderate inflammation Marked inflammation	0 1 2 3

4.4.4.2 Arthritic index[109]:

Arthritic index is Disease Activity Index (DAI). There are different methods of estimating arthritic index which can be included primary and secondary lesions with percentage inhibition of paw volume. In current study, arthritic index was measured on 0-4 scale where all four limbs of animal were used to check the disease severity at target organ only (limbs) as in model control groups forepaw swelling redness and edema was clearly visible which was not injected site. Induction was done only in hind limb paw but these symptoms at symmetric organs shows the

human disease similarity. This assessment was again done by a blind observer who is unaware about the group's identity and who has provided these scores on the basis of severity of all four paws and four scoring patterns, so the maximum score was 16 in form of selected symptoms described in table 4.9.

Table 4-9 Evaluation score for arthritic Index

Observation	**Score**
No erythema or swelling	0
Swelling or erythema or toes joints	1
Swelling or erythema of toes joints and toes	2
Swelling from ankle to toe joints	3
severe swelling from ankle joint up to entire paw	4

4.4.4.3 Radiographic Changes:

Radiological examination of rats was one of the confirmatory tools for the estimation of disease progression and bone deformity occurred due to disease generation. The X-ray of left hind paw was taken majorly for RA and groups which showed the secondary lesion in form of edema and bone deformities were also opted for X-ray imaging. Some animals were showed the bone disruption and severe deformities in tail and spinal cord which were also selected for full body X-rays. The X-rays were done at Dr. Lobo's clinic, Vadodara and Vadodara Society for prevention of Cruelty to animals, Chapad, Vadodara. The animals were provided i.p. injection of pentobarbital (60mg/kg) as a mild anesthesia to immobilize the animal ant to take X-rays of desirable body parts. The radiographic assessment of animals in CFA induced groups was done on day 14 and 28 and in CIA groups it was performed on day 28 and 42.

4.4.4.4 Atherogenic index[(110)]:

Atherogenic index is a quantitative measure used as an indicator for dyslipidemia and associated cardiovascular diseases. It is a novel index composed of triglyceride and high –density lipoprotein cholesterol. Here the atherogenic index was measured using following formula-

$$\text{Atherogenic index of plasma (AIP)} = \frac{\text{Log TC}}{\text{HDLc}}$$

4.4.4.5 Histopathological examination[98]:

At the end of the experiment (CFA groups on day 28 and in CIA groups on day 42). The animals were euthanized humanly with overdose of anesthetic agent and left hind paw of rat was taken out using bone cutter from the RA induced animals and the ankle portion of animals was separated cleaned and immediately fixed using freshly prepared 10% buffered neutral formalin solution. From groups developed cardiovascular complications other than bone, heart, aorta, Vastus medialis and biceps Femoris muscles were also taken out and fixed with similar procedure. The samples were sent to Sakshi histopathology center, Vadodara for preparation of slides for histopathological examination. The sections were examined under light microscope and photomicrographs were taken in electron microscope.

4.4.5 Biochemical indicators

4.4.5.1 Blood collection and plasma/ serum separation:

Blood sample from the animals for different estimation was collected by retro orbital plexus of eye of animal. At each time point the animals were anaesthetized using mild isoflurane anesthesia blood samples were taken into micro-centrifuge tubes for serum separation and for plasma sample the tubes were previously filled with anticoagulant (di potassium EDTA 2%) and after collecting blood this was gently mixed with anticoagulant and store on ice. Samples were then centrifuged at 2500 rpm for 10 minutes. The plasma obtained was stored at -80°C till further use.

4.4.6 Indicators for inflammatory responses

4.4.6.1.1 C - Reactive protein[111]:

Quantitative determination of CRP was done weekly and this acute phase reactant was measured to assess inflammatory rise in animals in response to RA generation. Turbilatex kit purchased from Labcare was based on the principle of quantitative turbidimetric test principle in which specific anti-human CRP are coated on latex particles and the absorbance change occurs due to agglutination of sample concentration of CRP.

The kit contained Reagent I (tris buffer 20mmol/L, pH 8.2. sodium Azide 0.95g/L), Reagent II (Latex particles coated with goat IgG anti-human CRP) and CRP calibrator (sodium Azide, PH

7.3, 0.95 g/L serum). Working reagent is prepared using 9:1 of latex reagent and diluent. Calibrator is also diluted using 1ml of distilled water and incubated at room tem. for 10min. UV spectrophotometer was used for this estimation. The base of zero was adjusted using distilled water and the prepared reagent was taken in to cuvette. The first absorbance was reader at 10 sec. (A1) before adding sample and after adding sample, reading was taken after 2 min (A2). And calculation was done using formula-

$$\frac{(A2-A1)sample}{(A2-A1)calibrator} \times calibrator\ conc = \frac{mg}{L}\ CRP$$

4.4.6.2 Erythrocyte Sedimentation Rate[112]:

ESR is another primary marker of progression of inflammation when sample of whole blood is mixed with anticoagulants and it is kept for some in specially designed Westergren apparatus, three events will occurs; Roulex formation, which is aggregation of RBCs as these tubes are vertically placed due to gravitational force the settlement of RBCs takes place from plasma and clear plasma will comes on top of the tubes this process takes 10-15min. in second stage RBCs get settled down and stage is known as sedimentation which will takes 30-40 min or more depending on tube length. In last stage packing of RBCs occurs which is also known as stationary phase here, the large number of RBCs sediments at the same time which create overcrowded RBCs struggling to get settled down, this procedure takes last 10min of whole process.

- ESR was measures here weekly using western green apparatus which were placed on flat surface with the scale inserted into the stand.
- Freshly prepared 0.250 ml of 3.8% w/v trisodium citrate solution was filled in collection vials with the help of syringe up to the mark provided.
- After that the rats were mildly anaesthetized for retro orbital blood collection in these tubes which is about 0.8ml.
- The collection vials were closed using their cap provided and mixed slowly three four times to mix anticoagulant and blood by upside down movement.

- The glass tube (ESRITE tube) was inserted slowly with clock wise movement till bottom of the collection vial and this was placed again in stand the blood sample should be raised up to 200mm height to match the zero marking on scale.
- The time for placing these tubes was noted and after one hour the height of clear column with plasma only was again noted to get ESR values in mm/hr.

4.4.7 Immunological markers

4.4.7.1.1 Neutrophil Count[113]:

Neutrophil are the first cells produced by body in immune response at the site of inflammation to limit the growth of microorganisms at inflamed site. Furthermore, they play an essential role in proper resolution of inflammation. When these processes are not properly regulated, they can trigger positive feedback amplification that promotes neutrophil activation, responsible for tissue damage to initiate chronic diseases.

Biological sample collection for neutrophil was done in morning hours with standard procedure described in section 4.7.3.1 in collection tubes containing 2% di potassium EDTA. The blood was mixed with slow upside down movement and these tubes were kept in ice box to maintain their conditions and handed over to divine lab at earliest as possible.

Neutrophil count was estimated by divine lab, Vadodara, using Sysmex, XN-550 - Japan five part differential counter. It is an automated and compact, hematology analyzer which can be used for full blood count with a standard five-part white blood cell differential and an immature granulocyte count, as well as an optional reticulocytes testing using an aspiration sample volume of 25 µL.

4.4.7.1.2 TNF-α[114]:

Tumor necrosis factor-alpha (TNF-α) is a protein and a potent mediator of inflammatory and metabolic functions. TNF-α was originally detected as a highly cytotoxic cytokine for tumor cells, it causes tumor necrosis *in-vivo* and shows cytolytic activity against tumor cells in *in-vitro* conditions.

