Pharmacological Evaluation of some herbs in experimentally induced Rheumatoid arthritis along with cardiovascular complications

SYNOPSIS OF THE
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IAEC Approval of Animal Studies

Animal husbandry, handling and treatments were performed as per the CPCSEA guidelines 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.

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 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*.

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 mentioned below:

MSU/IAEC/2015-16/1661 dated 30/12/2016

MSU/IAEC/2018-19/1802 dated 29/12/2018

MSU/IAEC/2019-20/1904 dated 29/08/2019

All the protocols were designed according to PREPARE and ECVAM guidelines and data were recorded according to ARRIVE guidelines.

1 Introduction

Rheumatoid Arthritis (RA) is chronic systemic autoimmune disease in which body's immune system mistakenly attacks on joints, this causes inflammation that lines the inside of joints (Synovium) to thicken, resulting in swelling and pain in and around joints.[1, 2]

Inflammation associated with synovial membrane is primary cause for initiation of RA. Synovitis contributes to cartilage and bone erosion and uncontrolled inflammatory process leads to deformity of the joints.[3] On epidemiological account RA affects 1% of population worldwide with 0.5% to 1% prevention and in India the RA prevalence has been estimated to be 0.75%. RA can occur at any age, but is most likely to show up between ages 30-50[4]. Disease involves younger people, elderly people and females. Females have two folding risks for the outbreak of illness, but the disease's extra-articular manifestations are more prevalent in men, causing impairment and mortality.[5]

As per the pathogenesis and etiological accounts, Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint swelling, joint tenderness, and destruction of synovial joints, leading to severe disability and premature mortality.[6] Involvement of the other organs; Lung, renal, heart and skin are the major contributor in worsen the conditions of the RA patients.[7]

Proinflammatory cytokines such as interleukin (IL-6), Tumor necrosis factor-alpha (TNF-α) and NF-κB are central mediators in RA. As disease progression occur hematological markers such as Anti- citrullinated protein (ACCP), RF- factor, ESR and C-RP becomes major criteria for diagnosis and interventions.[8] RA differs clearly from person to person due to auto immunity and presence of genetic variants. The prevalence of cardiovascular complications in patients suffering from Rheumatoid Arthritis (RA) is one of the major concerns today which is associated with both morbidity and mortality. There are **two main reasons** of occurrence of cardiovascular disorders (CVD) in RA: The **first being** inflammation, driven by NLR (Nod Like Receptors) and TLR (Toll Like Receptors) receptor activation, works as underpinning factor in both the diseases and long term cardiovascular complications[9]. Second one is the side effects and drawbacks of existing therapies.[10] According to the European League Against Rheumatism (EULAR) and FDA the drug regimen (NSAIDs, DMARDs and Biologics) of RA also contributes in progression of CVD.[11]

Toll like receptors plays a vital role in initiating the cascade of events in the form of activation of Neutrophills and Macrophages (the first guardian of the innate immune system). These cells have TLR epitopes on their surfaces which are responsible for the activation of the events for primary defense mechanism. On activation of TLR receptors proinflammatory cytokines and activation of T cell B cell and lymphocyte activation occurs[12]. In result endotoxemia, gut permeability microbial burden and cell infiltration are stimulated which results in impaired Immunity. This process is mediated by an interdependent network of cytokines, prostanoids, and proteolytic enzymes.

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Secondly the basic problem in the long term diseases like Rheumatoid is the continuation of the same therapy over the period of time. Being as an auto immune disorder there are some problems in the current treatment therapy. To overcome increased evidences of progression of disease different organization and committees in the field of Rheumatology as well as FDA are not mutually satisfied with the RA regimen or standards in treatment.[11, 13] Treatment plan in allopathic system for management of Rheumatoid Arthritis includes NSAIDs (Ibuprofen, COX inhibitors), Steroidal regimen (glucocorticoids) and most established DMARDs (Methotrexate, and Biologics).[14] These drugs are also proved contributor in the progression of cardiovascular complications in some clinical studies done by FDA, apart from their own side effects in patients.

Traditional medication is one of the most accepted and adopted area from early ages. Each and every community and culture have evidences of use of plants and other natural components as medicinal drug and therapy. Ayurveda is one of the ancient and traditional medicine systems which provides alternative treatment options on the basis of the three doshas (*vata*, *pitta* and *kapha*) which are physiological balancing elements of the constitution of individual (*Prakriti*). The disbalances between these three elements by metabolic dysbiosis can affect the *Prakriti* of an individual.

In Ayurveda Rheumatoid Arthritis is known as *Amaavata*, a metabolic disorder generated due to imbalance between *Ama* and *Vata*. *Ama* is substances produced by malfunctioning of the digestive system, circulates in the blood and finally accumulates within the joint cavity, resulting in the pain and loss of function of joints. *Vata* is air and ether elements which control the movement of fluid and cells through the body and coordinates between muscles, organ and nerves.

RA is one of the diseases which have more home remedy then the allopathic treatment. In Ayurveda and traditional Chinese and Indian medicines, *Curcuma longa, Boswellia serrata, Tripterygium wilfordil* and *Tanacetum parthenium* are some of the herbs which are accounted for relief against Rheumatoid Arthritis.[15] In present scenario commonly available Ayurvedic proprietary products have so many options in the form of ointments, Tailas and liniments to relive pain and inflammation. Maha Narayan Tel and Rheumasyl are most popular oils available in India for management of RA with local application. Therapeutic regimen in Ayurvedic marketed products includes purified herbs (Turmeric powder, Boswellia powder and tablets).

That being the case the potential of using medicinal herbs, common spices and condiments as anti arthritic drug is promising to increase life expectancy and patient compliance in long term diseases like RA. In-depth literature survey reveals anti-arthritic and cardio protective properties of *Nigella sativa* seed[16], *Carica papaya* seed[17] and *Momordica charantia* seed[18]. Herbs chosen in line for the management of the condition is promising due to their characteristic attributes on blockage of the pathways interconnected to pathophysiology of Rheumatoid Arthritis. These herbs are having proved toxicity accounts which shows them safe for management of RA[19, 20]. The combination of such potential herbs can be considered as an independent drug therapy or can be given in combination with the already existing therapies with better compliance and frugal option to solve the problem in designing a set regimen of therapeutic agents for RA which are targeted on the systemic approach only and are devoid of having ability to correct the root cause of basic disease generating elements (*Doshas* and *Prakriti*) due to the complex involvement of metabolic, inflammatory and autoimmune factors.

The select herb, *Nigella sativa* is commonly known as Kalonji contains Thymoquinone possessing anti inflammatory, anti arthritic, anti Atherogenic which corrects endothelial dysfunction and inhibits TNF-α production. *Carica papaya* seed commonly known as paw-paw seed is categorized as super food, flavanoids are major constituent with analgesic, anti

inflammatory, wound healing and cardio protective properties. *Momordica charantia* (Karela or bitter gourd) having account of anti inflammatory and wound healing properties due to presence of flavanoids. Thus the aim and objective of this study was to evaluate the potential of traditionally used spices and condiments as stand alone or add on therapy for management / treatment of RA and its long-term cardiovascular complications.

Objectives -

1. ***In-vitro* evaluation of extracts on various cell lines RAW 264.7 Macrophage cell lines, HEK 293 TLR-4 expression cells and HIG-82 Synoviocyte cells.**
2. **Development of RA models-**
   1. Model I (CFA induced Rheumatoid Arthritis in rat)
   2. Model II (Collagen induced Rheumatoid Arthritis in rat)
3. **Development of RA along with Atherosclerosis using CFA**
   1. Model III (CFA + HFD) cardiovascular complications in RA using diet modification
   2. Model IV (CFA+HFD+LPS) Cardiovascular complications in RA using Diet modification with endotoxine challenge

      (*LPS is given in different doses to select suitable model)
4. **Development of RA along with Atherosclerosis using CIA**
   1. Model V (CIA + HFD) cardiovascular complications in RA using diet modification
   2. Model VIII (CIA+LPS+HFD) Cardiovascular complications in RA using Diet modification with endotoxine challenge

      (*LPS is given in different doses to select suitable model)
5. **To evaluate effect of aqueous extracts of *Nigella sativa, Carica papaya* and *Momordica chirantia* seed in model of RA.**
6. **To evaluate effect of aqueous extracts of *Nigella sativa, Carica papaya* and *Momordica chirantia* seed in model of RA along with CVD complications.**
7. **To evaluate the effect of aqueous extracts of *Nigella sativa, Carica papaya* and *Momordica chirantia* seed in validated RA model CIA+ LPS (0.1ml+l0µg/ml) with standard treatment (Mtx 0.6mg/kg)**
8. **To evaluate the effect of aqueous extracts of *Nigella sativa, Carica papaya* and *Momordica chirantia* seed in validated RA along with CVD model CIA+ LPS**

(0.1ml+l0µg/ml)+HFD with standard treatment (Mtx 0.6mg/kg)

2 Methodology

2.1 *In-vitro* Studies

2.1.1 Material for *in-vitro* study

RAW 264.7 cell line, HEK 293 cell line and HIG 84 cell lines were obtained from NCCS Pune, Maharashtra. LPS (Lipopolysaccharide) was purchased from Sigma Aldrich (Co. St. Louis. MO. USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fetal Bovine Serum, Trypsin –EDTA solution, Propidium Iodide and antibiotic solution, DMSO and 6-, 24- and 96 well plates were purchased from Himedia.99% IPA (Isopropyl Alcohol/propane-2-ol) and Acetone was purchased from Fisher Scientific.

