5 Results

The results for this study is divided in to five sections where-

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

Section II- Results of *in-vivo* studies adopted for model development

a) Results of study design I

b) Results of study design II

Section III- Results of model validation methods

Section IV- Results of pharmacological evaluation of NSAE, CPAE, and MCAE in optimized and validated model

Section V- Results of formulation development and standardization.

5.1 Results of section I

(Outcomes of pharmacognostic, physicochemical and phytochemical evaluation of selected crude drugs, powders and their extracts)

5.1.1 Results of Pharmacognostic studies (Macroscopic characterization):

The procured plant materials (seeds of *Nigella sativa, Carica papaya and Momordica charantia*) were studied before extraction by their appearance on the basis of organoleptic and morphological characteristics such as size, shape, taste, texture, as well as for color, odor The results of this characterization are as follows-

Table 5-1 Macroscopic characterization of selected herbs

Organoleptic Characteristics of Selected Plant material				
Sr. no	**Characters**	**Herb 1 (*Nigella sativa* seed)**	**Herb 2 (*Carica papaya* seed)**	**Herb 3 (*Momordica charantia* seed with and without seed coat)**
1	**Visual appearance**			
1	**Shape**	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

5.1.2 Results of Physico-chemical studies of plant material:

Physico-chemical studies of aqueous extract of all three selected herbs according to WHO standardization values. The results summarized in Table 5-2, where values of extracts were evaluated against reference standard from AMSAR Pvt. Ltd.

5.1.3 Results of Proximate analysis

Proximate analysis of aqueous extracts were done for basic standardization in which organoleptic characterization of extract was done and specific methods of detection of moisture content, LOD, ash value, crude protein were also performed. The results compiled in following table where standard methods according to guidelines were followed and standards of AMSAR Pvt. Ltd. were taken for the estimation.

Table 5-2 Results of physico-chemical analysis for NSAE, CPAE, MCAE

	Basic evaluations for Standardization						
	Tests	**Standard to which compared**			**Actual result**		
		NSAE	**CPAE**	**MCAE**	**NSAE**	**CPAE**	**MCAE**
I	Appearance	Brown Powder with characteristic odour	Brown Powder with characteristic odour	Brown powder with characteristic odour	Complies	Complies	Complies
II	Organoleptic test	Bitter taste	Slightly bitter	Bitter taste	Complies	Complies	Complies
III	Alcohol soluble extractives in 90% alcohol by API method taking 1gm sample	- -	NLT 30% (sample in 50% alcohol)	NLT 40% (Sample in 90% alcohol)	88.24%	30.62%	42.36%
	Water Soluble extractives by API method taking 1 gm sample	NLT 70%	- -	- -		- -	- -
IV	Bitter	NLT 1.5%	NA	NLT 3.5%	1.78%	NA	3.52%
V	Loss on drying	NA	NMT 20%	NLT 8%	NA	17.56%	4.24%

Sr. no	**Proximate Analysis Parameter**	***Nigella sativa* Linn**		***Carica papaya* Linn**		***Momordica charantia* Linn**	
		Experimental value	**Reference value from AMSAR Pvt. Ltd.**	**Experimental value**	**Reference value AMSAR Pvt. Ltd.**	**Experimental value**	**Reference value AMSAR Pvt. Ltd.**
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	9± 0.05	9.73 ± 2.34
3.	Acid insoluble ash	0.17	0.15 % w/w	022%	0.44%	7.2± 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

5.1.4 Results of preliminary phytochemical studies

Preliminary tests were performed for the qualitative measures of the phytochemicals of interest such as Alkaloids, Saponin, Carbohydrate, Terpenoids, Phenolic acids, Glycosides, reducing sugar, proteins, fats and oils and phytosterols. Estimation was done in the supernatant collected from the aqueous extracts of *Nigella sativa, Carica papaya* and *Momordica charantia* seed. The results of this primary data Indicated in table as + for Present and – for Absence as mentioned in table 5-3

Table 5-3 Results of preliminary phytochemical estimation in extracts

Phytochemical estimation for metabolites				
Sr. no	**Active Metabolite**	***Nigella sativa* Linn**	***Carica papaya* Linn**	***Momordica charantia* Linn**
1	Alkaloids	(P) ++	(P) +	(N) --
2	Bitters	(N) -	(N) -	(N) -
3	Tannins	(P) +	(P) ++	(N) --
4	Saponins	(N) -	(N) -	(P) +
5	Carbohydrates	(P) +	(P) +	(P) +
6	Flavonoids	(N) -	(P) +	(N) --
7	Terpenoids	(P) +	(P) +	(P) +
8	Phenolic acids	(P) +	(P) +	(P) +
9	Anthocyanins	(N) -	(P) +	(N) -
10	Glycosides	(P) +	(P) +	(P) ++
11	Reducing sugars	(P) +	(P) +	(P) +
12	Protein	(N) -	(N) -	(P) +
13	Fat and oil	(P) +	(P) +	(P) +
14	Steroids	(P) +	(P) +	(N) --
15	Phytosterols	(P) +	(P) +	(P) +
** Presence of metabolite is indicated by (P) + and Absence is indicated by (N) – sign here				

5.1.5 Results of heavy metal analysis

Heavy metal analysis is also one of the important standardization criteria for herbal preparations as per the WHO guidelines where heavy metals in the herbal components and polyherbal formulations should be in given in limit. Here the estimation of heavy metals such as arsenic, lead, cadmium and mercury were estimated which were in the limit specified in the monograph as summarized in table 5-4

Table 5-4 results of Heavy metal analysis in NSAE, CPAE and MCAE

Heavy metals							
Sr. no.	**Tests**	**Standard to which compared (As per herbal monograph)**			**Actual result**		
		NSAE	**CPAE**	**MCAE**	**NSAE**	**CPAE**	**MCAE**
1	Arsenic (As)	NMT 03PPM	Complies	NMT 03PPM	Complies	NMT 03PPM	Complies
2	Lead (Pb)	NMT 10PPM	Complies	NMT 10PPM	Complies	NMT 10PPM	Complies
3	Cadmium (Cd)	NMT 0.3PPM	Complies	NMT 0.3PPM	Complies	NMT 0.3PPM	Complies
4	Mercury (Hg)	NMT 1PPM	Complies	NMT 1PPM	Complies	NMT 1PPM	Complies

5.1.6 Results of microbial Profile

Microbial profile evaluation is one of the important criteria for estimation before performing any preclinical as well as clinical studies utilizing herbs. In the herbal standardization WHO guidelines have given certain limits for microbial load as well as specific bacterial, fungal and other pathogen which should not be present in the selected medicinal herbs. The estimations were performed in aqueous extracts of *Nigella sativa, carica papaya* and *momordica charantia* seed against standards provided at AMSAR Pvt. Ltd.

Table 5-5 Microbial profile of NSAE, CPAE, MCAE

Microbial Profile							
Sr. no	**Tests**	**Standard to which compared**			**Actual result**		
		NSAE	**CPAE**	**MCAE**	**NSAE**	**CPAE**	**MCAE**
1.	Total plate count	NMT 1000 CFU/ G	410 CFU/ G	NMT 1000 CFU/ G	340 CFU/ G	NMT 1000 CFU/ G	360 CFU/ G
2.	Yeast and Moulds	NMT 100 CFU/ G	Absent	NMT 100 CFU/ G	Absent	NMT 100 CFU/ G	Absent
3.	E. Coli	Absent	Absent	Absent	Absent	Absent	Absent
4.	Salmonella	Absent	Absent	Absent	Absent	Absent	Absent

***CFU/ G- Colony-forming unit per gram, NMT- Not More Than**

5.1.7 Results of qualitative determination of markers of interest using Thin Layer Chromatography

Several TLC runs were performed in different series of solvent prepared as mobile phases (listed in methodology section) and were optimized with different runs using silica gel plates. Thymoquinone, gallic acid and chlorogenic acid were taken as standards against *Nigella sativa, Carica papaya* and *Momordica charantia* respectively. TLC run for NSAE showed the spot (indication of thymoquinone/ standard presence) in mobile phase where, mixture of Benzene: Chloroform (50: 50) was taken. Chlorogenic acid was taken as standard against *Momordica charantia* sample and the spot was detected in ethylene acetate: water: formic acid (7.7:1.3:0.9) as mobile phase. Gallic acid was detected in carica sample when TLC was run using mobile phase using toluene: ethyl acetate: formic acid: methanol (3.3: 0.8: 0.2). The R_f value was calculated using previously discussed formula. The plats were read under UV chamber to mark the spot clearly and digestion with concentrated sulfuric acid was also done for confirmation of chlorogenic acid. The results summarized in table 5-6 and fig. 5.1

Table 5-6 Results of R_f values for test components

Test compound	**Mobile Phase**	**R_f value (distance travelled by solute/distance travelled by solvent)**
Herb 1 (*Nigella sativa*)	Benzene: Chloroform (50: 50)	0.33 (2/6)
Herb 2 (*Carica papaya*)	Ethylene acetate: Water: Formic acid (7.7:1.3:0.9)	0.5 (3/6)
Herb 3 (*Momordica charantia*)	Toluene: Ethyl acetate: Formic acid: Methanol (3.3: 0.8: 0.2).	0.5 (3.1/5.9)

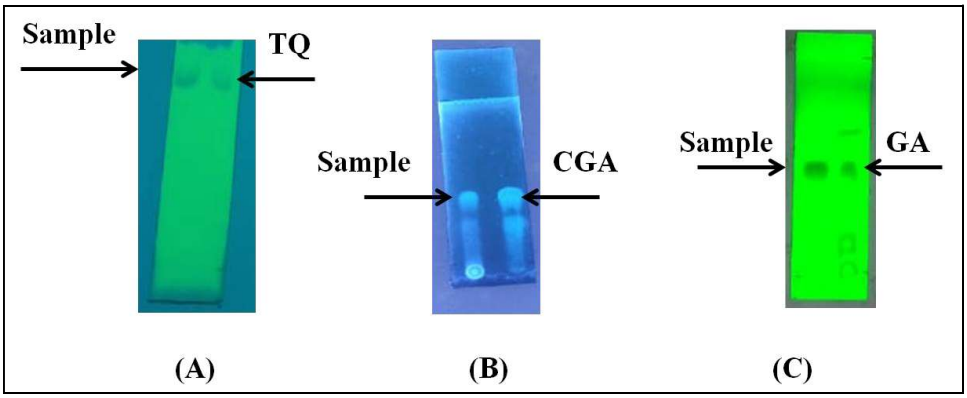


Fig. 5-1 TLC of A (TQ and *Nigella sativa*), B (CGA and *Momordica charantia*), C (GA and *Carica papaya*)

5.1.8 Results of quantitative determination of markers of interest using UV spectroscopy

5.1.8.1 Results of determination of Thymoquinone

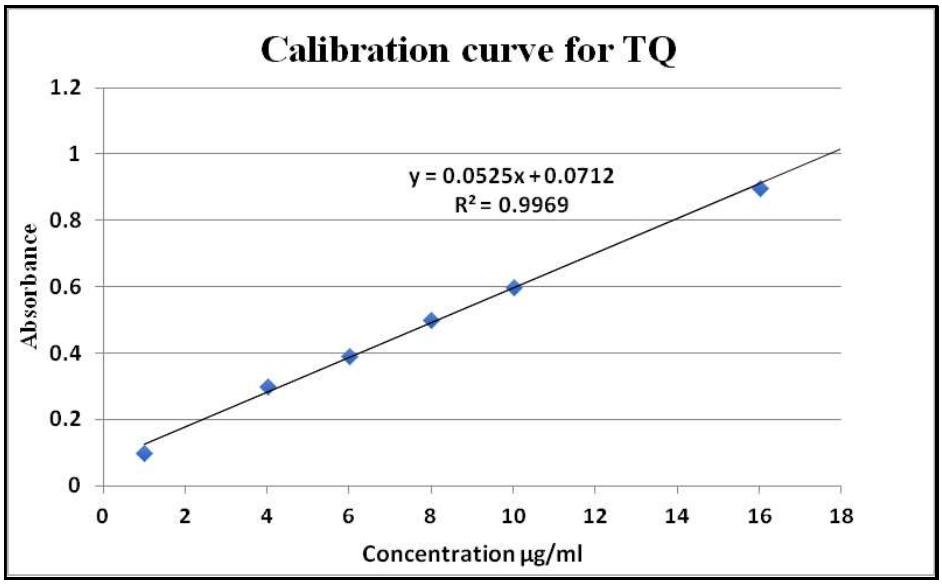
Thymoquinone (TQ) is one of the potential phytoconstituents present in *Nigella sativa* seeds. UV visible spectrometry showed the presence of TQ in extract sample against the standard curve at 254nm wavelength. Fig. 5.2 depicts the standard calibration curve for TQ in different dilutions series from 1, 4, 6, 8, 10 and 16µg/ml. The absorbance for TQ in test compound (extract) at 254nm was found as 0.899. The results of determination, absorbance at different concentration, regression analysis, LOD and LOQ are summarized in table 5-7