This estimation was done by using ELISA kit for rat TNF-α purchased from Krishgen biosystems. The kit contained: Micro titer Coated Plate (96 wells) – 1 no, Recombinant Rat TNF-α Standard, 1µg/ml, 25µl – 4 vial, Rat TNF-α Biotin Conjugated Detection Antibody, 25µl

– 2 vials, Concentrated Streptavidin Horseradish Peroxidase, 50µl - 1 vial, Wash Buffer (20X) – 25ml, Assay Diluent (5X) – 10ml, TMB Substrate – 12ml and Stop Solution – 12ml.

Preparation of reagents:

All the reagents were prepared as per the instructions given in the instruction manual received with the kit. And to estimate the results accurately samples were seeded in duplicated=s or triplicates and a standard curve was plotted according to described calculations.

As per the instructions, upon first use, 1µg tube of recombinant standard and quick-spin was thawed, aliquot into polypropylene vials, and stored at -20°C. To run the assay, the recombinant protein was thawed and diluted by adding 20µl of standard solution in 10ml of Assay Diluent (1X) to prepare the top standard solution (2000pg/ml).

Working solution of 1X was prepared by using 5ml of wash buffer added to 95ml of DI water

To make **Assay Diluent (1X)**, add 1ml of Assay Diluent (5X) was added to 4ml of DI water.

Detection Antibody Dilution 1:200, 25µl of Detection Antibody solution was added to 4975µl of Assay Diluent (1X) to make final volume to 5ml.

Streptavidin-HRP Dilution 1:200, 50µl of Streptavidin: HRP solution was added to 9950µl of Assay Diluent (1X) to make final volume to 10ml.

Assay Procedure:

- All the reagents were brought to room temperature prior to use.
- Standards and samples were added in 100µl/well quantity to the plate.
- Six Two fold serial dilutions of rat TNF alpha having standard concentrations of 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, and 31.3pg/ml were prepared and Assay Diluent (1X) was taken as zero standard (0 pg/ml). These concentrations were seeded in plate and incubated for 2 hrs at 18-25°C temperature.
- After incubation plate was washed using Wash Buffer (1X) and blot residual buffer 3-4 times and tapped it upside down with mild action on absorbent paper. The plate was wiped to remove residue which can be interfere in reading.
- Previously diluted detection antibody (100µl) was added to each well and plate was sealed and incubated for 2 hours at 18-25°C. after incubation washing procedure was again performed.
- Now at this stage previously diluted Streptavidin-HRP solution in100µl quantity was introduced to each well and again incubated for 30 minutes at 18-25°C.

- Plate was again washed for final time. Here wells were soaked in between the washes given 3-4 time with Wash Buffer for 30 seconds to 1 minute.
- At this stage 100µl of TMB Substrate solution was added and plate was incubated in dark environment for 15 minutes. After incubation the positive control wells showed bluish color.
- At this stage Stop Solution (100µl) was used to stop the reaction which changes the color of positive wells from blue to yellow. Absorbance was taken at 450 nm within 30 minutes of stopping the reaction.

Calculation:

The mean absorbance for each set of duplicate or triplicate standards and samples were taken and the mean absorbance of the zero standards was subtracted from each well. The standard curve was plotted by taking cytokine concentration on the x-axis and absorbance on y-axis. The best fit straight line was drawn through the standard points. The unknown cytokine concentrations were determined, by mean absorbance value on the y-axis and a horizontal line to the standard curve was drawn. At the point of intersection, a vertical line was drawn to the x-axis and cytokine concentration was measured.

4.4.7.1.3 IL-6[115]:

Interleukin-6 (IL-6) is a major constitute of defense mechanism initiates in inflammatory responses as it is a multi-functional cytokine involved in regulation of immune responses, acute phase reactions and hematopoiesis. This estimation was done by using ELISA kit for rat TNF-α purchased from Krishgen biosystems. The kit contained: Microtiter Coated Plate (96 wells) – 1 no, Recombinant Rat IL-6 Standard, 1µg/ml, 25µl – 4 vials, Rat IL-6 Biotin Conjugated Detection Antibody, 25µl – 2 vials, Concentrated Streptavidin Horseradish Peroxidase, 50µl - 1 vial, Wash Buffer (20X) – 25ml, Assay Diluent (5X) – 10ml, TMB Substrate – 12ml and Stop Solution – 12ml.

Preparation of reagents:

Upon first use, 1µg tube of recombinant standard and quick-spin was thawed, aliquot into polypropylene vials, and stored at -20°C. To run the assay, the recombinant protein was thawed

and diluted by adding 20µl of standard solution in 10ml of Assay Diluent (1X) to prepare the top standard solution (2000pg/ml).

1. **Wash Buffer (1X):** Dilution: To make Wash Buffer (1X), 5ml of Wash Buffer was added (20X) to 95ml of DI water. This was used as working solution.

2. **Assay Diluent (1X):** Dilution: To make Assay Diluent (1X), add 1ml of Assay Diluent (5X) was added to 4ml of DI water. This was the working solution.

3. **Detection Antibody** Dilution 1:200, 25µl of Detection Antibody solution was added to 4975µl of Assay Diluent (1X) to make final volume to 5ml.

4. **Streptavidin-HRP Dilution** 1:200, 50µl of Streptavidin: HRP solution was added to 9950µl of Assay Diluent (1X) to make final volume to 10ml.

Assay Procedure:

- 100µl/well of Standards and Samples were added to the plate.
- Six two-fold serial dilutions of Rat IL-6 having standard concentrations of 2000pg/ml, 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 312.5pg/ml, 156.25pg/ml, and 78.125 pg/ml. were prepared and Assay Diluent (1X) was taken as zero standard (0 pg/ml). These concentrations were seeded in plate and incubated for 2 hrs at 18-25°C temperature.
- After incubation plate was washed using Wash Buffer (1X) and blot residual buffer 3-4 times and tapped it upside down with mild action on absorbent paper. The plate was wiped to remove residue which can be interfere in reading.
- Previously diluted detection antibody (100µl) was added to each well and plate was sealed and incubated for 2 hours at 18-25°C. after incubation washing procedure was again performed.
- Now at this stage previously diluted Streptavidin-HRP solution in100µl quantity was introduced to each well and again incubated for 30 minutes at 18-25°C.
- Plate was again washed for final time. Here wells were soaked in between the washes given 3-4 time with Wash Buffer for 30 seconds to 1 minute.
- At this stage 100µl of TMB Substrate solution was added and plate was incubated in dark environment for 15 minutes. After incubation the positive control wells showed bluish color.

- At this stage Stop Solution (100µl) was used to stop the reaction which changes the color of positive wells from blue to yellow. Absorbance was taken at 450 nm within 30 minutes of stopping the reaction.
- Absorbance was read at 450 nm within 30 minutes of stopping reaction.

Calculation:

The mean absorbance was determined for each set of duplicate or triplicate standards and samples. The mean absorbance of the zero standards was subtracted from each well. The standard curve was plotted by taking cytokine concentration on the x-axis and absorbance on the y-axis. The best fit straight line was drawn through the standard points. The unknown cytokine concentrations were determined, by mean absorbance value on the y-axis and a horizontal line to the standard curve was drawn. At the point of intersection, a vertical line to the x-axis was drawn and cytokine concentration was measured.

4.4.7.1.4 Homocysteine [116-118]:

Homocysteine (Hyc) is an amino acid and homologue of cysteine. It differs with cysteine due to an extra side chain methylene bridge. This amino acid intermediate formed during production of methionine, counted as an essential dietary amino acid. 80% of Hyc is found in plasma in protein bound form. When any inflammatory insult leads the immune responses this amino acid increases in free form in blood, which is a primary indication for premature incidences of vascular disease, heart disease and inflammatory conditions.