Experimental design[21, 22]

Cell line study is a primary tool for estimation of receptor activation in representative cells of the living being. Here in this study cell line study were performed for the estimation of preliminary data collection with reference to drug effect on the synovial cells (HIG 84 cell line), activation of Toll like receptors-4 in synovial membrane (RAW 267.4 cell line) and in the specific cells (HEK 293 cell line) which are major effective site for activation of TLR-4 via NLRP-3 receptor stimulation. These studies provide the toxicity account of the herbs and to check the receptor activation for hypothesis to develop *In-vivo* model via particular combination of sensitizing agents to activate the specific pathophysiological markers for a robust model. The following methods were used for fulfillment of the study-

- MTT assay (For dose selection and cytotoxicity profile of selected herbs in different concentrations)
- Biomarker studies with reference to different activation pathways (Macrophage activation with LPS,IL-6 assay and TNF-α assay)

2.2 *In-vivo* studies

Ethical approval

All the studies were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Dept, Faculty of Pharmacy. All experimental procedures are mentioned below and materials were procured by different sources to initiate the work –

2.2.1 Material for *in-vivo* study

2.2.1.1 Drugs and chemicals

Methotrexate was obtained as gift sample from Alembic pharmaceuticals, Vadodara. CFA (Complete Freund's adjuvant) and LPS were obtained from Sigma-Aldrich, India. Bovine Collagen (Type II) was purchased from Condrex Krishgen Biosystem Mumbai.

Casein, corn starch and sucrose were obtained from Spectrochem Pvt. Ltd, Mumbai, India. Vitamin mix was purchased from veterinary shop from Vadodara.

2.2.1.2 Sources of diagnostic kits

Kits for estimation of C - reactive protein was obtained from ADI enterprises, Baroda. ELISA Kits for estimation of TNF-α and Interleukin-6 and TLR- 4 purchased from Krishgen biosystems Mumbai. Kits for estimation of total cholesterol, triglycerides, HDL-C were purchased from Span Diagnostics Pvt. Ltd., Surat.

2.2.1.3 Plant collection and Authentication

Nigella sativa seeds were purchased from a local grocery store from Siyagunj Indore, Madhya Pradesh, and *Carica papaya* seeds 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 at The Maharaja Sayajirao University of Baroda, Vadodara. Reference no. for the authentication certificate is Ref: Bot/30317/aut/1.

2.2.1.4 Preparation of aqueous extracts of the selected herbs

The procured plant material was studied morphologically 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. Simple maceration technique was used to extract out all the components of seed in aqueous extract of *Nigella sativa*, *Carica papaya* and *Momordica charantia* seeds. Dry seeds procured were powdered in grinder individually. Coarsely 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. Than the filtrate was evaporated using water bath till get dry. The practical yield of *Nigella sativa* extract was found to be 10% and was

labeled as NSAE (*Nigella sativa* aqueous extract). The practical yield of extract was found to be 8% and was labeled as CPAE (*Carica Papaya* Aqueous Extract). The practical yield of extract was found to be 8% and was labeled as MCAE (*Momordica charantia* aqueous extract). Standardization of the prepared aqueous extracts were done using qualitative phytochemical screening of plant material and proximate analysis tests were performed for the qualitative measures of the phytochemical components present in extracted material.

2.2.1.5 Standardization of the prepared aqueous extracts

The procured plant material was studied morphologically 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. After confirmation crude plant material in powdered form is also analyzed for the determination of moisture, total ash, acid-insoluble and water-soluble ash and protein contents were done in the sample. Other analysis (loss on drying, heavy metals, microbial profile) was done.

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. 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. After estimation of primary data for presence (Indicated in table as + for Present and – for Absence) of metabolites the serial dilution of extracted materials were prepared to confirm the major metabolites against markers using UV visible photo spectrometer and TLC.

Table: 1 Compilation of standardization data for selected plants and their extracts

Organoleptic Characteristics of Selected Plants				
Sr. no	**Characters**	**Herb 1 (Nigella sativa seed)**	**Herb 2 (Carica papaya seed)**	**Herb 3 (Momordica charantia seed)**
1	Appearance	Ovate	Oval	Oblong
2	Color	Black	Black	Seed coat is Yellowish brown and inner kernel is creamy to off white
3	Odor	Characteristic and pungent	Characteristic	Characteristic
4	Uniformity	Uniform and crystalline	Uniform	Uniform
5	Visual identification	Rough surface	Hairs on seeds	Grooved margins and a sculptured surface
6	Taste	Characteristic with aroma	Slightly Bitter	Slightly bitter with characteristic smell

Phytochemical estimation for metabolites				
1	Alkaloids	++	+	- -
2	Bitters	-	-	-
3	Tannins	+	++	--
4	Saponins	-	-	+
5	Carbohydrates	+	+	+
6	Flavonoids	-	+	--
7	Terpenoids	+	+	+
8	Phenolic acids	+	+	+
9	Anthocyanins	-	+	-
10	Glycosides	+	+	++
11	Reducing sugars	+	+	+
12	Protein	-	-	+
13	Fat and oil	+	+	+
14	Steroids	+	+	--
15	Phytosterols	+	+	+
**** Presence of metabolite is indicated by + and Absence is indicated by – sign here**				

Proximate analysis results

Sr. no	**Proximate Analysis Parameter**	**Nigella sativa Linn**		**Carica papaya Linn**		**Momordica charantia Linn**	
		Experimental value	**Reference value**	**Experimental value**	**Reference value**	**Experimental value**	**Reference value**
1.	Moisture Content	1.99	2.91 %	4.6± 0.05	5.4 ± 0.05	17.8± 0.05	20.69 ± 5.85
2.	Total Ash	3.87	4.82 % w/w	6.7± 0.05	8.2 ± 0.08	7.2± 0.05	9.73 ± 2.34
3.	Acid insoluble ash	0.17	0.15 % w/w	022%	0.44%	9± 0.05	8.31 ± 0.19
4.	Water Soluble extractive	9.35	11.59 % w/w	72%	84.27%	22± 0.05	28.42 ± 0.36
5.	Crude Protein	18.45	27.19	19.5± 0.05	25.1 ± 0.08	20.5± 0.05	19.50 ± 0.73
6.	Heavy metals						
	a) As	NMT 03PPM	Complies	NMT 03PPM	Complies	NMT 03PPM	Complies
	b) Pb	NMT 10PPM	Complies	NMT 10PPM	Complies	NMT 10PPM	Complies

	c) Cd	NMT 0.3PPM	Complies	NMT 0.3PPM	Complies	NMT 0.3PPM	Complies
	d) Hg	NMT 1PPM	Complies	NMT 1PPM	Complies	NMT 1PPM	Complies
7.	Microbial Profile						
	Total plate count	NMT 1000 CFU/ G	410 CFU/ G	NMT 1000 CFU/ G	340 CFU/ G	NMT 1000 CFU/ G	360 CFU/ G
	Yeast and Moulds	NMT 100 CFU/ G	Absent	NMT 100 CFU/ G	Absent	NMT 100 CFU/ G	Absent
	E. Coli	Absent	Absent	Absent	Absent	Absent	Absent
	Salmonella	Absent	Absent	Absent	Absent	Absent	Absent

2.2.1.6 Dose selection and preparation of doses

Dose of nigella for this study was selected from literature which has treatment indications for hypolipidemia and anti-arthritic activity, due to this said reason 100, 200 and 400 mg/kg[(23-25)] for rats were selected for evaluation of anti arthritic and anti atherosclerotic properties. Carica 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 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 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 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.

All the aqueous extracts were prepared and stored at 2-8 ^{0}C with labels NSAE (*Nigella sativa* aqueous extract) for nigella seed, CPAE (*Carica papaya* aqueous extract) for carica seed and MCAE (*Momordica charantia* aqueous extract) for momordica 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.