Table 5-7 Data for concentration vs. absorbance of Thymoquinone

Sr. no	Concentration	λ max (nm)	Absorbance
1	1µg/ml	251	0.1
2	4µg/ml	251	0.3
3	6µg/ml	252	0.39
4	10µg/ml	252	0.5
5	14µg/ml	254	0.6
6	16µg/ml	254	0.9

Table 5-8 Data of calibration curve of Thymoquinone

Regression equation	R^2 value	LOD (µg/ml)	LOQ (µg/ml)
y = 0.0525x + 0.0712	0.9969	1.78	7.2



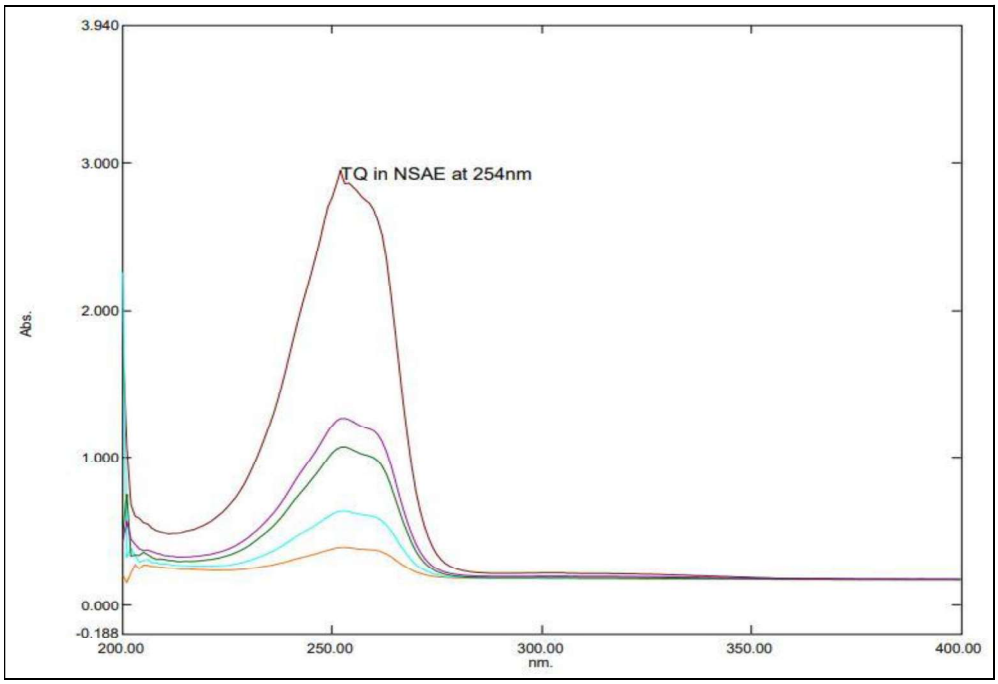


Fig. 5-2 Calibration curve and λmax determination of Thymoquinone

5.1.8.2 Results of determination of Chlorogenic acid

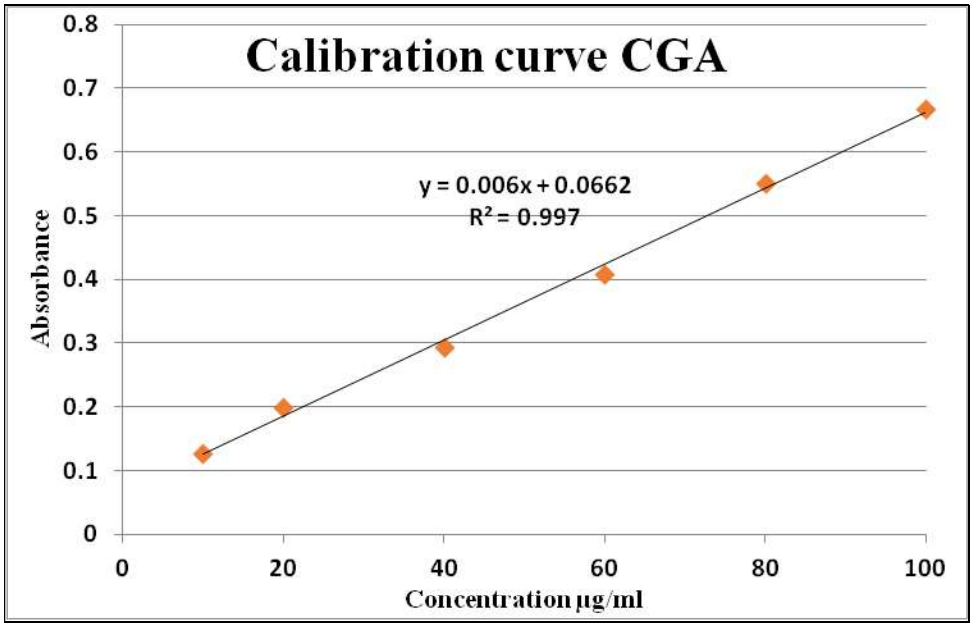
Chlorogenic acid (CGA) is phenolic component and it is principally present in bitters like melon and papaya seed. CGA was detected in all the three sample extracts; *Nigella sativa, Carica papaya* and *Momordica charantia* at 297nm wavelength. Fig. 5.2 depicts the standard calibration curve for CGA at 10, 20, 40, 60, 80 and 100µg/ml. Absorbance of all three components was tested against the standard wavelength and the absorbance for *Nigella sativa* was found as 0.668 at 290nm. Absorbance for *Carica papaya* was found as 0.552 at 290nm in and for *Momordica charantia* the absorbance 0.515 detected at 290nm.

Table 5-9 Data for concentration vs. absorbance of CGA

Sr. no	Concentration	λ max (nm)	Absorbance
1	10µg/ml	268	0.127
2	20µg/ml	268	0.199
3	40µg/ml	270	0.293
4	60µg/ml	270	0.409
5	80µg/ml	290	0.552
6	100µg/ml	290	0.668
7	500 µg/ml	290	0.515

Table 5-10 Data of calibration curve of CGA

Regression equation	R^2 value	LOD (µg/ml)	LOQ (µg/ml)
y = 0.006x + 0.0662	0.997	15.6	63.9



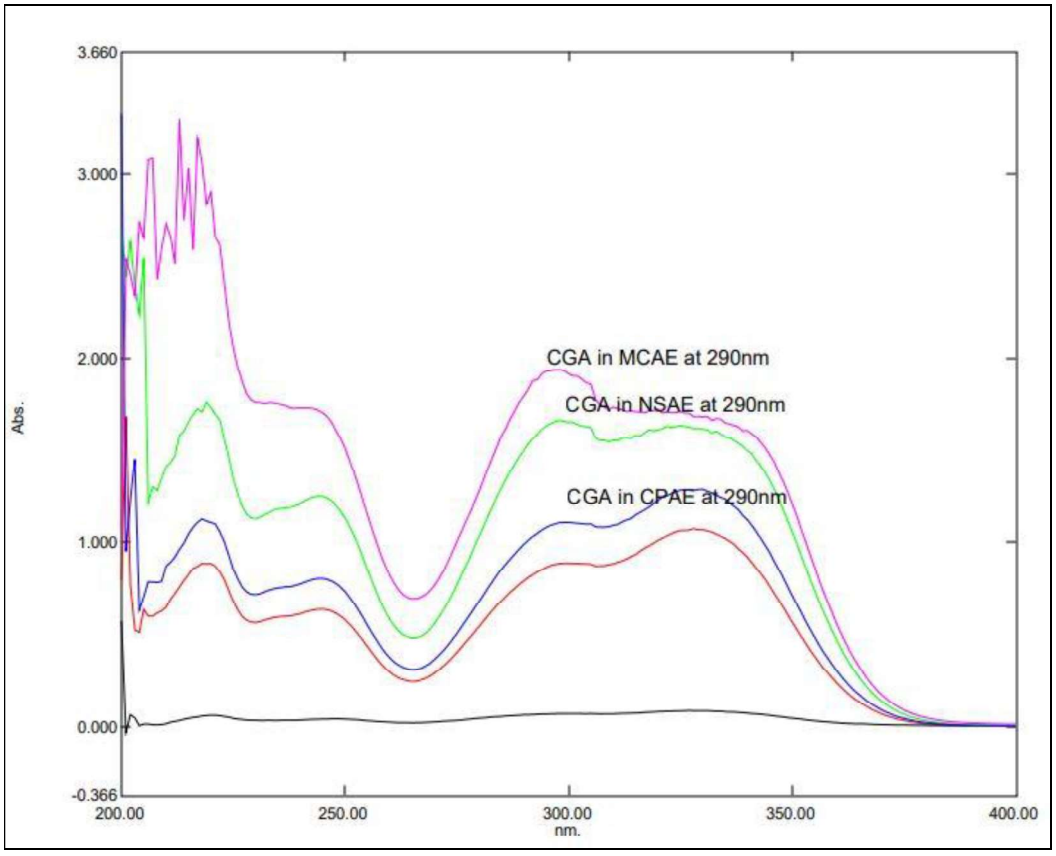


Fig. 5-3 Calibration curve and λmax determination of CGA

5.1.8.3 Results of determination of Gallic acid

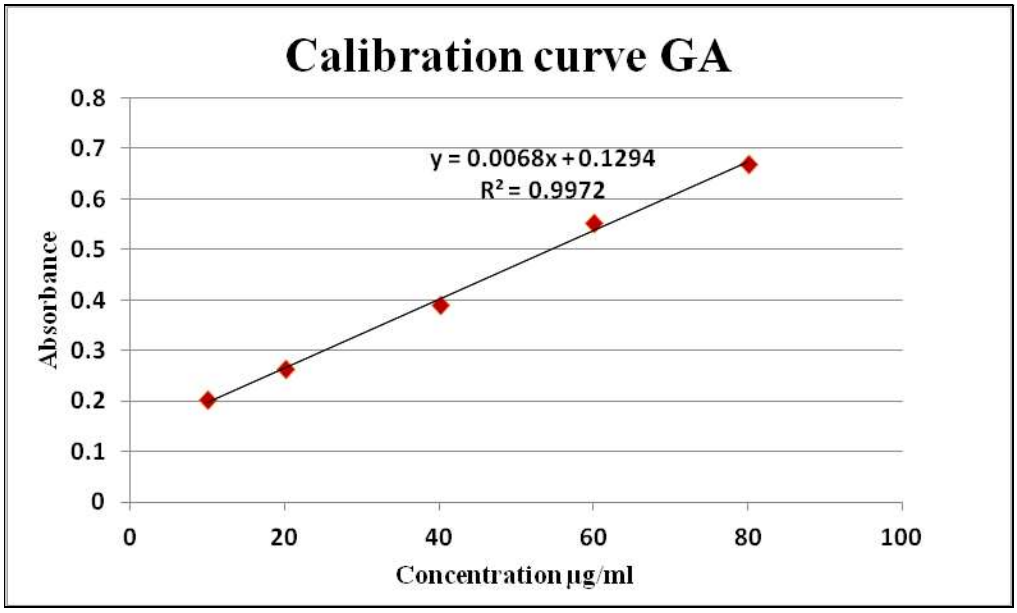
Gallic Acid (GA) is most widely found phenolic acid derivative in fruits and vegetables. The phytochemical is accounted to give immunomodulatory, anti inflammatory and cardioprotective effects. Here Gallic acid was also found in all three herbs; *Nigella sativa, Carica papaya* and *Momordica charantia* seeds at wavelength of 270 nm. The standard calibration curve was taken at different concentrations of 10, 20, 40, 60, 80 and 100 µg/ml. The absorbance of GA in *Nigella sativa* was found as 0.812 at 270nm and for *Carica papaya* the absorbance was found as 0.799 at 270nm and for *Momordica charantia* absorbance was found as 0.263 at 270nm.