Homocysteine was measured using ELISA estimation kit procured from Krishgen biosystems. The kit contained: 96 Well Protein Binding Plate, Anti-Homocysteine Antibody (500X), One 15 µL vial. Secondary Antibody, HRP Conjugate (1000X), One 20 µL vial. Assay Diluent, One 50 mL bottle. 10X Wash Buffer, One 100 mL bottle of Substrate Solution, One 12 mL amber bottle and Stop Solution.

Preparation of Reagents:

Homocysteine Conjugate Coated Plate: Determine the number of wells to be used, and dilute the Homocysteine Conjugate 1:1000 into PBS.

Anti-Homocysteine Antibody and Secondary Antibody, HRP Conjugate: Immediately before use dilute the Anti-Homocysteine Antibody 1:500 and the Secondary Antibody, HRP

Conjugate 1:1000 with Assay Diluent. Prepare a dilution series of Homocysteine-BSA standards in the concentration ranges from 0 to 40 µg/ml.

Assay Procedure:

- All reagents were prepared and mixed thoroughly before use. Each sample, Homocysteine-BSA standard and blank was assayed in duplicate.
- 100 µL of 1X homocysteine conjugate was added to each well of the 96-well Protein Binding Plate and Incubated for 2 hrs. at 37ºC. Diluted homocysteine conjugate was removed by blotting the plate on paper towels to remove excess fluid.
- Wells were washed 3 times with 200 µL of PBS and blotted on paper towels to remove excess fluid.
- 200 µL of assay diluent was added to each well and block for 1 hour at room temperature. The plates were transferred to 4ºC until ready to begin the assay.
- Assay diluent was removed from the plate and 50 µL of unknown sample or standard was added to the homocysteine conjugate coated plate and incubated at room temperature for 10 minutes.
- 50 µL of diluted anti-homocysteine antibody was added to each well and Incubated at room temperature for 1 hour on an orbital shaker.
- Micro well strips were washed for 3 times with 250 µL 1X wash buffer with thorough aspiration between each wash in the wells. After each wash, wells were emptied and excess 1X wash buffer was removed by tapping.
- 100 µL of diluted secondary antibody and HRP conjugate were added to each well and incubated at room temperature for 1 hour and substrate solution was warmed to room temperature.
- The wells were washed again and 100 µL of substrate solution was added immediately to each well, including the blank wells with Incubation at room temperature.
- Enzyme reaction was stopped by adding 100 µL of stop solution into each well, including the blank wells.
- Absorbance of each micro well on a spectrophotometer using 450 nm as the primary wave length was read to get results.

4.4.7.1.5 Anti- CCP[9, 119]:

Anti-cyclic peptide (ACCP/ACPA) is an auto antibody. These are major marker to differentiate RA with other inflammatory diseases. These autoantibodies are directed against peptide and proteins (citrulline). During inflammation citrullination is a common process activated by posttranslational modification of arginine to citrulline by the enzyme Peptidyl Arginine Deiminase (PAD) [120]. Several citrullinated proteins, including fibrinogen and fibronectin, are present in RA synovium, and other citrullinated epitopes have been identified as targets of highly RA specific autoantibodies. The peptides processed by antigen presenting cells (APCs) shared epitopes with the MHC molecules significantly alter the shapes of these autoantibodies and the proteins may be seen as antigens by the immune system, thereby generating a self-destructive immune response Through T cell and B cell guided immune responses. Anti CCP test can be performed by qualitative or semi quantitative method. In this study test was performed by enzyme-linked immunosorbent assay, which is semi quantitative test.

Assay Procedure-

The wells of the microtiter strips were coated with a highly purified synthetic cyclic citrullinated peptide containing modified arginine residues.

During the first incubation, specific autoantibodies in diluted serum or plasma bind to the antigen-coated surface. The wells were then washed to remove unbound components.

In the second incubation the conjugate, an enzyme-labeled polyclonal antibody to human IgG, binds any surface-bound autoantibodies. After further washing, specific autoantibodies were traced by incubation with the substrate.

Addition of stop solution terminated the reaction, resulting in a colored end-product and the amount of conjugate bound was measured in absorbance units.

4.4.7.1.6 TLR-4 receptor [45, 121]:

This sandwich enzyme linked immunosorbent assay (ELISA) used to estimate the levels of TLR-4 in samples. Addition of standard or samples to microtiter well which is pre coated with human TLR 4 monoclonal antibody and addition of biotin labeled human TLR 4 antibodies, followed by addition of HRP conjugate to form immune develops blue color during incubation period and reaction will get stop after addition of stop solution with development of yellow color. The

concentration of the human TLR-4 sample is directly proportional to the yellow color developed in well and will be positively correlated.

Reagent Preparation

All kit components and samples were brought to room temperature (18-25^{o}C) before use.

Standard– Standard was reconstituted with 1.0mL of standard diluent, and kept aside for 10 minutes at room temperature (concentration of the standard in the stock solution is 80ng/ml).

Stock solution was diluted to 40ng/ml and then prepared 7 tubes containing 0.5mL and the diluted standard was used to produce a double dilution series.

Content of tube thoroughly mixed before the next transfer. Diluted standard Set up 7 points such as 40ng/mL, 20ng/mL, 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL were prepared and the last tubes were filled with standard diluent as blank with 0ng/mL

Detection Reagent A and Detection Reagent B - Stock detection were centrifuged and diluted to the working concentration 100-fold with Assay Diluent A and B, respectively.

Wash Solution - 20mL of Wash Solution concentrate (30×) diluted with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).

TMB substrate - Aspirate the needed dosage of the solution with sterilized tips.

Procedure:

- The kit was stabilized for 30 minutes at room temperature and then used.
- Standard dilution were prepared using standard in different concentration from 8 ng/ml to 0.5 ng/ml as per the manual.
- 50 µl of standard and 40 µl of samples were added in respective wells and 10 µl of biotin conjugate was added into each sample well.
- 50 µl of HRP conjugate was added in to plate and incubated for 1hr at $37^{0\,C}$
- After incubation, content was aspirated and plate was washed for 4 times with 1X wash buffer and residual buffer.
- 50 µl substrate A and 50 µl substrate B were added as stop solution and kept aside till well turned blue to yellow in color and absorbance was taken at 450 nm.

4.4.8 Lipid Profile[122]

4.4.8.1 Total Cholesterol:

Cholesterol is essential part of cell membrane formation. This fat like substance has a protective effect for cells and if liver increases its production and level of free formed cholesterol increases in blood it will be an alarming situation. High cholesterol levels are significant marker for risk of cardiovascular disorders. In this study cholesterol levels were taken as a marker to estimate the cholesterol levels after providing high fat diet to the animals in groups observed for CVD complications with RA.

Colorimetric analysis was done for estimation of cholesterol in this study on weekly basis. This colorimetric endpoint detection of cholesterol in serum or plasma sample is based on absorbance of metabolites of cholesterol in the form of quinoneimine which is a red dye which is detected in UV spectrophotometer at 490-550nm.

The kit contains Reagent l : Enzyme Reagent and Cholesterol Standard : 200 mg/dl.

In this reaction firstly, cholesterol esters are hydrolyzed to produce cholesterol. And hydrogen Peroxide is then produced from oxidation of cholesterol by cholesterol oxidase. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxide. The absorption of the red quinoneimine dye is proportional to the concentration of cholesterol in the sample.