In-vivo Experimental design

The in-vivo experimental study was designed on the following three lines-

1) Development and validation of an experimental animal model for RA and its cardiovascular complications. 2) Evaluation of efficacy of selected herbs in the developed experimental model

of RA and its complications 3) Determination of nature of interactions for combination of herbs in ameliorations of RA and its complications with standard drugs

a) Development and validation of experimental models

All experiments were carried out on male wistar rats weighing 150 - 200g, obtained from registered breeders. Animals were divided in required groups for model development, containing six animals each. The animals had free access to conventional laboratory diet in all groups and high fat diet in specific groups with purified water *ad libitum*.

CFA induced RA

Group I served as normal control and not sensitized with any adjuvant. CFA induced RA study was designed for 28 days and group IIa, IIIa and IVa considered as CFA model and received CFA 0.1ml by sub planter route on left hind paw on day 0 for induction of RA. Group IIIa and IVa further sensitized with LPS (10µg/ml) from day 14 to day 28. In addition Group IVa received HFD[(26)] for induction of cardiovascular complications with LPS (10µg/ml) for aggravating the induction of immune components and cardiovascular complications. Group Va, VIa and VIIa were considered as standard groups. Group Va considered as treatment group and received CFA 0.1ml +LPS 10µg/ml+ HFD + combination of NSAE200mg/kg + CPAE 50mg/kg and MCAE 400mg/kg. Group VIa and VIIa considered as standard group where, group VIa (Std I) received CFA 0.1ml +LPS 10µg/ml+ HFD+ Mtx 0.6mg/kg and group VII (Std. II) received CFA 0.1ml +LPS 10µg/ml+ HFD+ Mtx 0.6mg/kg + combination of NSAE200mg/kg + CPAE 50mg/kg and MCAE 400mg/kg.

CIA 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.

CIA induced RA protocol was designed for 42 days and group IIb, IIIb, IVb and Vb were sensitized with collagen 0.1ml (CFA1: CIA1). 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[(27)]. Group IIb received CIA 0.1ml only on day 0. Group IIIb, IVb a Vb, VIb, VIIb and VIIIb received CIA 0.1ml on day 0 and these groups were further sensitized with LPS from day 14 to day 42 by s.c. route. Group IIIb received CIA +LPS, group IVb

received CIA 0.1ml sub planter + HFD +LPS 10µg/ml, group Vb and VIb considered as treatment groups where, group Vb received CIA 0.1ml sub planter + LPS 10µg/ml+ NSAE200mg/kg + CPAE 50mg/kg and MCAE 400mg/kg, group VIb received CIA 0.1ml sub planter + HFD +LPS 10µg/ml+ NSAE200mg/kg + CPAE 50mg/kg and MCAE 400mg/kg. Group VIIb and VIIIb and IXb considered as standard treatment groups where, group VII received CIA 0.1ml sub planter + HFD +LPS 10µg/ml+ Mtx 0.6mg/kg, group VIIIb received CIA 0.1ml sub planter + HFD +LPS 10µg/ml+ Mtx 0.6mg/kg + NSAE200mg/kg + CPAE 50mg/kg and MCAE 400mg/kg and group IXb received CIA 0.1ml sub planter + HFD +LPS10µg/ml+ Mtx 0.6mg/kg +Telmisartan 40mg/kg as standard drug for treatment of cardiovascular complications.

Here for better understanding the groups were further renamed according to model, standard and treatment plans. Models were divided as-

Model I (CFA 0.1 ml sub planter)

Model II (CFA0.1 ml sub planter + LPS 10µg/ml s.c.)

Model III (CFA0.1 ml sub planter + LPS 10µg/ml s.c. + HFD)

Model IV (CIA 0.1 ml sub planter)

Model V (CIA0.1 ml sub planter + LPS 10µg/ml s.c.)

Model VI (CIA0.1 ml sub planter + LPS 10µg/ml s.c. + HFD)

Standard treatment was done with Methotrexate (MTX 0.6mg/kg i.p.) and these groups were denoted as-

Std. I (CFA 0.1ml sub planter +LPS 10µg/ml s.c. + HFD+ Mtx 0.6mg/kg i.p.

Std. II (CFA 0.1ml sub planter +LPS 10µg/ml s.c. + HFD+ Mtx 0.6mg/kg i.p. + NSAE200mg/kg + CPAE 100mg/kg +MCAE 400mg/kg p.o.)

Std. III (CIA 0.1ml sub planter + HFD +LPS 10µg/ml+ Mtx 0.6mg/kg i.p)

Std. IV (CIA 0.1ml sub planter + HFD +LPS 10µg/ml+ Mtx 0.6mg/kg i.p + NSAE200mg/kg + CPAE 100mg/kg and MCAE 400mg/kg p.o.)

Std. V (CIA 0.1ml sub planter + HFD +LPS10µg/ml s.c.+ Mtx 0.6mg/kg i. p. +Telmisartan 40mg/kg p.o.)

Preventive treatment plan was performed with aqueous extracts of three selected seeds; NSAE, CPAE and MCAE and were named as-

Test I (CFA 0.1ml sub planter +LPS 10μg/ml s.c.+ HFD + NSAE200mg/kg + CPAE 100mg/kg +MCAE 400mg/kg p.o)

Test II (CIA 0.1ml sub planter +LPS 10μg/ml s.c.+ NSAE200mg/kg + CPAE 100mg/kg +MCAE 400mg/kg p.o.)

Test III (CIA 0.1ml sub planter +LPS 10μg/ml s.c.+ HFD + NSAE200mg/kg + CPAE 100mg/kg +MCAE 400mg/kg p.o.)

Different evaluation parameters were performed where protocol for CFA induced groups was followed for 28 days and protocol for CIA induced group was followed for 42 days.

Biological parameters- ESR and CRP were performed on day 7, 14, 21, 28 in CFA groups and 7, 14, 21, 28, 35, 42 in CIA groups. Neutrophil, total WBC, Anti-CCP and Homocysteine were performed on day 14 and 28 in CFA and 14 and 35 in CIA sensitized groups. TNF-α, NF-κB and IL-6 were performed on day 14 and 28 in CFA and on day 14 and 42 in CIA groups. Expression studies for NLRP-3 and TLR-4 receptors were performed at the end of the study.

Physical parameters- Paw volume of left hind paw was measured on day1, 3, 5, 7, 11, 14, 17, 21, 28 in CFA sensitized groups and 1, 3, 5, 7, 11, 14, 17, 21, 28, 35, 42 (CIA groups). Primary and secondary lesions were assessed on day 5 and 21 and 35 in all groups. Arthritic score was calculated. Arthritic Index was estimated on the basis of paw volume, secondary lesions and score gained on day 5, 21 and 35. Photographic assessment was also done on alternate days, Radiographic examination (X-Ray) was performed on day 14 and 28 in CFA groups and on day 28 and 42 in CIA groups.

Confirmatory tests- At end of the study Histopathology of bone was performed in CFA sensitized groups and in CIA sensitized groups bone, heart, aorta, Vastus Medialis muscle and Biceps Femoris muscle were collected for histopathological evaluation.

Statistical assessment

All the data of quantitative analysis of Paw volume, Arthritic Score, Arthritic index, CRP, ESR, Anti-CCP, Homocysteine, IL-6, TNF-α were evaluated statistically applying one way ANOVA for end point comparison and Repeated measure ANOVA for interval data gathered by weekly analysis. Values are expressed as mean ± SEM. Values are statistically evaluated using one way ANOVA analysis followed by Bonferroni's, Dunnett's and Tukey's post hoc tests for the assessment of model development and treatment on different time points with respect to normal

control, model control standard and test group comparison. Significant values were compared with, (*P<0.05, **p<0.01, *** P<0.001).

b) Model Validation

The results were compiles on the basis of validation parameters which show the disease severity in different groups on the basis of evaluation parameters.

Two methods for model validations were selected where-

Prevalidation- The scoring based method was adopted for this validation where each and every criterion has a score. The total of score is combined score for one model on the basis of different parameters which shows the total severity of disease or effectiveness of the treatment.

Comparison of experimental models with four different inducing agents alone and in combination followed by ELUAR and Rheumatology guidelines validation scoring systems containing three criteria; predictive validity (graded for pharmacological parameters Neutrophil count, CRP, ESR, Anti-CCP, IL-6, TNF-α, RF Factor), 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).using scoring system based on face validity-The similarity in biology and symptoms between the animal model and human disease.Predictive validity-Clinically effective interventions demonstrate a similar effect in model and Constrict/Target validity- The target under investigation should have a similar role in the disease model as in clinical situation.

Validation using FIMD- The results of the scoring system were not found satisfactory as they are score based on more predictive side. The developed models were further validated to get an optimized model for both the conditions individually (RA models/ CV complications in RA) as well as with one another (RA models compared with final RA along with CV complications) using eight domain based criteria for face validity, constrict validity and predictive validity measures using comparison and validation of developed models by FIMD (Framework to identify Models of Disease) method given by Guilherme S. Ferreira *et.al.* On the basis of all above criteria the findings of this study showed that CIA+LPS (0.1ml +10µg/ml) induced model closely fit for preclinical events of RA, and CIA+LPS (0.1ml+10µg/ml)+HFD model represents validated model for co-morbid cardiovascular conditions in RA.