Table 5-11 Data for concentration vs. absorbance of GA

Sr. no	Concentration	λ max (nm)	Absorbance
1	10µg/ml	268	0.202
2	20µg/ml	268	0.263
3	40µg/ml	270	0.389
4	60µg/ml	270	0.552
5	80µg/ml	270	0.668
6	100µg/ml	270	0.799

Table 5-12 Data of calibration curve of Thymoquinone

Regression equation	R^2 value	LOD (µg/ml)	LOQ (µg/ml)
0.0068x + 0.1294	0.9972	13.9	50.31



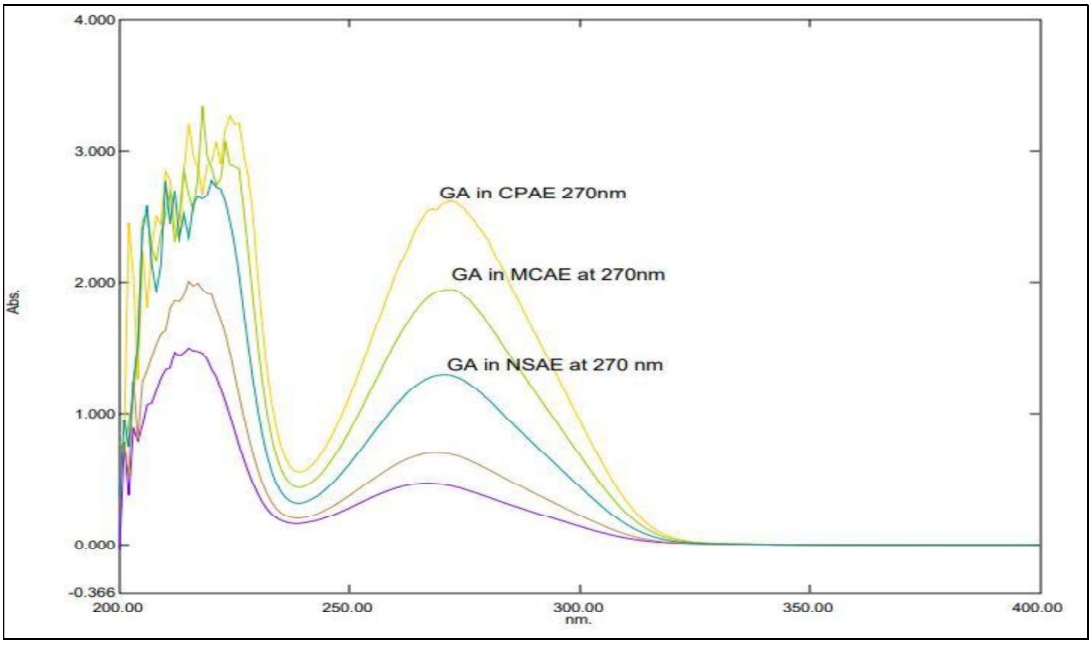


Fig. 5-4 Calibration curve and λmax determination of GA

5.2 Results of Section II (*in- vivo* model development):

5.2.1 Results of Study design-I (Development of animal models for replication of Rheumatoid arthritis)

5.2.1.1 Results of RA model developed using CFA and LPS

In this section, results of RA induction using CFA and CFA with LPS compiled where, LPS was utilized as secondary inducing agent. LPS is an endotoxine, so before incorporating it in animals already sensitized with CFA, it was individually evaluated in different set of animals, where LPS (0.1µg/ml, 0.5µg/ml and 10µg/ml) in absence of any inducing agent (CFA or collagen) was injected to each animal by s. c. route (0.1 ml). These groups were estimated for different parameters utilized in this study for estimation of RA but animals were unable to generate any effects correlating RA.

The results of only LPS sensitization were not significant in relation with RA generation so they are not discussed in detail here. LPS should generate inflammatory and immune responses as per properties of an endotoxine but, in this study LPS was observed to work together the primary inducers (CFA, collagen) and with HFD in later part. The possible reason for this can be very low dose of LPS to prevent sepsis like conditions or slow release of drug in this study. When LPS in same doses was used as a secondary inducer with CFA/Collagen it showed the elevation in the disease progression and symptomatology which supports the hypothesis.

In this part of model development for CFA induced RA; the comparison was done between-

Group I (normal control), group IIa (CFA 0.1ml), group IIIa (CFA 0.1ml + LPS 0.1µg/ml), group IVa (CFA 0.1ml + LPS 0.5µg/ml) and group Va (CFA 0.1ml + LPS 10µg/ml).

The study protocol for this model was 28 days and all data were compared using one way ANOVA and repeated measure ANOVA for three major disease indicators; Physical indicators, biochemical indicators, confirmatory indicators.

a) Results of physical indicators in CFA and CFA with LPS induced RA

Results of physical indicators are valuable in disabling diseases like RA because when biological insult occurs, with the severity of disease, physical symptoms on target organ are visible and

they are important for staging of disease. Here in current investigation, **paw volume, arthritic score, arthritic index, photographic assessment** were examine for this purpose.

Paw volume *is one of the important physical estimations in RA which indicates edema and inflammation in disease generation as a first sign for initiation of alteration in biological system. The results of paw volume from model groups were compared with normal control group to evaluate the increase in inflammation and edema. Group IIa, IIIa, IVa and Va served as model control for this particular study design.*

There was a maximum and constant rise in high grade inflammation from day 1 to 28 in ***group IIa, CFA 0.1ml (0.82±0.14)*** *and* ***group Va, CFA 0.1ml + LPS 10µg/ml (1.70±0.05)*** *which was significantly different with* ***group I normal control (0.24±0.01)*** *as well as with other developed models;* ***group IIIa, CFA 0.1ml + LPS 0.1µg/ml (0.33±0.04)*** *and group* ***IVa, CFA 0.1ml + LPS 0.5µg/ml (0.31±0.07)*** *which are having low grade inflammation and it was not persistent throughout the study(fig.5.5 A)*

Arthritic Score *is also one of the important physical measures in RA, as it gives an insight about inflammation and disability generated during progression of disease. The score were compared among the models for day 5 and for day 21. Initially on day 5 all models showed the signs of disease generation in terms of secretions, inflammation and patches on different parts of animals. The data were compared with* ***group I, normal control group (0.0±0.0),*** *which did not demonstrate any change in arthritis on day 5 and 21.*

Arthritic score of all other models developed was increased initially but ***group IIIa, CFA 0.1ml+LPS 0.1µg/ml (5.8±0.44), group IVa, CFA 0.1ml + LPS 0.5µg/ml (4.8±0.28)groups*** *did not showed any further increase in scoring after 7 day of study. Whereas group* ***Va CFA 0.1ml + LPS 10µg/ml*** *showed increase in the arthritic scoring on* ***day 5 (5.8±0.44)*** *and on* ***day 21 (9±0.64)*** *which was significantly higher among all groups as well as compared to scoring of* ***group IIa CFA 0.1ml*** *on* ***day 5 (5.5±0.36)*** *and on* ***day 21(7.1±1.08)*** *as depicted in fig.5.5B.*

Arthritic index *is one of the evaluation parameters, which can give accounts of disease progression in terms of primary and secondary lesions as well as increase in arthritic score which shows the visual as well as systemic changes in model animals as severity of disease increases and immune responses generates in diseased animals.*

Again, comparison of arthritic index was done with ***group I, normal control group (0.0±0.0),*** *which did not showed any changes in arthritic index as these animals were not given any*

inducing agent. Arthritic index, significantly increased in ***group Va CFA 0.1ml + LPS 10µg/ml (14.5±1.88)*** *as compared to other groups sensitized with CFA and LPS and* ***group IIa CFA 0.1ml (15±1.25)****which was sensitized with CFA only (fig 5.5C).*

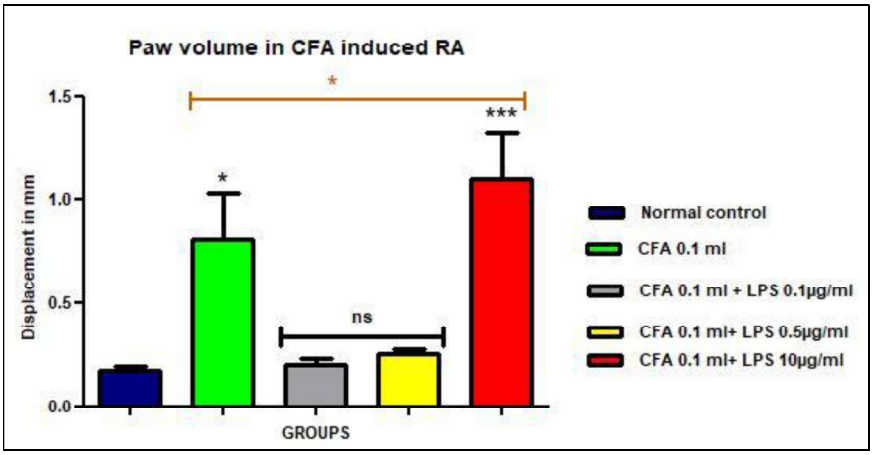
Photographic assessment *was done to check gradual physical changes at the site of disease in animals. The photographs of all the groups were taken on alternate days and weekly to check the progression of disease. The comparison of model control groups was done with normal control to evaluate the change in physical appearance and disease progression in the form of inflammation, primary, secondary lesions as well as major deformities as mentioned in fig. 5.6.*

Notation:

In succeeding figures the group labeling will be as follows-

Group I - Normal Control
Group IIa - CFA 0.1ml
Group IIIa - CFA 0.1ml + LPS 0.1µg/ml
Group IVa - CFA 0.1ml + LPS 0.5µg/ml
Group Va - CFA 0.1ml + LPS 10µg/ml

(A)



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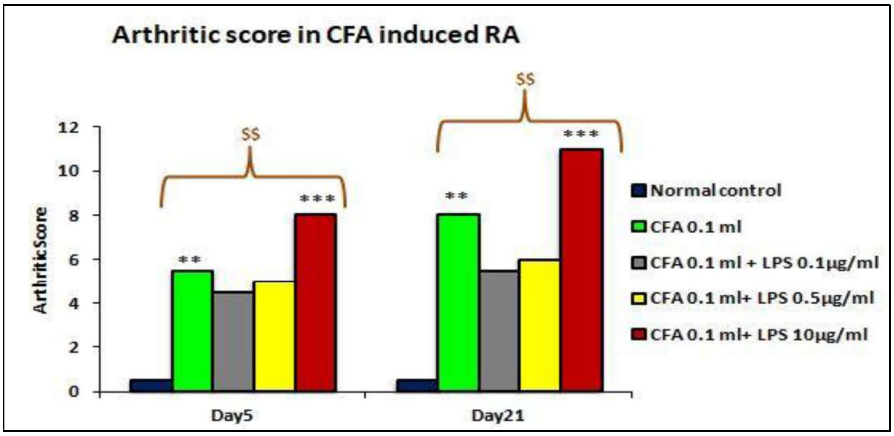


(C)

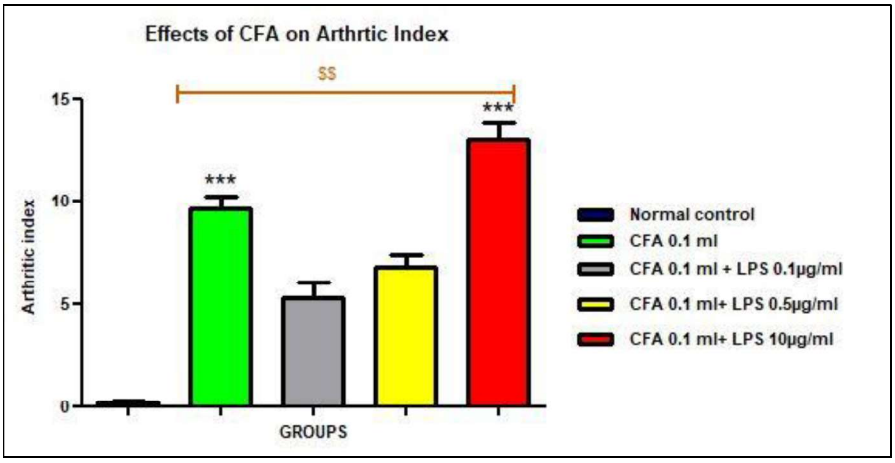


Fig. 5-5 Graphical representation of Physical parameters in CFA and CFA + LPS induced RA

(A) Effects of CFA and LPS on Paw volume (B) Effects of CFA and LPS on Arthritic score

(C) Effects of CFA and LPS on Arthritic index

Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis and repeated measure ANOVA for paw volume. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. $, $$, $$$ showed comparison between CFA 0.1ml vs. CFA 0.1ml+ LPS10µg/ml. ns showed non-significant differences between normal and other groups.