Assay procedure

- Standard reagent was prepared using cholesterol reagent 10µl and 1000µl of reagent1
- Reagent 1 was taken as blank and instrument was set on zero
- Sample was prepared using 10 µl of sample and 1000 µl of reagent 1
- The samples were prepared and incubated for 5min at 37^0 C
- Absorbance of sample (AT) was measured against standard (AS) against reagent blank at 505 nm. Using following formula-

AT/AS x conc. Standard = mg/dl Total Cholesterol

4.4.8.2 Triglyceride:

Triglycerides are also fats like cholesterol and provide energy to the cells by lipoproteins in the blood. High fat content diet is responsible for elevation of triglyceride levels in blood. High TG levels are another indicator for vascular disorders and dysfunction of body in the form of dysfunction in cholesterol metabolism.

In this study endpoint detection method was utilized to detect TG in samples by colorimetric analysis.

Triglycerides are determined after enzymatic hydrolysis with lipases. The quinonemine indicator is formed from hydrogen peroxide, 4- aminophenazone, and 4-chlorophenol under the catalytic influence of peroxidase. The kit contained Reagent l: Enzyme reagent and Triglyceride standard: 200 mg/dl.

Assay procedure

- Standard reagent was prepared using triglyceride reagent 10µl and 1000µl of reagent1
- Reagent 1 was taken as blank and instrument was set on zero
- Sample was prepared using 10 µl of sample and 1000 µl of reagent 1
- The samples were prepared and incubated for 5min at 37^0 C
- Absorbance of sample (AT) was measured against standard (AS) against reagent blank at 505 nm. Using following formula-

AT/AS x conc. Std. = mg/dl Triglycerides

4.4.8.3 High Density Lipoproteins (HDL):

HDL is denoted as good cholesterol because HDL particles carry cholesterol from the cells back to the liver for metabolism. High levels of HDL are thought to lower the risk of heart disease and low HDL cholesterol levels, is considered a greater heart disease risk.

In this study HDL was assessed on weekly basis and the kit contained Reagent I: Precipitating Reagent and Cholesterol Standard: 50 mg/dl. In this method low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated by the precipitating reagent. After centrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction remains in the supernatant in this phase and is determined by an enzymatic method.

Assay procedure

- Standard reagent was prepared using cholesterol standard 50µl and 1000µl of reagent1
- Reagent 1 was taken as blank and instrument was set on zero
- Sample was prepared using 50 µl of sample and 1000 µl of reagent 1
- The samples were prepared and incubated for 5min at 37^0 C
- Absorbance of sample (AT) was measured against standard (AS) against reagent blank at 520 nm using following formula-

Ac/As x conc. Standard x 2 = mg% Cholesterol HDL in serum

4.4.9 Statistical Analysis:

In biomedical research, the study design or conduct of an investigation can be observational studies, pilot studies, exploratory experiments, confirmatory studies, and experiments based on parameter estimates. Pharmacological experimentations involve some or all of these types depending upon the hypothesis and the requisite of final outcome.[123] The present study has observational outcomes (on the basis of physical estimations and the statistical data), pilot studies (for estimation of dose response and variability of different inducing agents), exploratory experiments (treatment responses, hypothesis correction via *p* value correction using post hoc tests in ANOVA), and confirmatory studies using the estimation parameters. Models with both the situation (RA and cardiovascular complications in RA) statistically analyzed applying one way ANOVA and Repeated measure ANOVA using GraphPad Prism software where values are expressed as Mean ± SEM. Boneferroni's, Dunnett and Tukey's Post hoc tests were used for comparison between each group. Significant values were compared at *$p<0.05$, **$p<0.01$, *** $p<0.001$. Here three different post hoc tests were applied-

Boneferroni's post hoc test- This test is considered as a simplest comparative test in ANOVA by this analysis we can get results of comparison between each pair of groups. Boneferroni test gives the list of *p* values (significant difference) and confidence intervals in comparison to each and every pair selected for the analysis.

Dunnett's post hoc test- This comparison allows to compare means of each and every selected group with mean of control group. The confidence interval in this method is also 95% for significant difference with degree of freedom but it will not give liability for multiple comparisons.

Tukey's post hoc test- This test is also known as Honest Significant Difference (HSD) analysis. This is one of the most used post hoc tests for multiple comparisons like Bonferroni's and compares every mean with every other mean. In this test the confidence interval is 0.00 which shows the significant difference via P values in multiple mean comparisons and we can apply it on unequal sample size also.

4.4.10 Methods for final model validation:

As these statistical data are predictive values and there outcome is insufficient to give a model which can be called as validated, so among all nine groups, four groups; group Va (CFA 0.1ml + LPS 10µg/ml), group Vb (CIA0.1ml+ LPS 10µg/ml), VII (CFA0.1ml+ HFD+ LPS 10µg/ml) and group IX (CIA0.1ml+ HFD+ LPS 10µg/ml) were carried forward for external validity measures. Here, Decision regarding selection of an optimum/appropriate dose of LPS (among three concentrations 0.1, 0.5 and 10µg/ml) was made by processing the obtained data for significance using ANOVA as primary evaluation tool for pharmacological comparison. The selected models were carried forward for validation based on the Framework to Identify Models of Disease (FIMD) given by Guilherme S. Ferreira et.al. This is a questionnaire based validation system where were models were further compared and evaluated for eight domains; epidemiology, symptomatology and natural history–SNH, biochemical validation, aetiological validation, pharmacological validation, histological validation, endpoint validation and genetic validation.

The framework was adopted and the questions were framed using reference of the framework but some extra points were added according to the need of the study. The questions were more focused on the Rheumatoid Arthritis and cardiovascular complications in the selected models with the questions of clinical relevance.

Notation: For better understanding in succeeding sections the final compared groups will be denoted as-

Va (CFA 0.1ml + LPS 10µg/ml) as Model I

Vb (CIA0.1ml+ LPS 10µg/ml) as Model II (Representing RA models)

VII (CFA0.1ml+ HFD+ LPS 10µg/ml) as Model III

IX (CIA0.1ml+ HFD+ LPS 10µg/ml) as Model IV (Representing RA along with CVD)

4.4.11 Same weight score method using FIMD[(124)]:

To validate these final scrutinized groups on the basis of FIMD, one common questionnaire was formed. The questions were framed according to disease severity and progression using the mentioned domains. Each domain has given the same weighing (score) which was calculated using 100 as total score. Questions were also answered separately for all subsections on the basis

of five answer pattern suggested (yes, yes completely, yes partially, unclear and no). All the values calculated in this method were attached as supplement file (Annexure VII).

4.4.11.1 Design of questionnaire

Questionnaire were made for two situation as per the need of study-

1) ***Questionnaire prepared on the basis of FIMD for Rheumatoid Arthritis model***
2) ***Questionnaire prepared on the basis of FIMD for Rheumatoid Arthritis with cardiovascular complications.***

Steps for questionnaire preparation and to provide same weight score-

The FIMD questionnaire was prepared and each section and sub section was answered using the following instructions to get final radar score.

4.4.11.2 FIMD for best model developed for RA:

To finalize the model/models with human resemblance for Rheumatoid Arthritis were now compared and optimized on higher level of validation tools. The internal and external validity criteria were used here to closely fit the model with clinical relevance on the basis of fine tuned preclinical data. This purpose of minimizing the translational gap was done by designing a questionnaire for assessment of best model for RA according to FIMD. The main eight domains framework was adopted and the questions were framed using reference. The questions were more focused on RA and associated pathogenic events of clinical disease development and parameters of evaluation.