3 Results

3.1 Results of *in-vitro* studies-

The results for *in-vitro* studies were compiled according to two separate studies performed in three different cell lines where cell viability assay was performed in all three cell lines (RAW-267.4, HEK-293 and HIG 84) to check the negative effects of the select herbs if , any. Biomarker activation was seen in RAW 267.4 cell lines to check the activation of cytokine release after LPS challenge in different concentrations against control cells denoted as control group. Receptor expression studies for NLRP-3

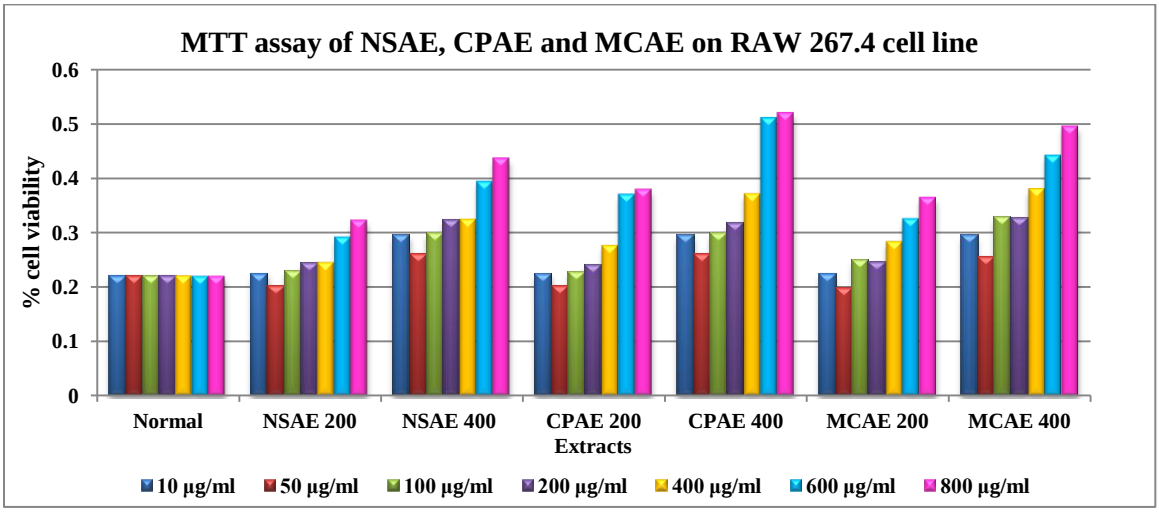
and TLR-4 were performed in RAW 267.4 cell lines to correlate the linking pathway between cytokine and receptor activation which was primary tool for bridging the hypothesis in correlation with inflammatory and immune responses.

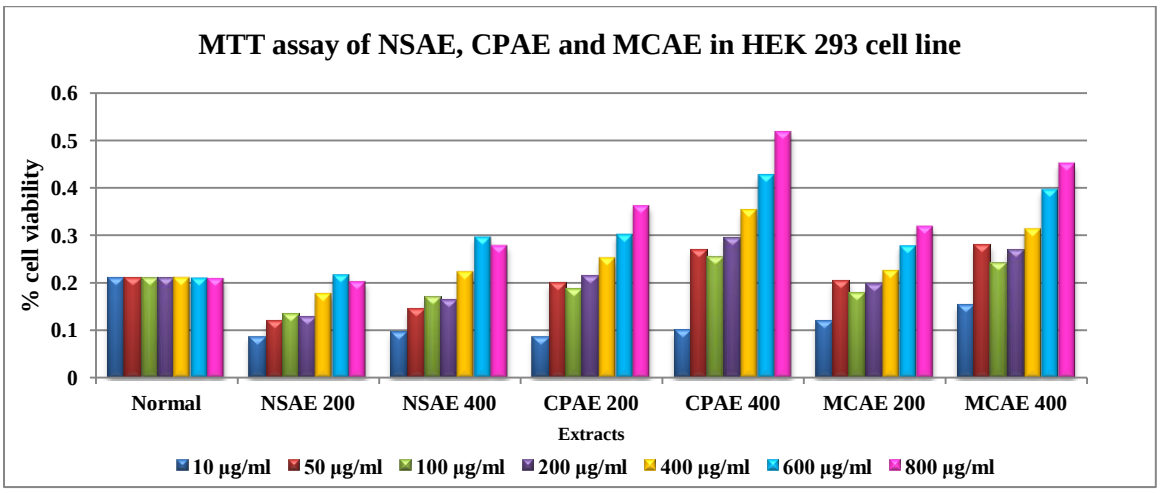
3.1.1 Results of MTT assay for cell viability in RAW-267.4, HEK-293 and HIG 84 cell lines cell lines

MTT assay was performed to check the percentage viability of cells in all three selected cell lines. Aqueous extracts of the test herbs (NSAE, CPAE and MCAE) were prepared according to dilution series and the assay was performed by standard method for assessment of percentage cell survival in cells upon adding the different concentrations of the herbs. The outcome of this estimation showed in following table and the graphical representation of these data is depicted in Fig.1

Table: 2 Compilation of cell viability assay (MTT assay) in respective cell lines

S. No.	Estimation	Observation	Remarks
1.	**MTT Assay**		
a)	**RAW-267.4**	Cell proliferation was seen in drug treated cell lines Cells were showed the inhibition with LPS in different concentrations	Cytoprotective effects of NSAE was seen ($EC_{50=}$ 3.77) CPAE cytoprotective effect was seen ($EC_{50=}$ 3.83) MCAE showed cell proliferation ($EC_{50=}$ 3.51)
b)	**HEK-293**	Cell proliferation was seen in drug treated cell lines	Cell proliferation was seen by all three herbs at different concentration; NSAE(600µg/ml) with $EC_{50=}$ 3.0023 CPAE (800µg/ml) with $EC_{50=}$ 2.586 MCAE (800µg/ml) with $EC_{50=}$ 2.929
c)	**HIG 84**	Cell proliferation was seen in drug treated cell lines	Cell proliferation was seen and no toxicity was observed NSAE with $EC_{50=}$ 3.042 CPAE with $EC_{50=}$ 3.46 MCAE with $EC_{50=}$ 3.52





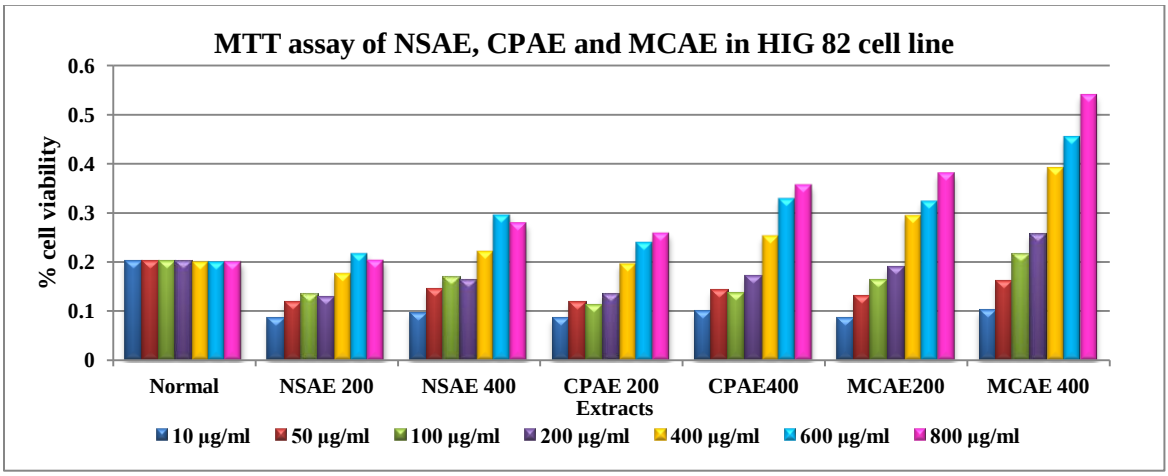


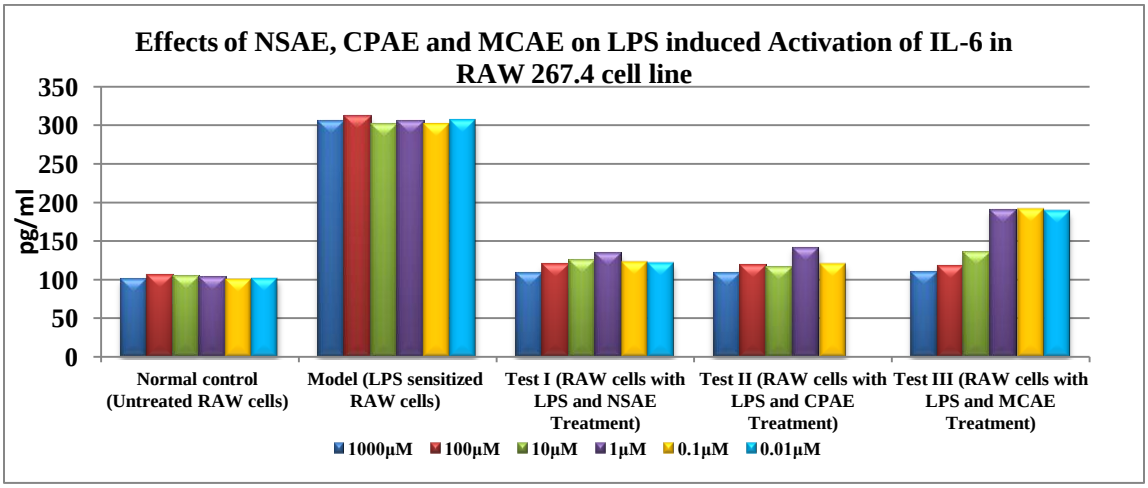
Fig. 1 Graphical representation of MTT assay