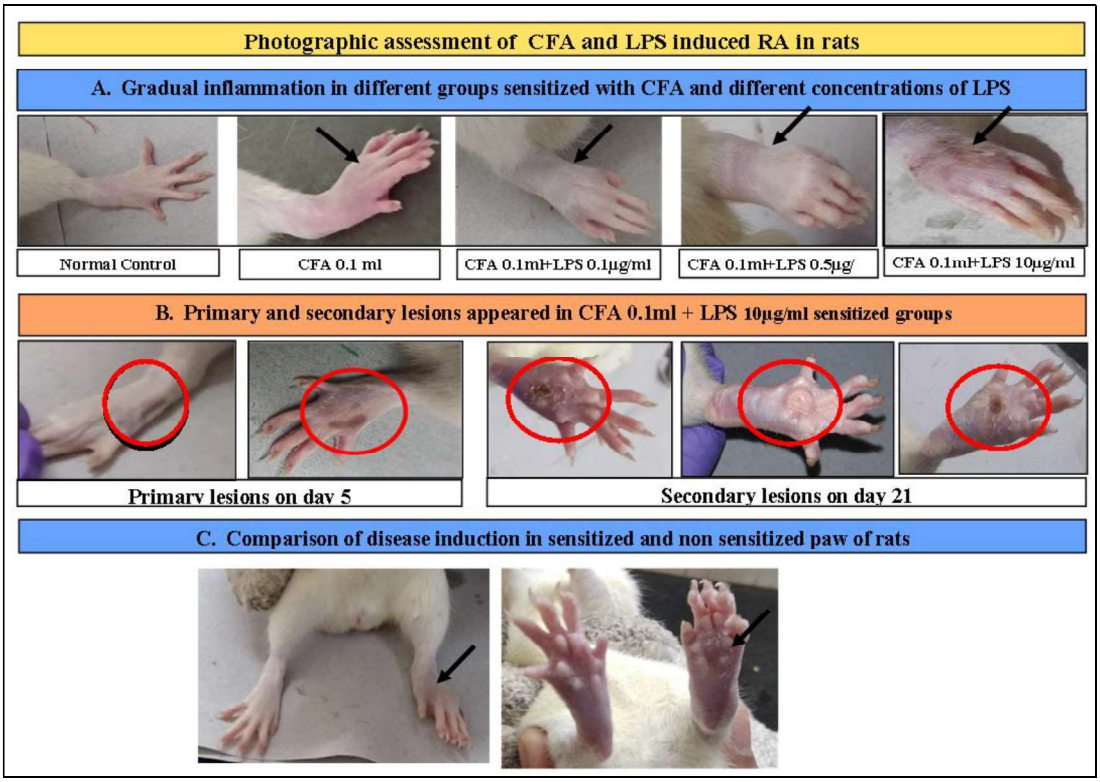


Fig. 5-6 Photographic assessment of CFA and LPS induced RA in rats

Figure 5-6A- Shows gradual inflammation in different groups sensitized with CFA and CFA with LPS induced groups where arrow shows the inflammatory responses.

Figure 5-6B- Shows the primary and secondary lesions where on day 5 mild lesions were observed on day 21 boil and wounds showed in sensitized paw only.

Figure 5-6C- Shows the comparison of sensitized and non sensitized paw where only sensitized paw showed inflammation and there were no symmetrical progression of RA.

Interpretations of photographic assessments

In this figure we can clearly assess the primary and secondary lesions in sensitized rat's paws.

(A) Primary lesions in CFA induced RA in rats

When model control groups compared with normal control group on day 5 the primary lesions were observed in the form of scars, bone deformity and swelling in left hind paw of rats.

(B) Secondary lesions in CFA induced RA in rats

When normal control group compared with CFA and different doses of LPS induction on day 21 they clearly showed the increased disease severity as secondary lesions but it was not that much severe as compared to collagen induced model.

*These models were further compared in validation section for proper comparison.

b) Results of biochemical indicators in CFA and CFA with LPS induced RA

Biochemical parameters (ESR, CRP, Anti-CCP, RF, and homocystein, TNF-α, IL-6, NF-κB and TLR-4) were estimated as markers generating in response to disease stimulation, progression and disease severity. Here results of markers combined as-

i) Inflammatory markers- ESR, C-RP, homocysteine

ii) Immunological markers- Neutrophil count, IL-6, TNF-α, NF-κB

iii) Disease specific markers- RF, Anti-CCP, TLR-4

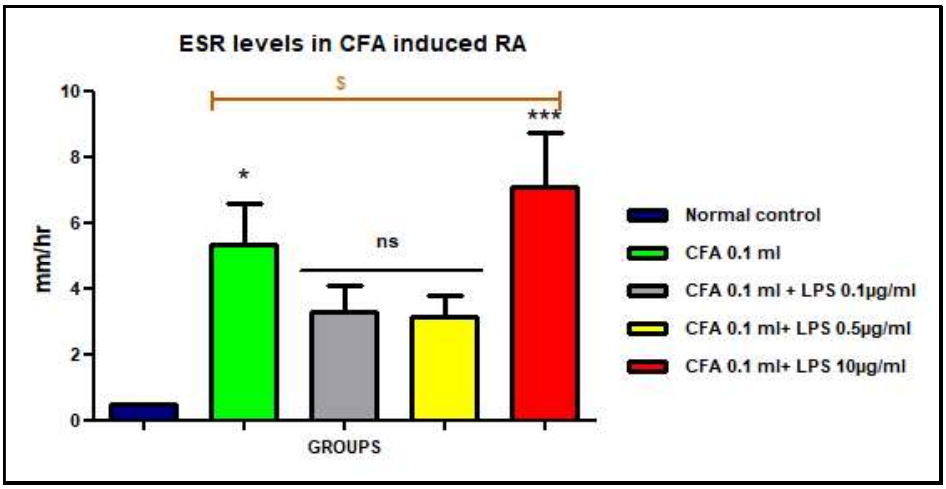
i) Inflammatory markers- ESR, C-RP, homocysteine

ESR *is one of the important parameters to analyze the rate of infection and inflammation via sedimentation of erythrocytes. In this study ESR was performed on day 1, 7, 14, 21 and 28. Model control groups were compared with ESR of* ***group I normal control group (0.66±0.22)*** *was compared with all the model control groups where,* ***group IIa, CFA 0.1ml (9±0.25)*** *showed the significant increase in ESR on day 28. LPS induced groups were compared,* ***group Va CFA 0.1ml + LPS 10µg/ml (10±0.61)*** *showed the maximum increase among all LPS groups (fig 5.7 iA).*

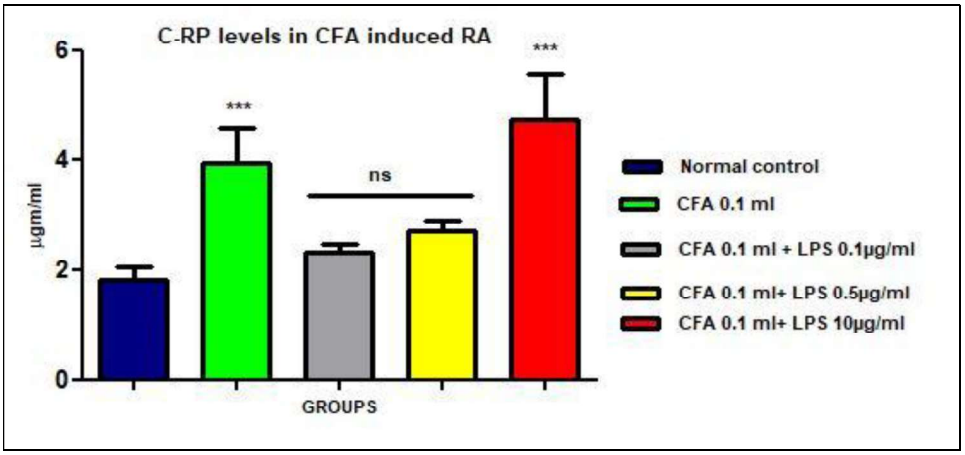
C-RP *is also one of the inflammatory markers of importance in RA to see the inflammatory responses in disease conditions. The results of CRP were found in increasing trends from day 7 to 28.in all model controls as per the comparison was done among normal control and models. When individual groups were compared,* ***group IIa, CFA 0.1ml (6.05±0.29)*** *and* ***group Va, CFA 0.1ml + LPS 10µg/ml (7.36±0.24)*** *showed the increase in CRP levels throughout the study period which was significantly different as compared to* ***group I, normal control (2.2±0.30).*** *The combination of LPS 10µg/ml and CFA 0.1ml induced RA represented the maximum effects on CRP levels when compared with other doses of LPS.(Fig 5.7 i B)*

Homocysteine *is associated with extra organ manifestations in the existing disease. Here in study flow the next objective is to assess cardiovascular complications in existing RA due to which Homocysteine levels of groups developed only for RA were also evaluated for this marker for comparison.* ***Group I, normal control (4.8±0.36)*** *compared with other groups where,* ***group IIa, CFA 0.1ml (6.7±0.30) and group Va, CFA 0.1ml + LPS 10µg/ml (7.3±0.29)*** *showed the significant increase in homocysteine levels as compared to other groups. Here group Va with LPS sensitization leads the systemic increase in homocystein which further supports the hypothesis.fig 5.7 iC*

i (A)



i (B)



i (C)

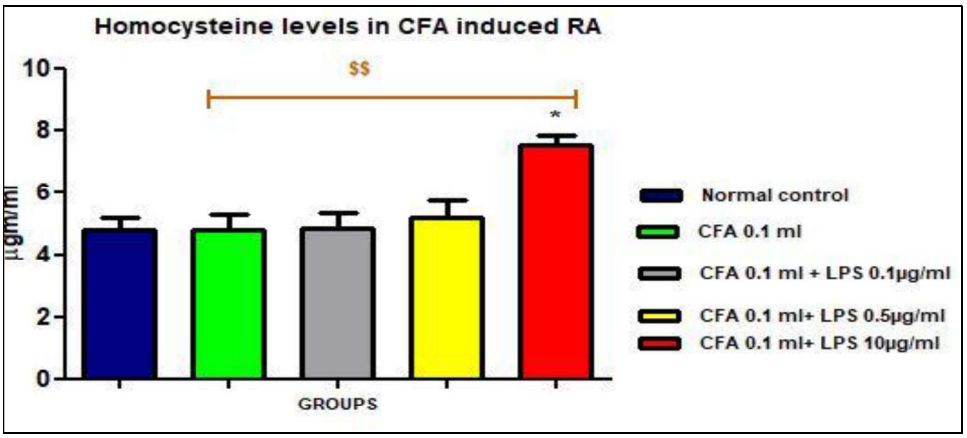


Fig. 5-7 Graphical representation of inflammatory parameters in CFA and CFA + LPS induced RA

(A) Effects of CFA and LPS on ESR (B) Effects of CFA and LPS on CRP (C) Effects of CFA and LPS on homocystein levels

Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis and repeated measure ANOVA for paw volume. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. $, $$, $$$ showed comparison between CFA 0.1ml vs. CFA 0.1ml+ LPS10µg/ml. ns showed non-significant differences between normal and other groups.

ii) Immunological markers- Neutrophil, IL-6, TNF-α, NF-κB

Immune responses are considered as a disease severity and aggravator for multiple complications in disease progression. In this study, immune indicators were estimated for confirmation of disease progression, severity and to analyze extra organ manifestations in existing RA.