Table 4-10 Questionnaire for comparison of models developed for RA

Questions for RA Model	SW
1. Epidemiology	**12.5**
1.1 Nature of population (Inbred/Outbred)	4.16
1.2 Is the model able to simulate the disease in the relevant age groups (Juvenile, adult or aging)	4.16
1.3 Is the model able to simulate in different genders	4.16
2. Symptomatology and natural history	**12.5**
2.1 Is model is able to Mimic human disease symptoms, if so which one	4.16
2.1.1 Inflammatory Markers	1.38
2.1.2 Immunological markers	1.38
2.1.3 Crosslinking markers for cardiovascular complications	1.38
2.2 Natural History criteria matching with human disease onset	4.16
2.2.1 Time of onset	0.83
2.2.2 Disease Progression	0.83
2.2.3 Duration of Symptoms	0.83
2.2.4 Severity	0.83
2.2.5 Metabolic dysbiosis (Obesity, TG, TC, LDL, HDL and fat accumulation in stool)	0.83
2.3 Co-Morbid Conditions replicated in model similar to human conditions? if yes which one	4.16
2.3.1 Secondary lesions	1.38
2.3.2 Overlap Syndrome (Digestion of digits)	1.38
2.3.3 Extra organ manifestation	1.38
3. Biochemical Validation	**12.5**
3.1 Pharmacodynamic biomarkers mimic the pathophysiology of the human disease	4.16
3.1.1 Increased inflammatory Markers (CRP, ESR, Arthritic Index)	2.08
3.1.2 Increased immunological markers (ACCP, IL-6, TNF-α)	2.08
3.2 Do these PD markers behave similarly to human?	4.16
3.3 Known prognostic markers related to pathophysiology of the disease	4.16
3.3.1 Walking disability	1.04
3.3.2 Symmetric progression of disease	1.04
3.3.3 Increase in secretions	1.04
3.3.4 Nodule Formation	1.04
4. Aetiological Validation	**12.5**
4.1 Is the aetiology of the disease similar to human for Rheumatoid Arthritis? If yes which one	6.25

4.1.1 Cytokine Activation (TNF-α, IL-6)	**2.08**
4.1.2 Cell infiltration (ACCP generation)	**2.08**
4.1.3 Radiographic changes	**2.08**
4.2 Is the aetiology of the disease similar to human for RA and co-morbid conditions? If yes which one	**6.25**
4.2.1 Disability	**2.08**
4.2.2 Receptor Activation (TLR-4)	**2.08**
4.2.3 Extra organ manifestation	**2.08**
5. Pharmacological Validation	**12.5**
5.1 Are effective drugs in humans also effective in this model? If yes which one	**4.16**
5.1.1 Which one (Methotrexate)	**4.16**
5.2 Are ineffective drugs also ineffective in this model?	**4.16**
5.3 Have drugs with different mechanisms of action and acting on different pathway been tested in this model? Which one (aqueous extracts of herbs)	**4.16**
5.3.1 Test drug 1	**1.38**
5.3.2 Test drug 2	**1.38**
5.3.3 Test drug 3	**1.38**
6. Histological Validation	**12.5**
6.1 Do the Histopathological structures in relevant tissues resemble the ones found in humans? If yes which one	
6.1.1 Histopathology of Bone	**12.5**
7. Endpoint Validation	**12.5**
7.1 Are the endpoints used in preclinical studies are same or translatable to the clinical endpoints?	**6.25**
7.1.1 Radiographic Changes	**2.08**
7.1.2 Perceptive changes	**2.08**
7.1.3 Cell infiltration in histopathology	**2.08**
7.2 Are the methods used to assess preclinical endpoints comparable to the ones used to assess related clinical endpoints?	**6.25**
7.2.1 Paw Volume	**1.56**
7.2.2 Walking Disability	**1.56**
7.2.3 Symmetric progression	**1.56**
7.2.4 Severity of disease	**1.56**
8. Genetic Validation	**12.5**
8.1 Does this species also have orthologous genes and /or proteins involved in the human disease	**4.16**

8.2 If so, are the relevant genetic mutations or alterations also present in the orthologous genes/proteins	**4.16**
8.3 If so, is the expression of such orthologous genes and/ or proteins similar to the human condition?	**4.16**

SW- Same weight score

4.4.11.3 FIMD for best model developed for cardiovascular complications in RA

Questionnaire for model of co morbid CV complications in RA was developed again using the standard format of eight domain intervention as mentioned above. As this model is combination of higher complexity in terms of cardiovascular complications in RA the questions were framed according to the symptoms and the pathogenesis involved in both the diseases. The groups optimized and validated on all the complexities and disease severity points and the three states were scrutinized. All the models were compared for the questions and were answered individually according to standard format.

Table 4-11 Questionnaire for comparison of models developed for cardiovascular complications in RA

Questions for RA with CVD Model	
1. Epidemiology	**12.5**
1.1 Nature of population (Inbred/Outbred)	**4.16**
1.2 Is the model able to simulate the disease in the relevant age groups (Juvenile, adult or aging)	**4.16**
1.3 Is the model able to simulate in different genders	**4.16**
2. Symptomatology and natural history	**12.5**
2.1 Is model is able to Mimic human disease symptoms, if so which one	**4.16**
2.1.1 Inflammatory Markers	**1.38**
2.1.2 Immunological markers	**1.38**
2.1.3 Crosslinking markers for cardiovascular complications	**1.38**
2.2 Natural History criteria matching with human disease onset	**4.16**
2.2.1 Time of onset	**0.83**
2.2.2 Disease Progression	**0.83**
2.2.3 Duration of Symptoms	**0.83**
2.2.4 Metabolic Dysbiosis	**0.83**
2.2.5 GUT infiltration	**0.83**
2.3 Co-Morbid Conditions replicated in model similar to human conditions? if yes which one	**4.16**
2.3.1 Secondary lesions	**1.04**
2.3.2 Overlap Syndrome (Digestion of digits)	**1.04**
2.3.3 Extra organ manifestation	**1.04**
2.3.4 Steatosis	**1.04**
3. Biochemical Validation	**12.5**
3.1 Pharmacodynamic biomarkers mimic the pathophysiology of the human disease	**4.16**
3.1.1 Increased inflammatory Markers (CRP, ESR, Arthritic Index)	**1.38**
3.1.2 Increased immunological markers (TNF-α, IL-6, ACCP, Hyc)	**1.38**
3.1.3 Increased Atherogenic markers (TG, TC, Cholesterol, Atherosclerotic index)	**1.38**
3.2 Do these PD markers behave similarly to human?	**4.16**
3.3 Known prognostic markers related to pathophysiology of the disease	**4.16**
3.3.1 Walking disability	**0.69**
3.3.2 Symmetric progression of disease	**0.69**
3.3.3 Increase in secretions	**0.69**
3.3.4 Nodule Formation	**0.69**
3.3.5 Obesity	**0.69**

3.3.6 Metabolic dysbiosis	**0.69**
4. Aetiological Validation	**12.5**
4.1 Is the aetiology of the disease similar to human for Rheumatoid Arthritis? If yes which one	**6.25**
4.1.1 Cytokine Activation (TNF-α, IL-6)	**1.25**
4.1.2 Cell infiltration (ACCP generation and Hyc activation)	**1.25**
4.1.3 fibre length of Vistus medialis	**1.25**
4.1.4 Fibre length of Biceps Femoris	**1.25**
4.1.5 Radiographic changes	**1.25**
4.2 Is the aetiology of the disease similar to human for RA and co-morbid conditions? If yes which one	**6.25**
4.2.1 Disability	**1.56**
4.2.2 Metabolic Dysbiosis (Obesity, TG, TC, LDL, HDL and fat accumulation in stool)	**1.56**
4.2.3 Receptor Activation (TLR-4, NLRP-3)	**1.56**
4.2.4 Extra organ manifestation	**1.56**
5. Pharmacological Validation	**12.5**
5.1 Are effective drugs in humans also effective in this model?	**4.16**
5.1.1 Which one (Methotrexate)	
5.2 Are ineffective drugs also ineffective in this model?	**4.16**
5.3 Have drugs with different mechanisms of action and acting on different pathway been tested in this model? Which one (aqueous extracts of herbs)	**4.16**
5.3.1 Test drug 1	**1.38**
5.3.2 Test drug 2	**1.38**
5.3.3 Test drug 3	**1.38**
6. Histological Validation	**12.5**
6.1 Do the Histopathological structures in relevant tissues resemble the ones found in humans? If yes which one	**4.16**
6.1.1 Histopathology of Bone	**4.16**
6.1.2 Histopathology of Heart	**4.16**
6.1.3 Histopathology of Vistus medialis and Biceps Femoris muscle	**4.16**
7. Endpoint Validation	**12.5**
7.1 Are the endpoints used in preclinical studies are same or translatable to the clinical endpoints?	**6.25**
7.1.1 Radiographic Changes	**2.08**
7.1.2 Perceptive changes	**2.08**
7.1.3 Cellular infiltration in Histopathology	**2.08**