3.1.2 Results of Biomarker studies for activation of IL-6 and TNF-α in LPS sensitization in RAW 267.4 cell line

The challenges given in the form of LPS were evaluated via different biomarker expressions. Major biomarkers IL-6 and TNF-α as immune response were evaluated in RAW 267.4 cell lines. The graphical representation of these data are represented in fig. 2 and the summary of obtained results are compiled in following table-

Table: 3 Expression of immunological biomarkers in selected cell lines

S. No.	Estimation	Observation	Remarks
2.	**Biomarker studies**		
a)	IL-6	Activation of IL-6 indicates the stimulation of macrophages and it was observed in presence of LPS sensitization as well as in absence of LPS challenge which was hypothesized to activate cytokine release.	The group which was kept as a normal (no sensitization) was represents the lowest concentration of IL-6 as compared to model group which contained the cells challenged with LPS only in different concentrations Test groups have lower activation of IL-6 which is non significant with normal control group in some concentrations
b)	TNF-α	Next step in cytokine release is activation of TNF-α which indicates the initiation of immune pathway.	The model group which has LPS sensitization showed the higher expression of TNF-α as compared to normal and treatment groups. This suggests the activation of immune responses of LPS sensitization and immunomodulatory effect of select herbs.



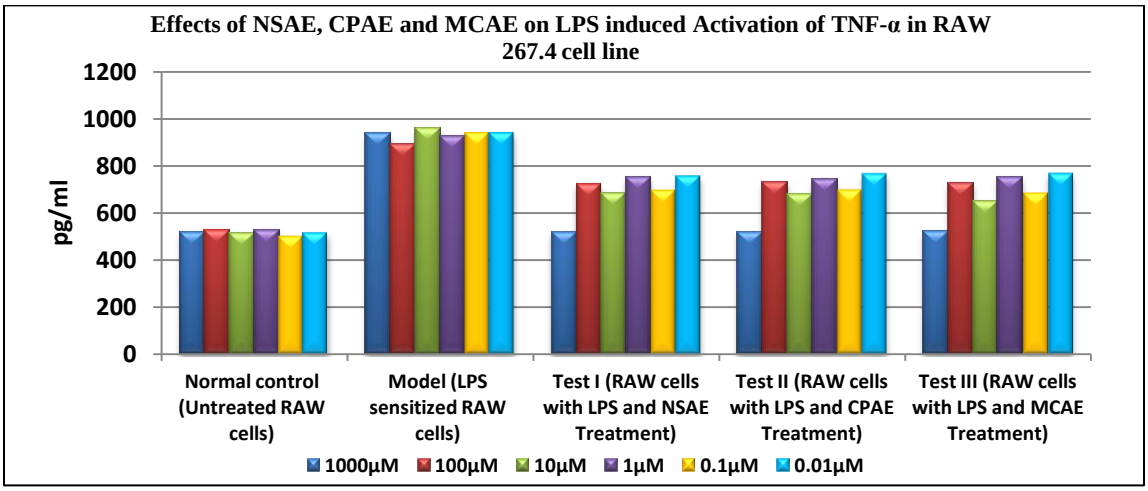
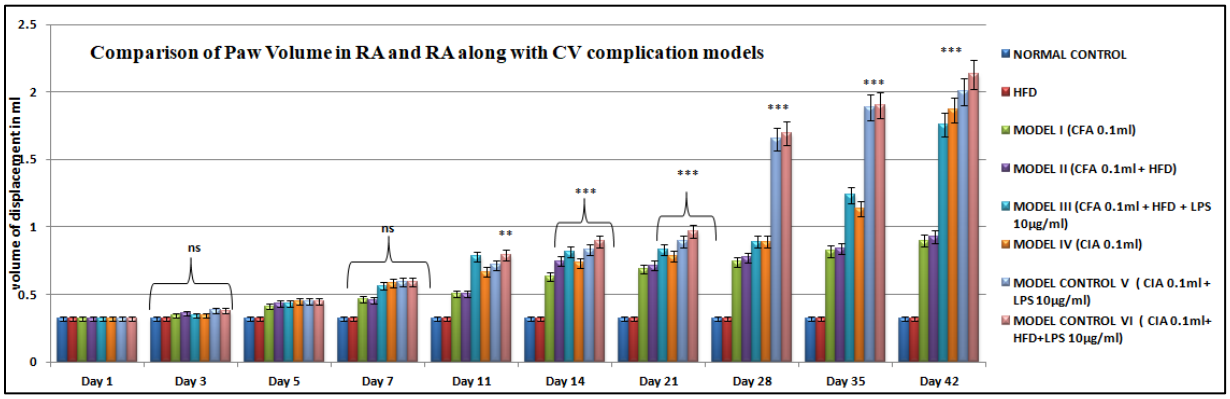


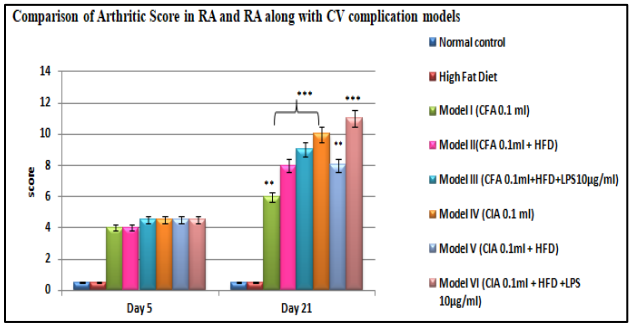
Fig. 2 Graphical representation of biomarker (IL-6 and TNF-α) activation

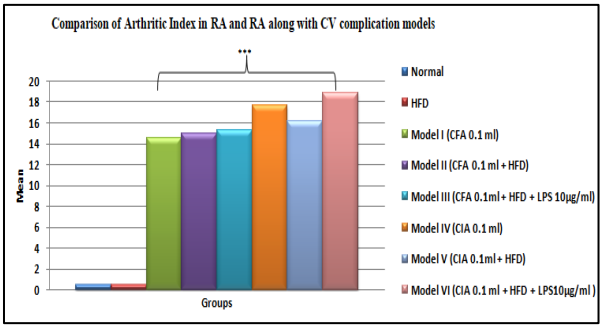
3.2 Results of *in-vivo* studies

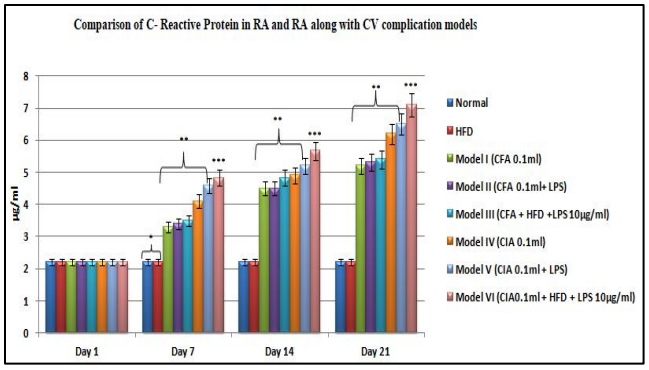
3.2.1 Results for comparison between developed models for RA and RA along with cardiovascular complications