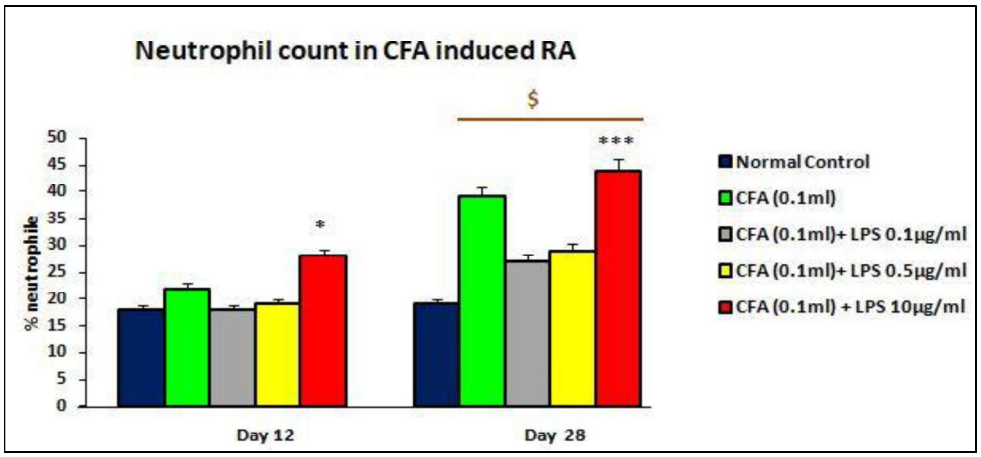
***Neutrophil count** was estimated on day 12 and 28 in this study to check the immune response activation in CFA induced RA models where **group Va CFA 0.1ml + LPS 10µg/ml (40-44)** showed the significant increase in neutrophile count as compared to normal control and other groups. This is may be due to addition of endotoxin LPS which stimulated the immune response in this group (fig. 5.8 ii A)*

***IL-6** was estimated in this study again to confirm cytokine stimulation via inflammatory and immune activation in RA and models were compared for IL-6 activation with normal control responses. **Group Va CFA 0.1ml + LPS 10µg/ml (51.3±14.6)** demonstrated significant increase in IL-6 as compared to **normal control group (36.32±7.7)** suggesting disease progression (fig.5.8 ii B).*

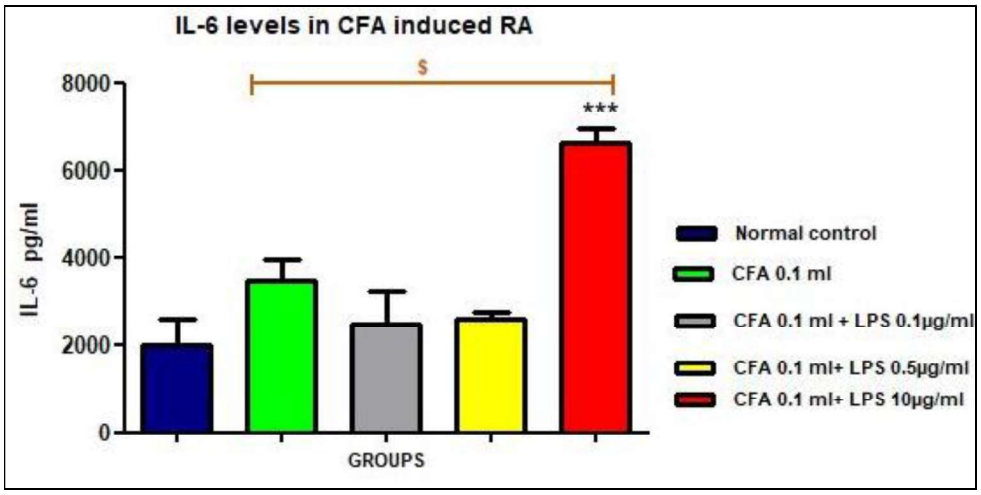
***TNF-α** is marker associated with inflammatory and immune response. The results of markers compared for day 28 where, model control groups were compared with **normal control group (1.76±1.05)** in this comparison **group IIa CFA 0.1ml (10.3±5.89)** and group **Va CFA 0.1ml + LPS 10µg/ml (10.3±1.08)** showed equal and significant increase in TNF-α levels indicating immune responses(fig.5.8 ii C).*

***NF-κB** stimulation in RA was also measures as one of the confirmatory and inter-connected molecules in immune activation via inflammatory pathway. Model control groups when compared with normal **control group (23.0±0.2)** showed higher disease stimulation in **group Va CFA 0.1ml + LPS 10µg/ml (0.51±0.05)** fig.5.8 ii D.*

ii (A)


Neutrophil count in CFA induced RA
$
***
*
% neutrophile
50
45
40
35
30
25
20
15
10
5
0
Day 12
Day 28
Normal Control
CFA (0.1ml)
CFA (0.1ml)+ LPS 0.1µg/ml
CFA (0.1ml)+ LPS 0.5µg/ml
CFA (0.1ml) + LPS 10µg/ml

ii (B)


IL-6 levels in CFA induced RA
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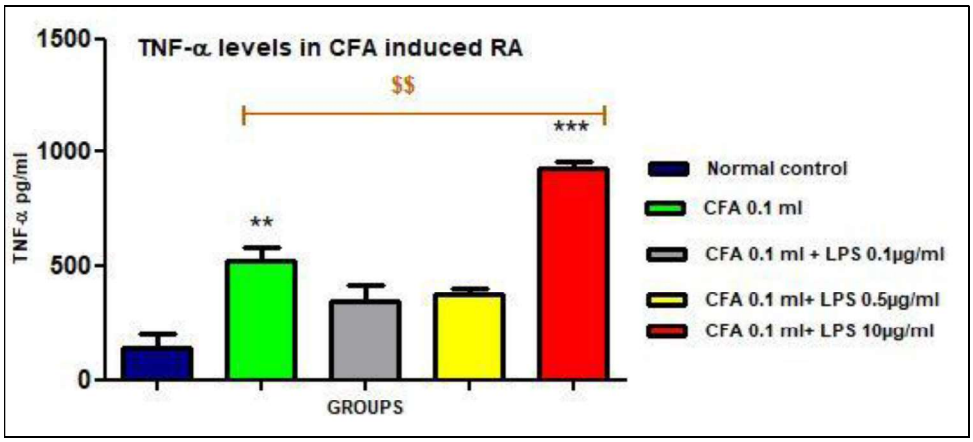
IL-6 pg/ml
8000
6000
4000
2000
0
GROUPS
Normal control
CFA 0.1 ml
CFA 0.1 ml + LPS 0.1µg/ml
CFA 0.1 ml+ LPS 0.5µg/ml
CFA 0.1 ml+ LPS 10µg/ml

ii (C)


TNF-α levels in CFA induced RA
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TNF-α pg/ml
1500
1000
500
0
GROUPS
Normal control
CFA 0.1 ml
CFA 0.1 ml + LPS 0.1µg/ml
CFA 0.1 ml+ LPS 0.5µg/ml
CFA 0.1 ml+ LPS 10µg/ml

ii (D)

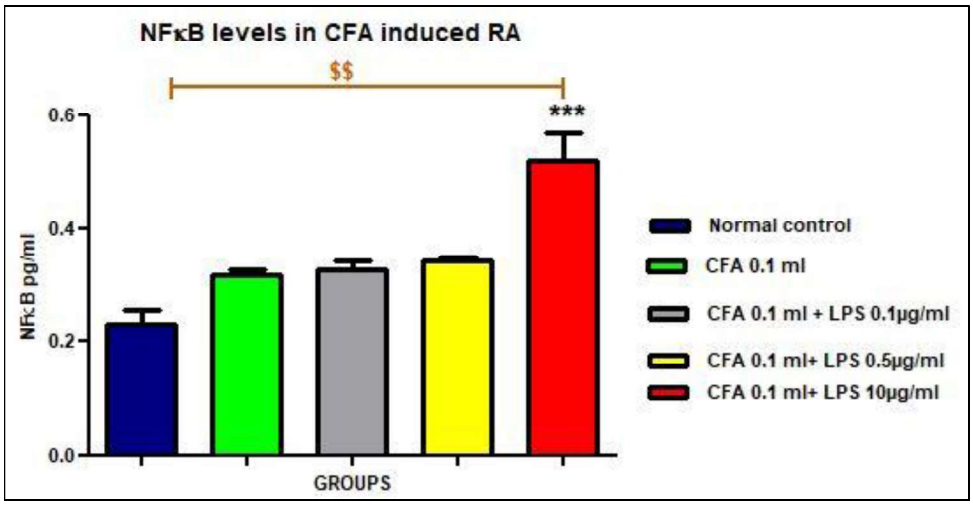


Fig. 5-8 Graphical representation of immunological parameters in CFA and CFA + LPS induced RA
(A) Effects of CFA and LPS on Europhile count (B) Effects of CFA and LPS on IL-6
(C) Effects of CFA and LPS on TNF-α (D) Effects of CFA and LPS on NFκB

Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis and repeated measure ANOVA for paw volume. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. $, $$, $$$ showed comparison between CFA 0.1ml vs. CFA 0.1ml+ LPS10µg/ml. ns showed non-significant differences between normal and other groups.

iii) Disease specific markers- RF, Anti-CCP, TLR-4

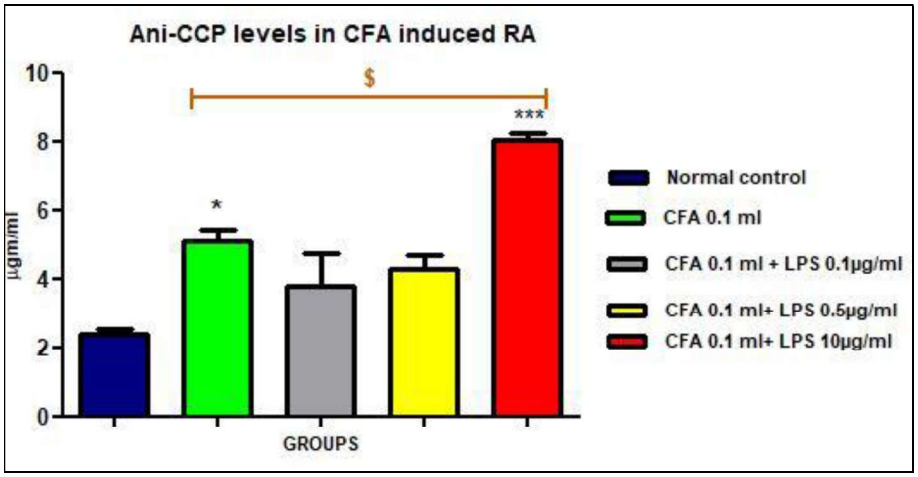
RF or Rheumatoid factor *is one of the identical disease markers for RA with genetic correlation of disease. The results for RF were compared on day 28 with the values of* ***normal control group (10.2±1.08).*** *The groups sensitized with CFA and low dose LPS (0.1, 0.5 µg/ml) as well as CFA alone were not produced any significant difference in RF values when compared with each other as well as with normal control group. Whereas* ***group Va, CFA 0.1ml + LPS 10µg/ml (15.5±0.31)*** *found significantly different from normal control group and the values of RF were significantly high among all models.*

Anti-CCP *is a major indicator for differentiation between RA and osteoporosis. This marker only stimulates in response to antibodies generates in body due to high grade inflammation and immune responses (fig 5.9* iii A*).*