7.2 Are the methods used to assess preclinical endpoints comparable to the ones used to assess related clinical endpoints?	**6.25**
7.2.1 Paw Volume (Pain and stiffness)	**1.04**
7.2.2 Walking Disability	**1.04**
7.2.3 Symmetric progression	**1.04**
7.2.4 Severity of disease	**1.04**
7.2.5 Atherogenic biochemical markers (Lipid Profile)	**1.04**
7.2.6 Obesity and metabolic dysbiosis	**1.04**
8. Genetic Validation	**12.5**
8.1 Does this species also have orthologous genes and /or proteins involved in the human disease	**4.16**
8.2 If so, are the relevant genetic mutations or alterations also present in the orthologous genes/proteins	**4.16**
8.3 If so, is the expression of such orthologous genes and/ or proteins similar to the human condition?	**4.16**

SW- Same weight score

b) How to score same weight score and interpreted it-

Instructions given for scoring and calculations for the final radar plot reading were followed to fit the validation criteria in the proposed models of RA and RA along with CVD using the questions framed according to disease severity and progression in the mentioned domains. Each domain has given the same weighing (score) and provided calculation based score to find out ratio for plotting radar chart to interpret the validity of model (details attached as supplement file at the end of thesis document).

c) How to prepare and interpret radar plot

Radar chart or web chart is one of the comparative tools for analyzing multivariate data. Here the radar chart give each domain an axis and we can compare the models by putting the ratio obtained after giving suitable score to each question and calculated through the steps mentioned above.

Interpretation of radar plot

- Here, Microsoft excel was used to generate a radar plot and values (calculated ratio) for each domain on different axis as this graph is 2 dimensional representations.
- On the basis of the ratio value which move towards the axis and the dispersion from the axis we can get results in the form of similarity factor and uncertainty factor.
- The values moves towards the axis and having the similar intersecting points with other domains values have the interconnectivity between them.
- The values which are not intersecting each other and having higher dispersion from the axis towards the edges of graph are having higher ratio and they are having higher dissimilar or uncertainty with compared groups.

4.4.11.4 How to validate models on the basis of FIMD method

After answering the questions with suitable grades, summation of all the scores of sections and subsections of individual domain was done to get the final score and it was subtracted with the actual domain score to get ratio for plotting radar chart which is one of the comparative tools for analyzing multivariate data. Here the radar chart give each domain an axis and we can compare the models by putting the ratio obtained after giving suitable score to each question and calculated through the steps mentioned above. To check the similar domains in models,

similarity factor (counted by points matched validation criteria on radar plot by different models) and uncertainty factors (counted by points on which validation criteria not matched on radar plot by models) were also calculated which can give an account for improvement of experimental design. The final score after radar analysis was compared to check the level of validation according to source reference which suggest the percentage of validation in different categories as validation levels; if model lies between 0-40% it will be *Insufficiently validated*, if model scores between 41-60% it will be *Slightly validated*, if model scores between 61-80% it will be *Moderately validated* and if model scores 81-100% it will be *Highly validated*.

4.4.12 Comparison of developed models to get a novel, validated and optimized model

The final outcome from the above two situations were interconnected at some level due to common disease RA and some points were differed according to increased complexities in the form of secondary inducers. So for culminating the study, final RA model II (CIA0.1ml+ LPS 10µg/ml) which gained higher radar value and represents the clinical resemblance for RA was compared with the model having highest extra organ manifestations in form of Atherosclerosis Model IV (CIA 0.1ml +LPS 10µg/ml+ HFD) for concluding the higher complexity, co morbid conditions and connectivity to introduce a single model which represents the clinical imprints of cardiovascular complications in RA. The comparison was done on the basis of Table I and Table II questions, which have analyzed individually for all the models and scored according to statistics of same weight scoring system, where the models were optimized again on similarity and uncertainty factors.

4.5 Methodology for section IV (Pharmacological evaluation of NSAE, CPAE and MCAE in validated model of Rheumatoid Arthritis)

4.5.1 Dose selection for test drugs for evaluation

In comparison of developed models for RA final validated model CIA0.1ml+ LPS 10µg/ml model was proved to give a human resemblance in terms of pathophysiological and biochemical events. Thus in treatment protocol this final model was carried out for pharmacological evaluation of NSAE, CPAE, MCAE in three different dose levels (100mg/kg, 200mg/kg and 400mg/kg) against the commonly used DMARDs Methotrexate as a clinical standard.

As per the toxicity accounts of all three herbs discussed in plant profile section and on the basis of past research, maximum tolerated animal dose for *Nigella sativa* seed was found as 800mg/kg so the selected dose was chosen as 400, 200 and 100mg/kg. *Carica papaya* seed aqueous extract dose was directly taken from research of *Nazneen Zehra and Lubna Naz* where these three doses; 100, 200 and 400mg/kg were selected for hepatoprotective effects in rats. There were no strong evidences were found for dose of *Momordica charantia* seed aqueous extract so the different doses were selected as 100, 200 and 400 mg/kg in accordance with other two drugs.

Moreover placebo groups were also selected where 600mg/kg doses of *Momordica charantia* were not compatible for animals which showed severe weight lose and diarrhea after one week of oral administration. In *Nigella sativa* placebo effect was compatible for animals and in *Carica papaya* also higher doses were not suitable for animals.

Clinical doses of all three herbs were also confirmed from Bhava prakasha and Ayurvedic Pharmacopeia according to mentioned doses also this dose selection found to be in limits of therapeutic range.

In the experimental schedule protocol was designed for 42 days and these treatments were compared with normal control and Model control groups from model development studies.

Group (Test I) received CIA0.1ml+LPS 10µg/ml+NSAE 100mg/kg

Group II (Test II) received CIA0.1ml+LPS10µg/ml+NSAE200mg/kg

Group III (Test III) received CIA0.1ml+LPS10µg/ml+NSAE400mg/kg

Group IV (Test IV) received CIA0.1ml+LPS 10µg/ml+CPAE 100mg/kg

Group V (Test V) received CIA0.1ml+LPS10µg/ml + CPAE 200mg/kg

Group VI (Test VI) received CIA0.1ml+LPS10µg/ml+CPAE400mg/kg

Group VII (Test VII) received CIA0.1ml+LPS10µg/ml+MCAE100mg/kg

Group VIII (Test VIII) received CIA0.1ml+LPS10µg/ml+MCAE200mg/kg

Group IX (Test IX) received CIA0.1ml+LPS10µg/ml+MCAE400mg/kg

Different parameters; biological (ESR, CRP, neutrophil, Anti-CCP, homocysteine), Physical (paw volume, arthritic scoring, arthritic index), photographic, radiographic (X-ray) evaluation were performed on regular intervals and at the end of the study histopathology of left hind limb bone and paws having secondary lesions was done. All the groups were compared statistically applying one way ANOVA and Repeated measure ANOVA using pot-hoc tests (Bonferroni's, Tukey's and Dennett's where applied) for comparison of model and treatment groups on different time points.