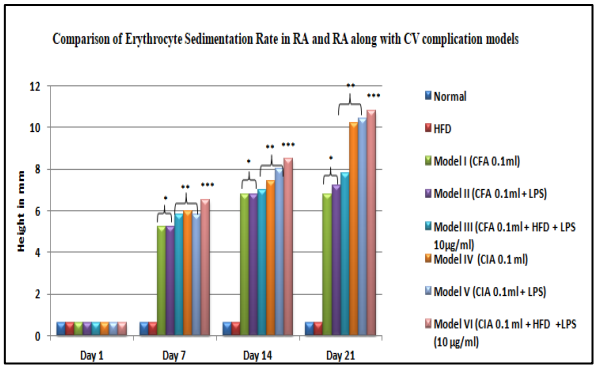
All developed models using primary inducing agents (CFA and CIA) and secondary inducing agents (HFD and LPS) were compared for both the situations; RA and cardiovascular complications developed in RA. Graphical representation of the statistical data is comprised in Fig. 3 and the summary of the results is given in Table: 4

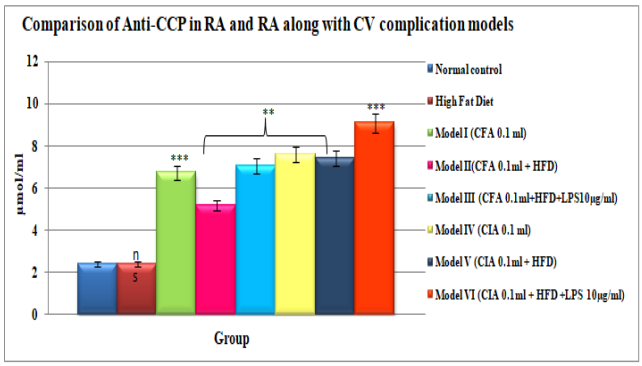












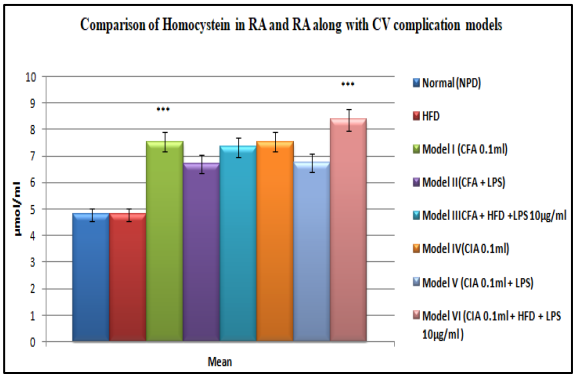


Fig. 4 Graphical representation of model development results

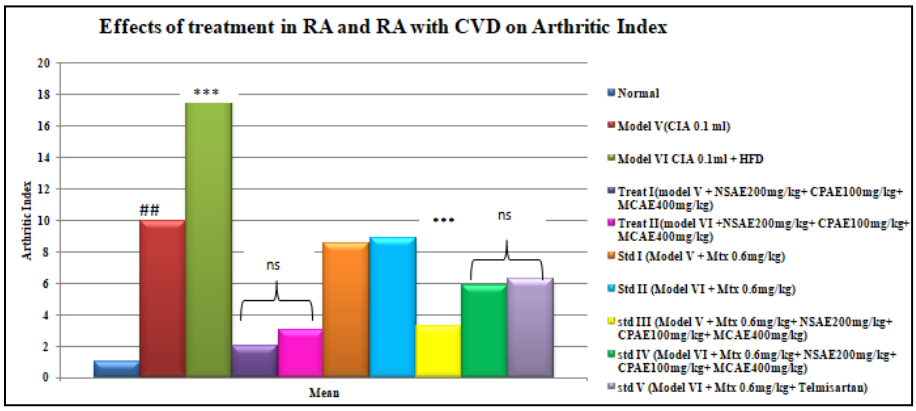
Values are expressed as Mean ± SEM. Values are statistically evaluated using repeated measure ANOVA (Paw volume) and one way ANOVA analysis followed by Boneferroni's Post hoc test for other tests the comparison between groups were done by Bonferroni's, Dunnett's and Tukey's post hoc test.
Significant values were compared with (*P<0.05, **p<0.01, *** P<0.001)
*,**, *** is used for comparison between normal and other groups
#, ##, ### is used for comparison between standard to other groups
@, @@, @@@ is used for comparison between Model and other groups
ns is non-significant differences between groups

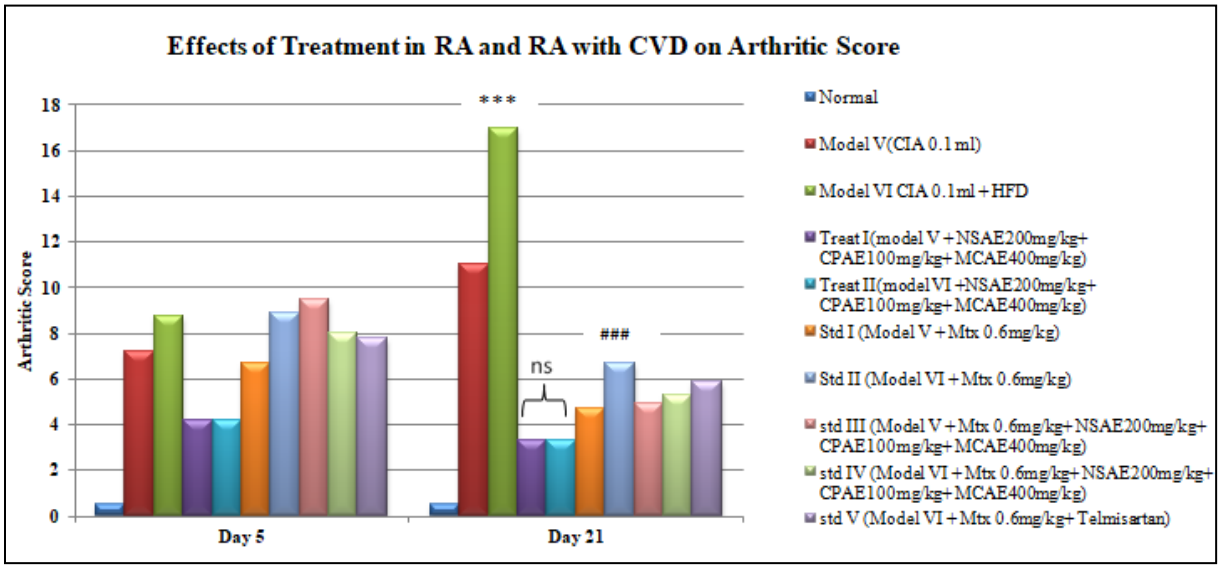
Table: 4 Summary of Results for developed models for RA and RA along with cardiovascular complications

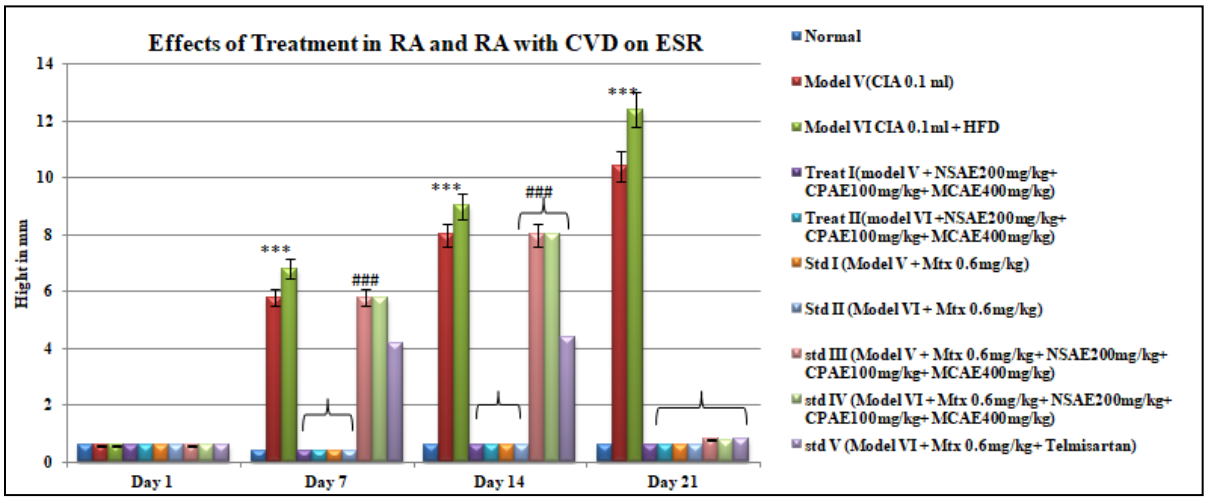
Groups	Physical					Inflammatory markers				Immunological markers				Confirmatory tests	
	Paw Volume	**Arthritic score**	**Arthritic Index**	**X-Ray**	**Walking difficulties**	**ESR**	**CRP**	**CBC**	**Rheumatoid Factor**	**IL-6**	**TNF-α**	**Hcy**	**TLR-4**	**ACCP**	**Histopathology**
Normal control	No change	No change	No change	No change	No change	No change	No change	No change	No change	No change	No change	No change	No change	No change	No change
Model I (CFA0.1ml)	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	No change	↑	↑
Model II (CFA0.1ml + LPS 10 µg/ml)	↑↑	↑	↑	↑	↑	↑	↑	↑	No change	↑	↑	No change	No change	↑	↑
Model III (CFA0.1ml + HFD + LPS 10µg/ml)	↑↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	No change	↑	↑
Model IV (CIA 0.1ml)	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑	↑↑	↑↑	↑	↑↑	↑↑	↑↑
Model V (CIA 0.1ml + LPS 10µg/ml)	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑
Model VI (CIA 0.1ml + HFD +LPS 10µg/ml)	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑↑↑