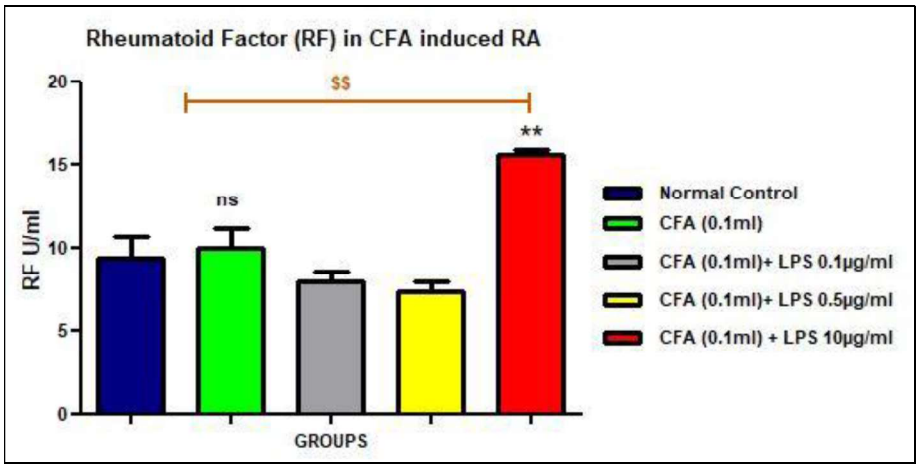
Here disease models were compared with ***normal control group (2.3±0.15)*** *and there were a significantly increased levels of Anti-CCP were marked in* ***group IIa, CFA 0.1ml (7.03±0.44) and group Va CFA 0.1ml + LPS 10µg/ml (7.4±0.36)*** *showed the significant difference in Anti-CCP levels as compared to normal control and other groups. Simultaneously both the groups were insignificant with each other which are indicator of RA generation in developed model (fig 5.9* iii B*).*

TLR-4 *marker associated with LPS secretion in body in response to leaky gut generated via multiple systemic insults in homeostasis. Here model control groups when compared with* ***normal control group*** *(**21.0±0.51**) suggests the activation of TLR-4 activation in response to additional LPS provided to* ***group Va, CFA 0.1ml + LPS 10µg/ml (37±0.80)*** *which was not present in any other model groups which also supports the hypothesis (fig 5.9* iii C*).*

iii (A)



iii (B)



iii (C)

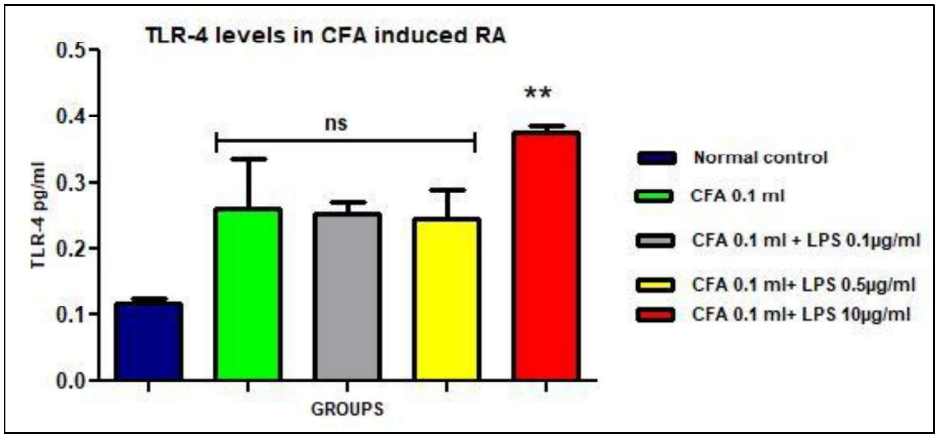


Fig. 5-9 Graphical representation of effects of CFA and CFA + LPS induced RA on disease specific markers

(A) Effects of CFA and LPS on Anti-CCP (B) Effects of CFA and LPS on RF (C) Effects of CFA and LPS on TLR-4 Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis and repeated measure ANOVA for paw volume. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. $, $$, $$$ showed comparison between CFA 0.1ml vs. CFA 0.1ml+ LPS10µg/ml. ns showed non-significant differences between normal and other groups.

C) Results of confirmatory indicators in CFA and CFA with LPS induced RA

In this study design the selected disease has specific target as bone and skeletal muscle tissues where- bone deformity, bone erosion, edema, cell infiltration and tissue damage can be confirmed with two estimations; **X-ray and histopathology**.

X-ray *of animals was done on day 28 where CFA sensitized paw (left hind limb) was taken and all groups were compared with normal control group. Assessment of x-ray film was done for edema, bone erosion, bone rupture and deformed joints as showed in fig 5.10. In this comparison mild edema and bone rupture was seen maximum in* ***group Va, CFA 0.1ml + LPS 10µg/ml*** *and there were no digestion of digits or secondary lesions and inflammation in upper limbs as generated in collagen induced RA shown in later part of study to compare disease severity.*

Histopathological examination *was also performed for confirmation of disease impact on tissue level and to estimate the changes in cell cytology. In this estimation the cellular infiltration and change in cell cytology was observed in model control groups as depicted in fig. 5.11and the disease severity was higher in* ***group Va, CFA 0.1ml + LPS 10µg/ml.***

X-ray and histopathology results were further compared with other inducing agents to check the disease progression through different inducers for which both the protocol of study I were cumulatively compared between all the groups of CFA and LPS as well as groups developed with CIA and LPS for better understanding on observational assessment.

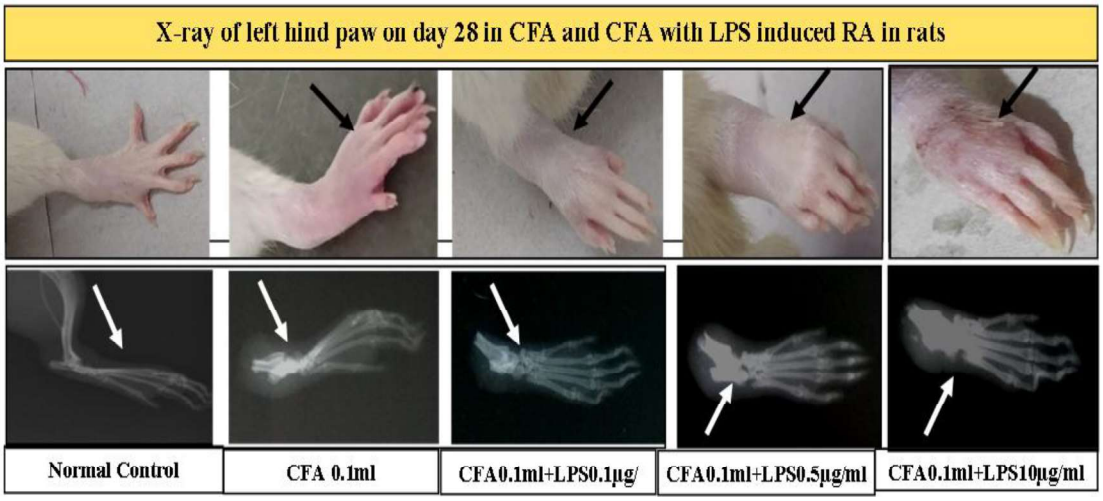


Fig. 5-10 Radiographic assessment of CFA and CFA with LPS induced RA in rats

The figure shows the normal morphology with x-ray of left hind paw

Picture 1- Depicts the normal control group and normal bone structure

Picture 2- Depicts group sensitized only with CFA 0.1ml

Picture 3, 4 and 5- Depicts CFA and LPS sensitized groups where the maximum effect of CFA was seen in CFA 0.1ml+ LPS 10µg/ml induced animals as shown in picture 5 with bone erosion and deformed ankle joint and metaphalangeal joints of paw fingers.

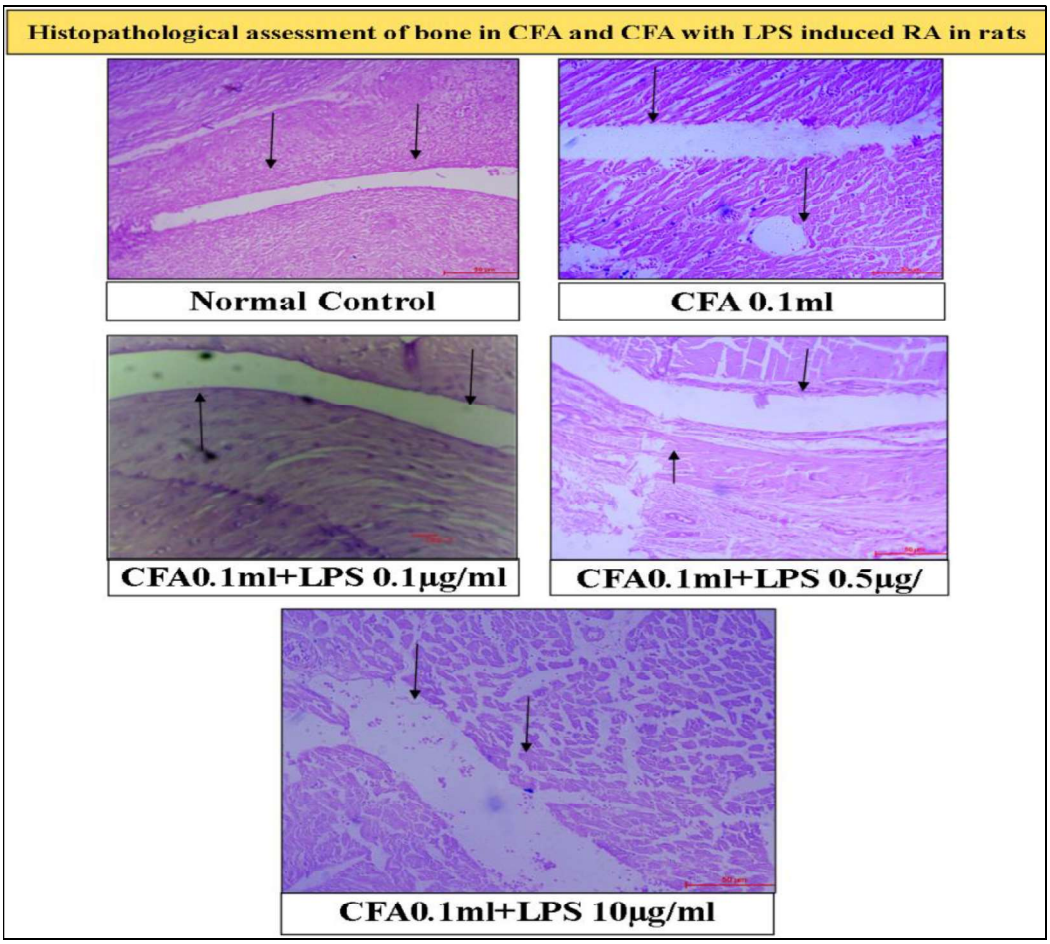


Fig. 5-11 Histopathological assessment of CFA induced RA in rats

Image I shows the cytology of normal rat paw bone

Image II shows the cytological changes in CFA 0.1ml induced group where cells showed infiltration and changes in cell structure

Image III, IV and V shows changes in bone cells on addition of LPS with CFA in different concentrations As arrow indicates there was a cell infiltration and rupture of cells seen in CFA 0.1ml + LPS 10µg/ml group

5.2.2 Results of RA model developed using collagen and LPS (CIA)

In this section, results of RA induction using collagen and collagen with LPS compiled where, LPS was utilized as secondary inducing agent with collagen. All the study design were similar as per CFA study and three doses of LPS (0.1, 0.5 and 10µg/ml) were evaluated with collagen (0.1ml) similarly but the study duration was of 42 days. Again as study proceeds, the best model for RA was taken for model validation for RA as well as RA with CVD (via incorporation of HFD and LPS).

In this part of model development for collagen induced RA; the comparison was done between-

Group I normal control, group IIb (CIA 0.1ml), group IIIb (CIA 0.1ml + LPS 0.1µg/ml), group IVb (CIA 0.1ml + LPS 0.5µg/ml) and group Vb (CIA 0.1ml + LPS 10µg/ml).