4.5.2 Combination dose selection for validated model of CVD in RA

As this protocol was designed to evaluate different doses in RA we get the concentrations which are effective in RA. Three dose levels for NSAE, CPAE and MCAE (100mg/kg, 200mg/kg, 400mg/kg) when evaluated the dose dependency and efficacy was proved here. To overcome this problem 3^3 was taken for further combine these doses for treatment protocol for cardiovascular complications in RA. Here lower dose combination for CPAE was opted as there was some dose sensitization was observed in 200 and 400mg/kg. to overcome this problem 50 and 100mg doses of CPAE were selected.According to this design total nine combinations were designed for further evaluation with low medium and high doses of all three herbs. In this study path run 1 is having concentrations of all three drugs as 0 so this particular combination was taken as a control group.

Table 4-12 Combinations of selected doses for treatment of CVD in RA

Drug 1	Drug 2	Drug 3
NSAE (mg/kg)	CPAE (mg/kg)	MCAE (mg/kg)
0	0	0
200	50	200
100	50	200
200	50	400
100	100	200
100	50	400
200	100	200
200	100	400
100	100	400

Rest of the groups were selected as test groups against CIA0.1ml+ LPS10µg/ml + HFD model and standard control Mtx. and HFD. The groups for study were divided as-

Group I Normal control (NC-1)

Group II High fat diet (NC-2)

Group III Standard control group CIA0.1ml+ LPS10µg/ml + HFD+ Mtx (0.6mg/kg)

Group IV Test I CIA0.1ml+ LPS10µg/ml + HFD+NSAE 200+CPAE 50 +MCAE200mg/kg

Group V TestII CIA0.1ml+ LPS10µg/ml + HFD+ NSAE 100+CPAE 50 +MCAE200mg/kg

Group VI Test III CIA0.1ml+ LPS10µg/ml + HFD+ NSAE 200+CPAE 50 +MCAE400mg/kg

Group VII Test IV CIA0.1ml+ LPS10µg/ml + HFD+ NSAE 100+CPAE 100 +MCAE200mg/kg

Group VIII Test V CIA0.1ml+ LPS10µg/ml + HFD+ NSAE 100+CPAE 50 +MCAE400mg/kg

Group IX Test VI CIA0.1ml+ LPS10µg/ml + HFD+ NSAE 200+CPAE 100 +MCAE200mg/kg

Group X Test VII CIA0.1ml+ LPS10µg/ml + HFD+ NSAE 200+CPAE 100 +MCAE400mg/kg

Group XI Test VIII CIA0.1ml+ LPS10µg/ml + HFD+ NSAE 100+CPAE 100 +MCAE400mg/kg

All these groups were screed for evaluation of combination of all three extracts; NSAE, CPAE and MCAE in validated model of cardiovascular complications in RA on all the parameters described in study design I and II for evaluation of both the disease markers and conclusions of the study were drawn on the basis of obtained results for pharmacological evaluation of some herbs in experimentally induced Rheumatoid arthritis along with cardiovascular complications.

4.6 Methodology for Section V (Formulation development)

After screening combination of final selected doses were converted to human doses and a formulation in the form of tablet was manufactured by **Vasu Healthcare Pvt. Ltd. Vadodara,**

Gujarat. All the standardizations for formulation were performed by Vasu Healthcare and discussed in results section.

4.6.1 Conversion of animal dose to human dose

Step 1 – Calculate the amount of active metabolite in experimental doses

The direct conversion dose from animal to human is always higher and could be toxic, sometimes converted preclinical doses to human dose are ten times higher as the body surface area of small animal is high as compared to human. These kinds of dosage are not acceptable as per the safety guidelines. As we are proving reverse pharmacology of the selected herbs, this particular dose should be under the limits of maximum effective concentration to give the effect with safety profile. For this reason extracts were sent to AMSAR Pvt. Ltd. Indore M. P. to analyze the total yield of active metabolite responsible for the activity in each extract so that the doses should be under limits of effective dose concentration after conversion of animal dose to human dose for novel combination, where-

1. *Nigella Sativa* extract showed presence of active metabolites in 10:1 ratio (10 gm of extract contains 1 gm of active metabolites prominently thymoquinone)
2. *Carica papaya* extract showed presence of active metabolites in 1:0.5 ratio (1 gm of extract contains 500 mg of active metabolites majorly saponines)
3. *Momordica charantia* extract showed presence of active metabolites in 5:1 ratio (5 gm of powder contains 1g of active metabolites calculated as active bitters and phenolic acids)
4. Now the human dose conversion was done on the basis of active metabolites present in the animal doses.

Step 2-- Calculate human dose on the basis of this final obtained dose using conversion factor (6.2) for converting rat dose to human dose in mg/kg

Table 4-13 Conversion of animal dose to human dose

Sr. no.	Name of aqueous extract	Experimental dose for rat	Amount of active metabolite in experimental doses of extract	Human dose conversion (70kg)	Human Equivalent Dose (HED)	Final dose for dosage form design
1.	*Nigella Sativa*	200mg/kg	20mg	20mg / 6.2 x body weight	225.4mg	1,184.82 mg
2.	*Carica Papaya*	100mg/kg	5mg	5 mg / 6.2x body weight	56.42mg	
3.	*Momordica Charantia*	400mg/kg	80mg	80 mg / 6.2 body weight	903mg	
Total						1,184.82 mg

* This calculation is on the basis of individual maximum effective concentration of each herb

Step 3—Combine the dose of all the extracts for final dosage form (formulation dose)

The final combination doses of drugs were found as-

Table 4-14 Doses of extracts for formulation development

Sr. no.	Herb	Final combination doses	Human dose without exciepients
1.	*Nigella sativa*	100mg/kg	112.7 = 113 mg
2.	*Carica papaya*	50mg/kg	28.21 mg
3.	*Momordica charantia*	200mg/kg	451.5 mg
Total amount of selected herbs incorporated in doses form			**592.71mg**

*Note- These are the combination doses without exciepients

The final weight of tablet formed is 750mg which contains 592.71mg of drug content and +156.5 mg of exciepients for single administration.

The dosage form is for BD (twice a day) dosage, where combination dose total is (592.71mg x 2= 1,185.42) which is under the limits of safety doses.

4.6.2 Formulation and development of tablets of aqueous extracts of *Nigella sativa, Carica papaya and Momordica charantia* seed aqueous extract

After converting the doses the major concern for formulation development was doses size and dose of each extract to be incorporated in OD or BD doses to reduce the pill burden if this tablet is used for clinical trial in future. On the basis of this account Dr. Hardik Soni Assistant General Manager (R&D) and Dr. Vishal Patel Deputy Manager (ADL) from Vasu Healthcare (Research centre) were suggest to design a doses form which can be compatible and final formula for preparation of tablet was designed as follows-

Table 4-15 Final formulation designing for divided dosages forms

Sr. No.	Ingredients	mg/Tablet	%
	Extracts		
1.	*Nigella Sativa* seed (Kalonji) DE	113	15.06
2.	*Carica Papaya* seed (Papaya)DE	28.21	3.76
3.	*Momordica Charantia* seed (Karela) DE	451.5	60.2
	Total	**592.71**	79.02
	Exciepients		
4.	Starch	101.79	13.57
5.	Kyron T – 314	45.00	6.00
6.	Talcum	3.75	0.5
7.	Colloidal Silicon Di Oxide	3.00	0.40
8.	Magnesium Stearate	3.75	0.5
	Total Core Tablet Weight	**750**	**100.00**

*DE- Dry Extract

4.6.3 Material used for formulation development

Development of tablet as dosages form was done in this study by incorporating the minimum excipients needed for herbal formulation. In each and every dosage form there are some additives are necessary with the main API (Active Pharmaceutical ingredient). In this preparation the APIs are dry extracts of select nutraceutical and the additives added here are diluents, lubricants and dinsintegrants.