3.2.2 Results of Effect of treatment drugs on final developed and validated models of RA and RA along with cardiovascular complications

After getting final validated models on the basis of statistical and validation methods – Model II (CIA 0.1ml + LPS 10µg/ml) was proved as validated model for RA and Model VI (CIA 0.1ml + HFD+ LPS 10µg/ml) was proved as validated model for RA along with cardiovascular complications which were further taken for evaluation of aqueous extracts of select seeds (NSAE, CPAE and MCAE) in different doses against Methotrexate as a standard treatment for RA and Telmisartan as std treatment for RA along with cardiovascular complications. The following are the results for the effects of aqueous extracts, standard drug in models and test drugs combined with standard in selected models.







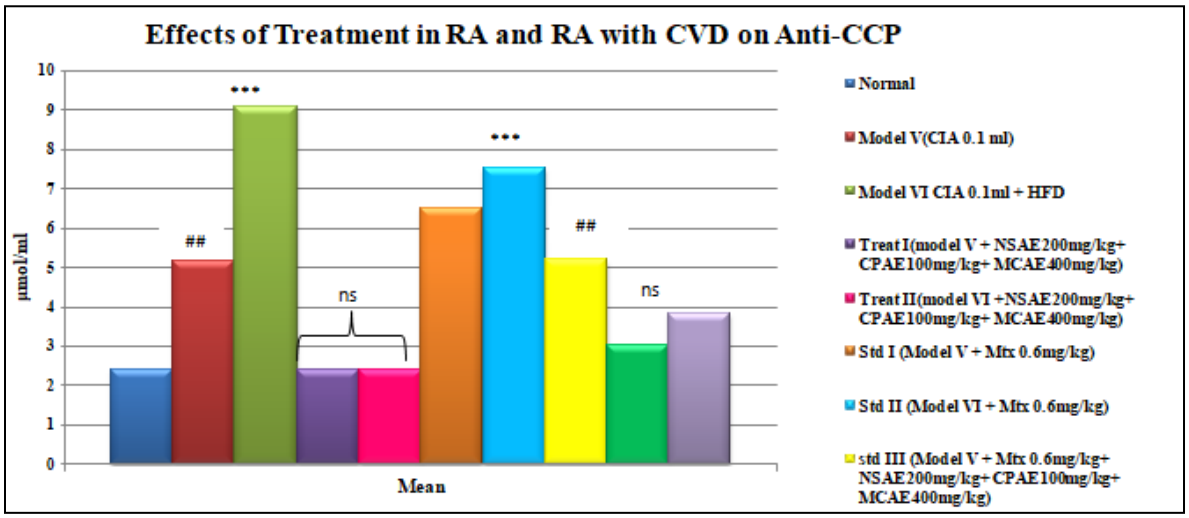


Fig. 5 Graphical representation of Treatment results

Values are expressed as Mean ± SEM. Values are statistically evaluated using repeated measure ANOVA (Paw volume) and one way ANOVA analysis followed by Boneferroni's Post hoc test for other tests the comparison between groups were done by Bonferroni's, Dunnett's and Tukey's post hoc test.
Significant values were compared with (*P<0.05, **p<0.01, *** P<0.001)
*,**, *** is used for comparison between normal and other groups
#, ##, ### is used for comparison between standard to other groups
@, @@, @@@ is used for comparison between Model and other groups
ns is non-significant differences between groups

Table: 5 summarized outcomes of the present study

Sr. No.	Parameter	Significance	Findings in our study
1.	Paw Volume	To check the inflammation of RA induced Paw of rat	Paw volume was found to be increased in Model control (CIA 0.1ml) induced animals and CIA + HFD + LPS 10µg/ml sensitized animals. There was a significant difference in paw volume from day 3 till day 28 and 42 as compared to normal control group. CIA induced animals showed sever changes on induced paw. Standard control I and II animals receiving MTX (0.6mg/kg) also showed increase in paw volume when compared with normal control but it was on the lower side when compared with model control. There was a significant decrease in Paw volume in NSAE 200mg/kg and CPAE 100mg/kg and MCAE 400mg/kg group as compare to model control
2.	Arthritic Index	To check disease progression through primary and secondary lesions	AI was found high in CIA sensitized groups as compared to CFA groups. Model control group CIA 0.1+LPS10µg/ml and CIA + HFD + LPS 10µg/ml was found to be increased as compare to normal control groups. Standard I and II showed increased arthritic index as compare to normal control group but lesser then model control groups. NSAE 200mg/kg and CPAE 100mg/kg and MCAE 400mg/kg treated groups. As well as in combination groups. Standard control groups showed increased arthritic index as compare to normal and treatment control group.
3.	Arthritic Scoring	To check walking disability of animals	Scoring in model control group (CIA) was found higher as compared to Model (CFA) and it was highest then all other groups in terms of secondary lesions and digestion of digits. Standard control group I and II were found to be increased on day 5 and 21 as compare to normal control and groups. But standard control group not shown any secondary lesions. Scoring in model control group (CIA 0.1+ LPS 10µg/ml) and CIA + HFD + LPS 10µg/ml and standard control group was found to be increased on day 5 and 21 as compare to normal control and200mg/kg and CPAE 100mg/kg and MCAE 400mg/kg groups.
4.	CRP	To check inflammatory responses	There was a significant increase in CRP levels in model and Standard control groups, where CIA induced groups showed higher inflammatory responses. as compare to normal and treatment groups from day 7 to day 21
5.	ESR	To check inflammatory Responses	ESR of model control I and II groups was found to be increased as compare to normal control and treatment groups. Model II was highest scored group for this parameter.

6.	Anti- CCP	To confirm RA in CFA induced arthritis	Model control and standard control groups shows increased levels of Anti-CCP which confirms that CFA induced model has developed Rheumatoid Arthritis in experimental animals. But the model II CIA induced group has highest elevated levels which also indicate the immunological intervention. ACCP levels were high in model control groups as compared to standard treated group. NSAE, CPAE and MCAE treated groups showed the decrease in Anti CCP which proves their immunomodulatory effects.
7.	Homocysteine	To check the intraarticular manifestation	There was no significant increase was observed in any other group except model control group (CIA 0.1+ LPS 10µg/ml) and CIA + HFD + LPS 10µg/ml which suggests that there was a extra articular manifestation in these models only and no extra articular manifestation of disease in other groups There was significant decrease observed in treatment groups.
8.	Neutrophil	To check immuno-modulatory effect	There was a significant increase in Neutrophil count in model and standard control group which shows immunological intervention in model development. The Neutrophil of normal control and treatment groups found to be normal which shows Immounomodulatory effects of200mg/kg and CPAE 100mg/kg and MCAE 400mg/kg .
9.	X-Ray	To confirm the evidences of RA	There was a significant change in edema and bone erosion was observed in X-Ray of model control animals as compare to normal control and treatment control groups. The X-Ray of standard group was also had some increased incidences of bone erosion.
10.	Histopathology	For confirmation	Synovial erosion and structural deformity of tissues were noticed in model control and standard control groups which was absent in normal and treatment control groups.