As discussed in previous section this protocol was followed to develop RA models with different inducers for comparison to select the best model on all three disease indicators *viz;* physical, biochemical and confirmatory indicators.

Notation-

Group I- Normal control
Group IIb- CIA 0.1ml
Group IIIb- CIA 0.1ml + LPS 0.1µg/ml
Group IVb- CIA 0.1ml + LPS 0.5µg/ml
Group Vb - CIA 0.1ml + LPS 10µg/ml

a) Results of Physical indicators for collagen and collagen with LPS induced RA

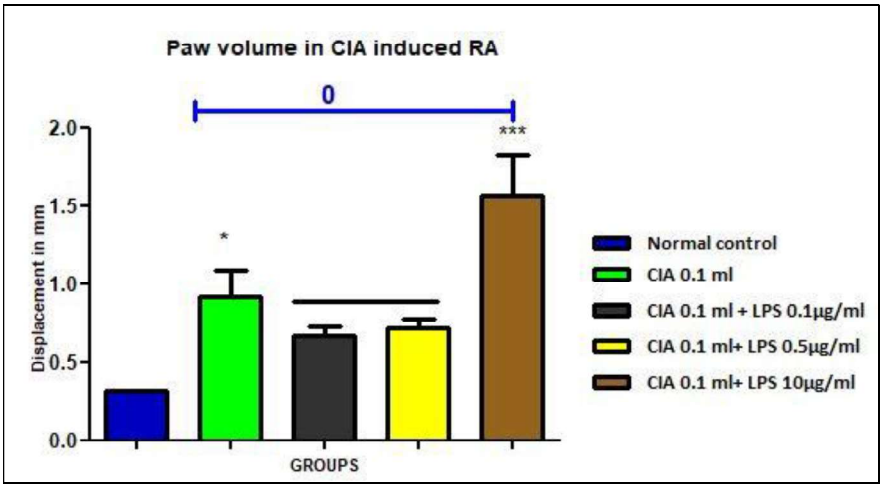
The results of physical indicators such as paw volume, arthritic score and arthritic index (fig. 5.12) with photographic assessment were performed for collagen induced RA models.

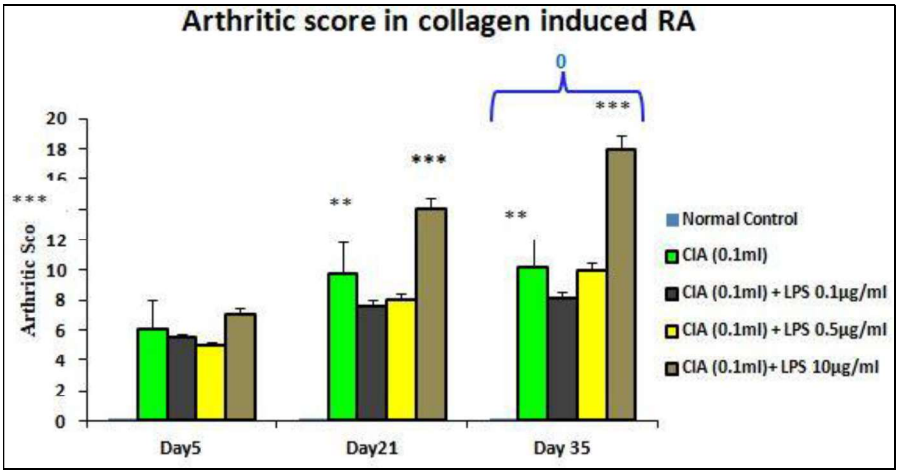
Paw volume *for this study was performed on different days as mentioned in methodology section and the results were compared for day 42. Model control groups compared with normal control and paw volume of model groups was high due to induction of disease. Among all groups there was no significant difference but the elevation of edema and inflammation of model* ***group Vb, CIA 0.1ml + LPS 10µg/ml (1.7±0.05) as compared to normal control (0.24±0.01)*** *suggesting that collagen induced RA is more severe and having human resemblance of RA in rats .*

Arthritic score *was taken on day 5 and 21 and 35 to confirm the disease progression on the basis of severity in the form of primary and secondary lesions and wound development in collagen induced animals as the impact of disease onset was very fast in this adjuvant which were appeared from day 5.*

When the data were compared, arthritic score of ***group Vb, CIA 0.1ml + LPS 10µg/ml (11±1.27)*** *on day 21 showed significantly high arthritic score as compared to* ***normal control (0.0±0.0)*** *and it was constantly high on day 35but here data of day 28 were compared with CFA group. The scoring of collagen and LPS in 0.1* ***group IIIb (7.6±0.61)*** *and 0.5 µg/ml doses* ***group IVb (8±0.34)*** *also showed the mild disease onset but it was lesser than the* ***group IIb Collagen 0.1 ml(9.8±0.53).***

Arthritic index *was also significantly increased in* ***group Vb, CIA 0.1ml + LPS 10µg/ml (19±0.28)*** *as compared to* ***group I, normal control (0.0±0.0)*** *which did not show any signs of arthritis throughout the study. Rest of the* ***groups; IIb, collagen 0.1(18±0.78), IIIb, collagen +LPS 0.1 µg/ml (18±0.71) and IVb, collagen +LPS 0.5 µg/ml (18.1±0.71)*** *also showed the elevated arthritic indexes in response to collagen and LPS induction.*





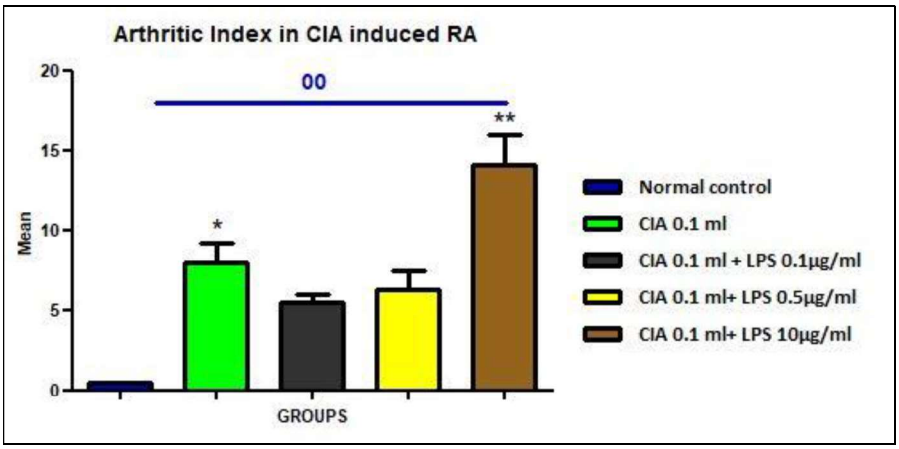
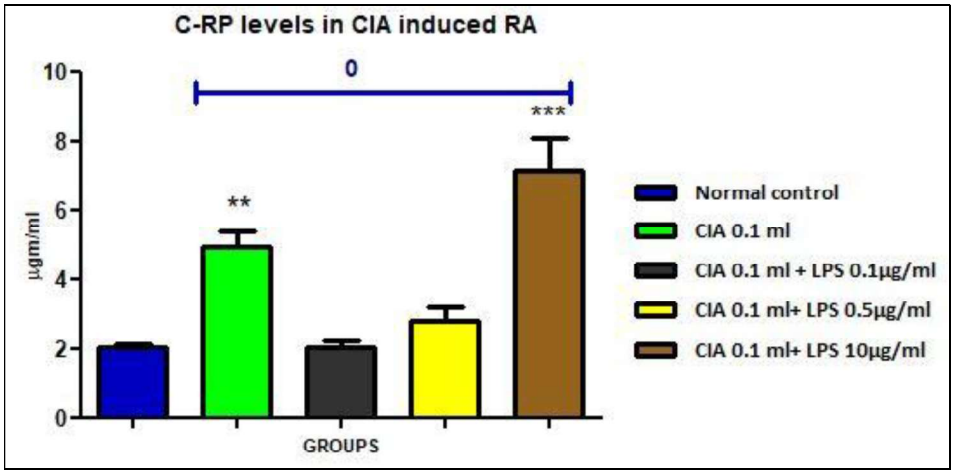


Fig. 5-12(A) Effects of collagen and LPS on Paw volume (B) Effects of collagen and LPS on arthritic score (C) Effects of collagen and LPS on arthritic index

Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. ns is non-significant differences between normal and other groups and 0, 00, 000 is comparison between models.

C-RP *as a marker of inflammation when evaluated, it showed the increased C-RP levels in all groups as compared to* ***normal control group (2.2±0.30).*** *These results indicate that the inflammatory markers were similarly increased in collagen as well as in collagen with LPS groups with the severity of disease. Among all the groups,* ***Vb, CIA 0.1ml + LPS 10µg/ml (7.7±1.08)*** *significantly represent the CRP elevation suggesting higher inflammatory responses*

ESR, *for inflammatory responses when evaluated, it was significantly high for* ***group Vb, CIA 0.1ml + LPS 10µg/ml (14.8±0.54)*** *and there was a significant increase in disease progression as compared to* ***normal control group (0.66±0.22). Group IIb, Collagen 0.1ml (10±0.68)*** *also showed a significant increase in ESR but it was low as compared to* ***group Vb*** *(fig. 5.13).*



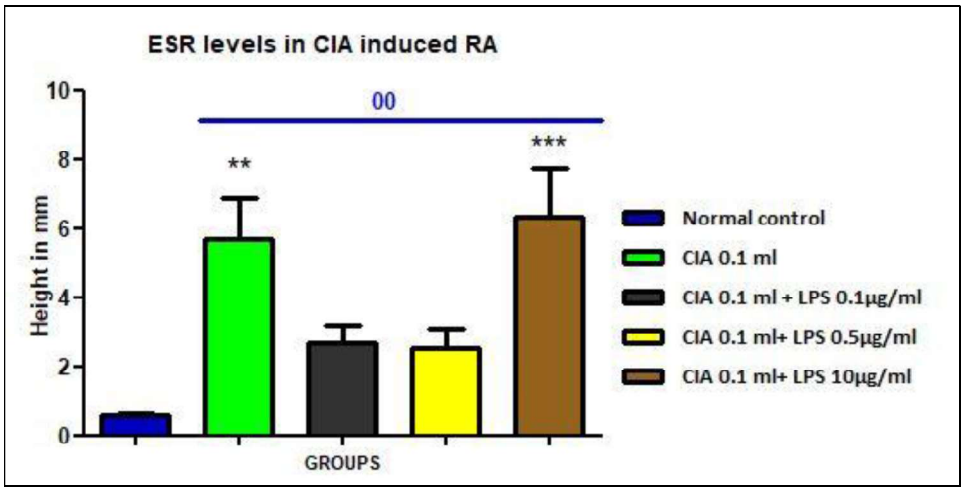


Fig. 5-13 (A) Effects of collagen and LPS on CRP (B) Effects of collagen and LPS on ESR

Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. ns is non-significant differences between normal and other groups and 0, 00, 000 is comparison between models

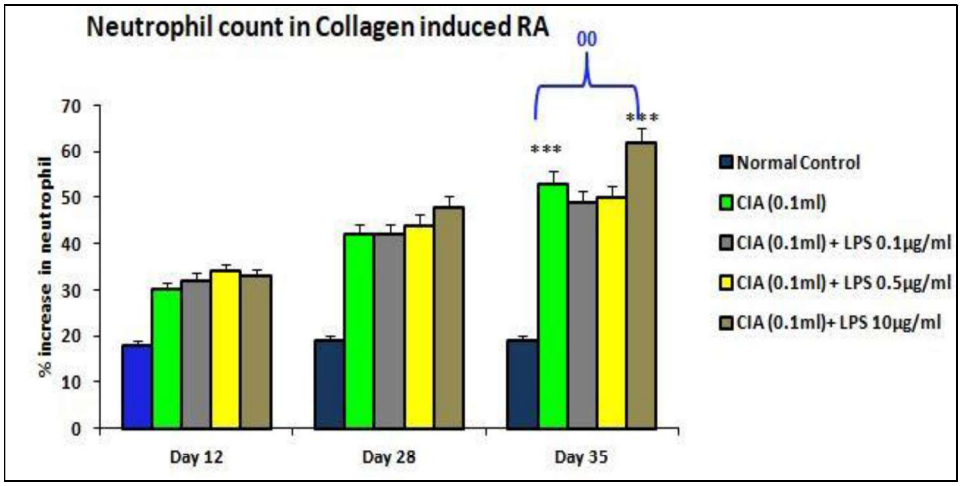
Neutrophil *count was performed on day 12, 28 and 35 and 42 of the study in collagen induced RA study, but as CFA induced study was of 28 days the results of neutrophil were compared for 12 and 21 days. The maximum effects of increase in neutrophil was observed on day 21 in* ***group Vb - CIA 0.1ml + LPS 10µg/ml (60-65)*** *as compared to* ***normal control(18-20)*** *and other groups and it was further increasing till end of the study. Group IIb- CIA 0.1ml (50-55) also showed the increase in neutrophil count but the maximum values was observed in LPS sensitized group.*