4.6.3.1 Role of each excipient in formulation development

Starch: starch is one of the most commonly used diluents, binder and disintegrating agents in pharmaceutical dosage forms generally obtained from the natural resources like *Cyperus esculentus*, Banana, rice and maize. As starch is polysaccharide and chemically it is polymer which contains anhydroglucose units linked with α-D glucosidic bonds. In different concentration and percentage the role of starch get changed in formulations. In this tablet formula quantity of starch is 13% which is preferable range for diluents as well as disintegrant[(125)].

Kyron T-314: Polacrillin Potassium is also known as Kyron T-314 which is a super disintegrant generally used for fast release of tablets especially in mouth dissolving tablets. Kyron T-314 is highly purified polymer and it has very high potency to swell quickly with contact of gastro intestinal fluid. The use of this super in this polyherbal formulation was required as all dry aqueous extracts used as dry powder were directly compressed where particles are highly compressed. In this situation this super disintegrant can increase absorption and bioavailability of drug when come in contact to fluids and simultaneously increases the effectiveness of prepared tablet which allowed us to reduce the dosage form on the basis of therapeutic effect of drug.

Lubricants: In pharmaceutical preparations, tablets are most common solid dosage forms and they required some additional substances to develop an API in to a dosage form. When we talk about herbal formulations the basic problem with these ingredients is hygroscopic nature or they form a sticky material which required proper flowability and lubrication to come out from the machine. In formulation development metallic salts of fatty acids such as magnesium stearate and stearic acid are widely used. Some times more than one lubricant is also necessary to incorporate in to the preparation to work synergistically.

Magnesium Stearate: Magnesium stearate is one of the frequently used lubricants in solid dosage form and it is well accepted by FDA as it is an inactive additive and it can be obtained from natural as well as synthetic origins. Magnesium stearate ($Mg(C_{18}H_{35}O_2)_2$) has very unique capacity to form different types of reversible hydration states when come in contact to moisture in surroundings which simultaneously affect the compressibility of material.

In this current formulation magnesium stearate is used to give stability and prevent hygroscopic characters of aqueous extracts. As well as it can act as a lubricant this increases the flow property of powders.

Talc: Talc or talcum is very basic but most important part of pharmaceutical industry. It can be used as lubricating agent in development of formulation at the time of preparation as well as in machine operation. Talc can act as a lubricant to increase the flow ability of mixture component as well as when tablet is going to be punched in tablet punching machine talc as an additive reduces the friction between solid component and machine.

Colloidal Silicon Di Oxide: Glidants are as equally important as flow property enhancers. Colloidal silicon dioxide is used in this preparation with lubricants and flow enhancers to increase the flow of dry powder extracts. Moreover it acts as moisture scavenger at the time of granulation to maintain the hardness of tablet dosage form.

4.6.4 Procedure for development of tablet

After selecting suitable ingredients for 100 tablet batch size dosage form was designed by following steps-

STEP 1: Weighing and Shifting

- Dried aqueous extracts of *Nigella Sativa* seed *Carica Papaya* seed and *Momordica Charantia* seed along with starch were weighted separately
- Weighed ingredients were passed by 40# Sieve to get uniform powder.

STEP 2: Binding and Granulation

- Sufficient quantity of starch after sieving was used to prepare starch paste using water.
- Dried aqueous extracts of *Nigella Sativa* seed *Carica Papaya* seed and *Momordica Charantia* seed were mixed and bind with starch paste.
- Wet granulation method was used to prepare granules by passing this mixture by 12# Sieve.

STEP3: Drying and Cutting

- Prepared uniform granules were dried in dryer at 60 ^{0}C.
- Granules were dried until 3% moisture level was achieved.
- If any oversized granules were formed they were cut by suitable method.

STEP 4: Lubrication and Blending

- Kyron T-314, Talcum and Colloidal silicon dioxide were weighted and passed through 40# sieve.
- All the additives were mixed in prepared dried granules

- Magnesium Stearate was weighted separately and passed from 40# sieve and mix into the granules.

STEP 5: Compression

- Compression was done using Double Rotarty Compression machine and proper cleanliness was maintained.
- The punch size was set up for 17.25 X 8.25 mm capsule shape tablets and tablets were punches
- The target weight of tablet was kept as 750mg ± 10 mg.

4.6.4.1 Standardization of prepared formulation

Standardization of prepared formulation was done on the basis of organoleptic analysis, physicochemical analysis, microbial analysis and comparative HPTLC fingerprinting for detection of all three extracts used for tablet formulation.

I) Organoleptic analysis: Prepared tablets were analyzed on the basis of their general description such as Size, shape, diameter and thickness of tablets. The outcomes of this section are summarized in section V in result section.

II) Physicochemical analysis: Physicochemical parameters on the prepared tablets were performed where hardness, friability, disintegration time, average weight of tablet, UV spectroscopy for Thymoquinone for *Nigella sativa* was analyzed. Gravimetric tests were performed for bitters and saponines present in *Momordica charantia* and *Carica papaya* aqueous extracts used for formulation development.

III) Heavy metal and microbiological analysis: analysis of heavy metals; lead, cadmium, mercury and arsenic were performed according to standards of Ayurvedic Pharmacopoeia of India. And total microbial load, total yeast and mould count, specific bacterial counts as suggested by WHO guidelines and Ayurvedic Pharmacopoeia of India for *S. aureus, Salmonella, P. aeruginosa* and *E. coli* were also performed on prepared tablets.

IV) Instrumental analysis: HPTLC fingerprinting was performed for comparison between dry aqueous extracts and formulated preparations for standards. Where different test solutions were prepared and run against the formulation with suitable spray reagent.

a) Preparation of test solution (T1, T2, T3 and T4): 1 g of each extract sample was weighed separately of (T1, T2, T3) and Tablet was powdered and 1 g weighed of (T4) accurately in a

Iodine flask. In this 20 mL methanol was added and refluxed for 15 minutes. After allowing the solution for 15 min the solution was filtered with help of Whatman filter paper No. 1. This obtained filtrate was further used for HPTLC fingerprinting.

b) Preparation of Spray reagent [Anisaldehyde – sulphuric acid reagent]: 0.5 mL Anisaldehyde is mixed with 10 mL Glacial acetic acid followed by 85 mL Methanol and 5 mL Sulphuric acid (98 %).

Table 4-16 Conditions for HPTLC fingerprinting

Chromatographic Conditions:	
Application Mode	CAMAG Linomat 5 – Applicator
Filtering System	Whatman filter paper No. 1
Stationary Phase	MERCK - TLC / HPTLC Silica gel 60 F254 on Aluminum sheets
Application (Y axis) Start Position	10 mm
Development End Position	80 mm from plate base
Sample Application Volume	8 µL
Distance Between Tracks	15.3 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Mobile Phase (MP)	Toluene : Ethyl acetate : Acetic acid (7 : 3 : 0.1 v/v)
Visualization	@ 254 nm, @ 366 nm and @ 540 nm (after derivatization)
Spray reagent	Anisaldehyde- Sulphuric acid reagent
Derivatization mode	CAMAG – Dip tank for about 1 minute
Drying Mode, Temp. & Time	TLC Plate Heater Preheated at 100± 5^0C for 3 minutes

All the results obtained in this procedure are described in result section (V)