3.2.3 Results of receptor activation studies for NLRP-3 and TLR-4 activation

The receptor activation studies were preformed in homogenate of hind limb paw of rats. The summary of the results is summarized in following table and the graphical representation of results is given in the Fig.6

Table: 6 Receptor expression studies in selected cell lines

S. No.	Estimation	Observation	Remarks
2.	**Receptor expression studies**		
a)	**TLR 4 expression studies**	Homogenate of synovial cells obtained by disease induced paw showed the different concentration of TLR 4 expression in different groups when estimated through ELISA	TLR-4 expression was higher in model control group Test controls and standard control showed the lower expression of TLR 4 receptor as compare to model control.
b)	**NLRP 3 expression studies**	Homogenate of synovial cells obtained by disease induced paw showed the different concentration of NLRP 3 expression in different groups when estimated through ELISA	NLRP 3 activation in model control showed the hypothesis of activation of TLR 4 then NLRP 3 due to activation of Neutrophil due to LPS sensitization

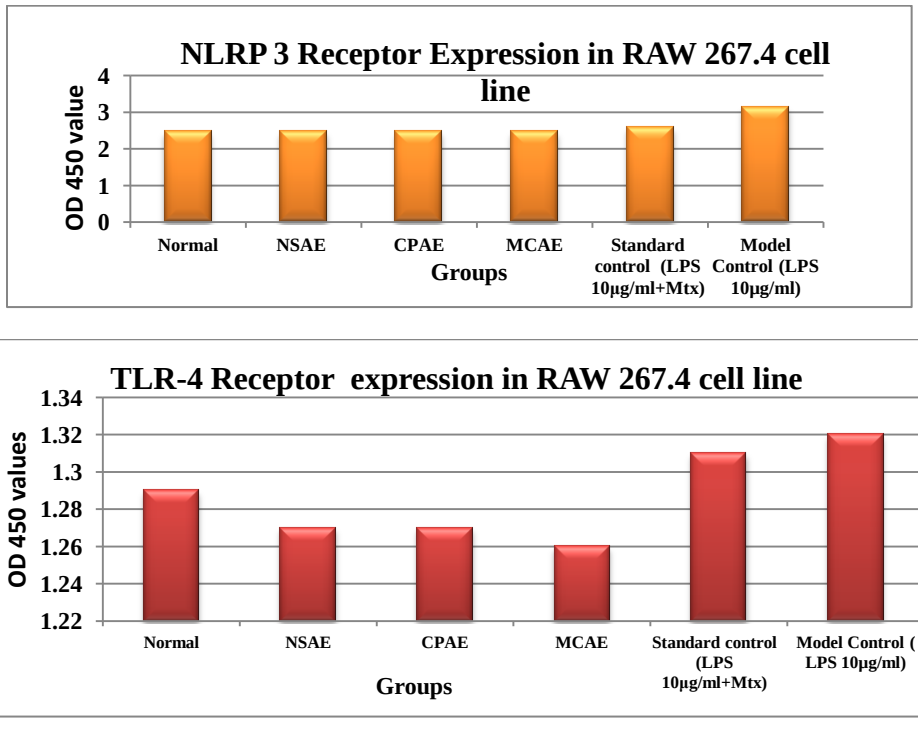


Fig. 6 Graphical representation of receptor expression studies in RAW 267.4 cell lin

3.2.4 Histopathological assessment

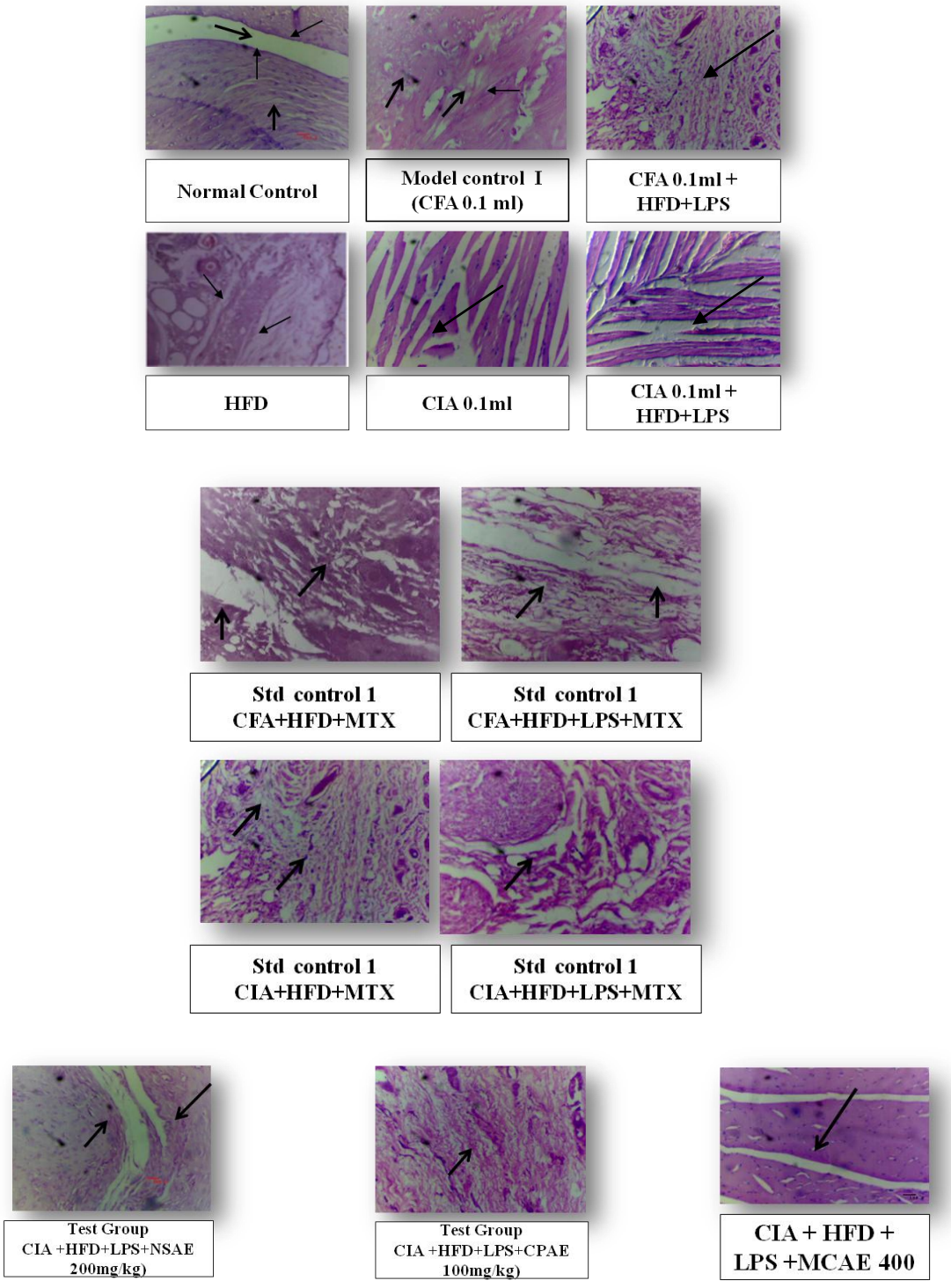


Fig.7 Histopathological comparison of treatment and model groups in respect to normal control where arrow indicates infiltration of cells and the deformities in comparison with normal control

4 Conclusions of the study

1. Model Development

1. Results of *In-vitro* studies prove that the LPS sensitization is responsible for macrophage activation which supports the hypothesis.

2. Results *of In-vivo* studies proved that CFA and CIA both are responsible for the RA induction but severity index is high in CIA group shows the immunological intervention along with inflammatory markers.

3. Sensitization of LPS in 10 µg/ml dose along with CFA and CIA caused RA +Athero respectively and the disease severity was noticed in CIA +HFD+LPS 10 µg/ml groups evident from face validity , constrict validity and predictive validity parameters

2. Treatment Study

1. Herbs selected for the treatment were found compatible to the cells in *in- vitro* study and proliferation in synovial cells supports the hypothesis to prevent synovial damage and increases the possibilities to treat RA.

2. *Nigella sativa* (200 mg/kg, p.o.), Carica papaya (100mg/kg) and *Momordica Charantia* (400mg/kg) dose were proved to prevented CFA and CIA induced Arthritis as evident from decrease in inflammation (Paw volume, Arthritic index, C-RP, ESR) and immunological response (Anti-CCP, IL-6 and TNF-α)

3. *Nigella sativa* (200 mg/kg, p.o.), Carica papaya (100mg/kg) and Momordica Charantia (400mg/kg) also prevented Atherosclerosis induced by dietary modification and LPS evident from comparison with MTX in decreasing inflammation and immunological responses and significant in amelioration of cardiovascular complications.

4. The combination of three aqueous seed extracts (NSAE 200mg/kg, CPAE 100 mg/kg and MCAE 400mg/kg) used in combination was proved to give synergistic Anti arthritic and Immounomodulatory actions coupled with prevention of cardiovascular complications associated with RA through inhibition of receptor activation.

Final Outcome of the proposed Research

The study suggests that CIA0.1ml+LPS10 µg/ml is a validated and optimized model replicating RA with maximum human resemblance and CIA0.1ml+LPS LPS10 µg/ml+HFD model proved as a novel validated and optimized model for replication of cardiovascular complications in RA.

The treatment part suggests that the combination of aqueous seed extracts (NSAE 200mg/kg, CPAE 100 mg/kg and MCAE 400mg/kg) has ameliorative potential to block the sequential pathways responsible for progression of RA as well as cardiovascular complications associated with RA due to their synergistic effect.

Publication Detail for present work

Converging a Preclinical validation approach for Comparing and evaluating diverse rat models for Rheumatoid Arthritis and associated long term complications

Trupti Dubey[a], Dr Kirti V. Patel[a*] (Communicated)

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