IL-6 *values were expressed higher among all groups as compared to* ***normal control group (36.3±7.7)*** *and the highest IL-6 expression was observed in* ***group Vb, CIA 0.1ml + LPS 10µg/ml (243±1.9)*** *which showed that collagen is more effective in activation of immuno and inflammatory both the markers.*

TNF-α expression *were high in* ***group Vb, CIA 0.1ml + LPS 10µg/ml (14.3±0.73)*** *which suggests the immunological intervention with both the inducers (collagen and LPS) as compared to* ***normal control group (1.76±1.05).*** *Other collagen and LPS sensitized groups were also showed the increase in TNF-α expression* ***group IIIb (12.8± 7.5)*** *and* ***group IVb (13.0± 7.5)*** *which is similar to* ***group IIb, collagen 0.1ml (12.8± 7.5)***

NF-κB *expression was also high in* ***group Vb, CIA 0.1ml + LPS 10µg/ml (98.8±0.1)*** *as compared to* ***normal control group (0.23±0.02).****The* ***groups IIb (0.63±0.06), group IIIb (0.57±0.08),*** *and* ***group IVb(0.53±0.02)*** *also showed the elevation in* ***NF-κB levels*** *and they are also significant in disease generation as compared to normal control group.*

Homocysteine *values were compared with* ***normal control (4.8±0.36)*** *there was a significant elevation in Hyc in* ***group IIb (7.5±0.29)*** *and* ***group Vb (8.3±0.14)*** *considered as factor for extra organ manifestations in existing RA. The result of Hyc suggests that there is initiation of disease severity in terms of extra organ manifestation (fig.5-14).*


Neutrophil count in Collagen induced RA
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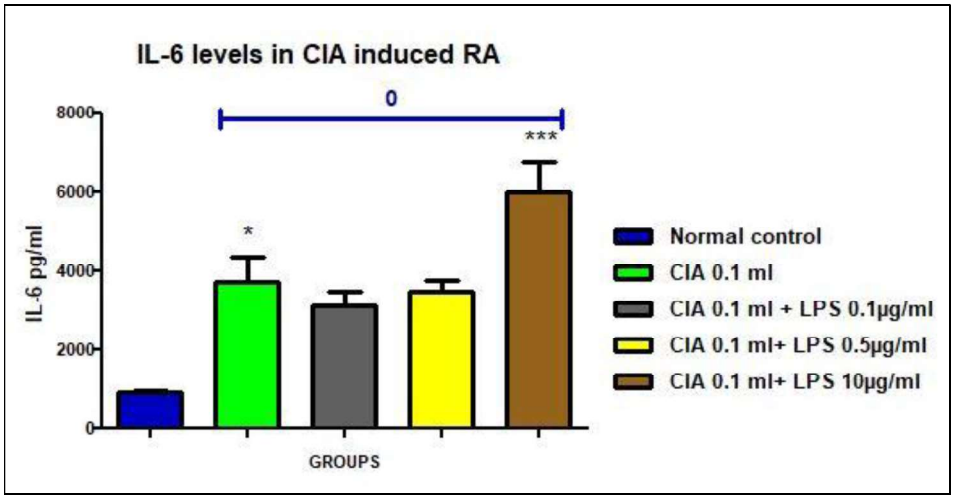
% increase in neutrophil
70
60
50
40
30
20
10
0
Day 12
Day 28
Day 35
Normal Control
CIA (0.1ml)
CIA (0.1ml) + LPS 0.1µg/ml
CIA (0.1ml) + LPS 0.5µg/ml
CIA (0.1ml)+ LPS 10µg/ml


IL-6 levels in CIA induced RA
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IL-6 pg/ml
8000
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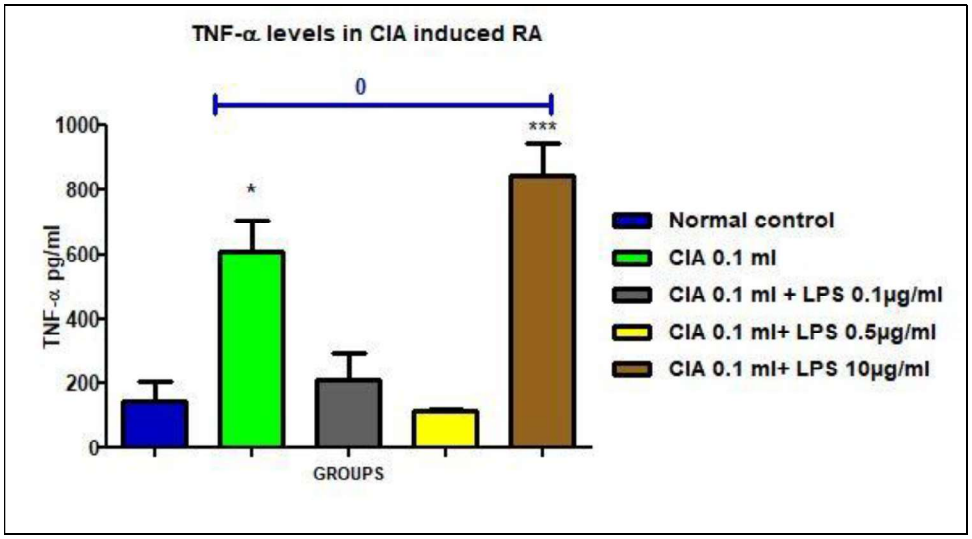
GROUPS
Normal control
CIA 0.1 ml
CIA 0.1 ml + LPS 0.1µg/ml
CIA 0.1 ml+ LPS 0.5µg/ml
CIA 0.1 ml+ LPS 10µg/ml


TNF-α levels in CIA induced RA
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1000
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400
200
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GROUPS
Normal control
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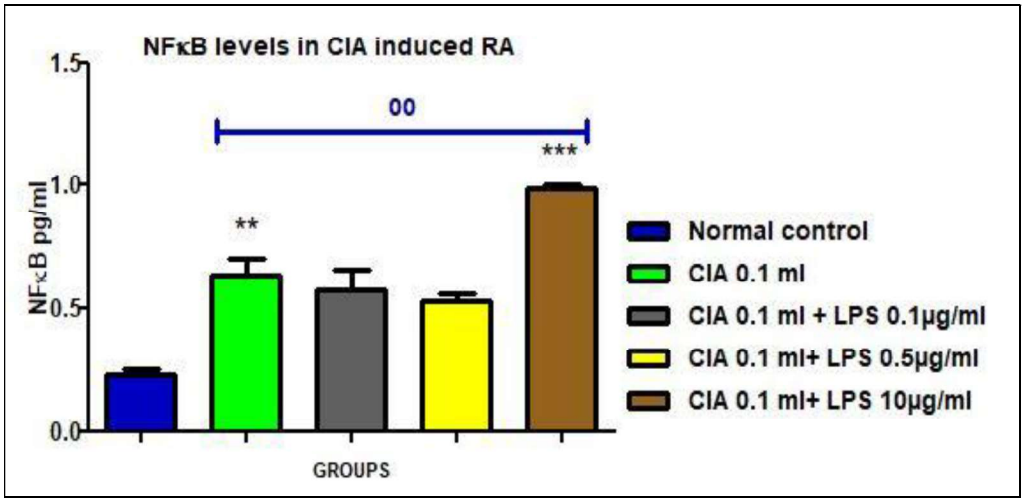
CIA 0.1 ml + LPS 0.1µg/ml
CIA 0.1 ml+ LPS 0.5µg/ml
CIA 0.1 ml+ LPS 10µg/ml



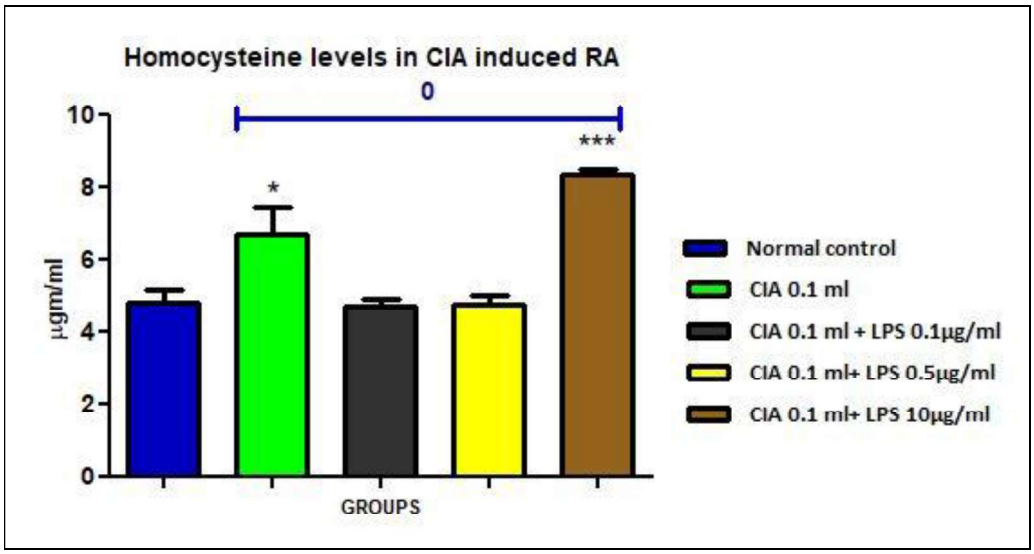


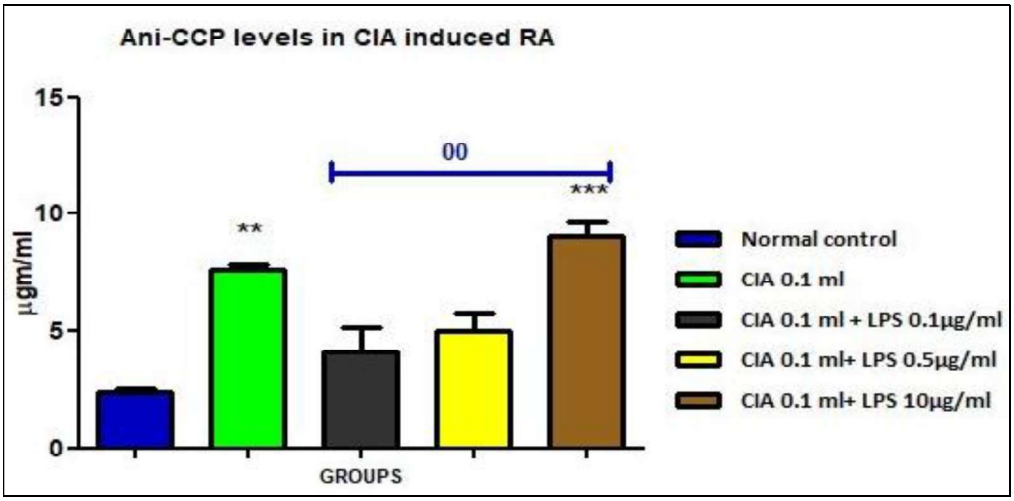
Fig. 5-14 (A) Effects of collagen and LPS on IL-6 (B) Effects of collagen and LPS on TNF-α (C) Effects of collagen and LPS on NFkB (D) Effects of collagen and LPS on Homocystein

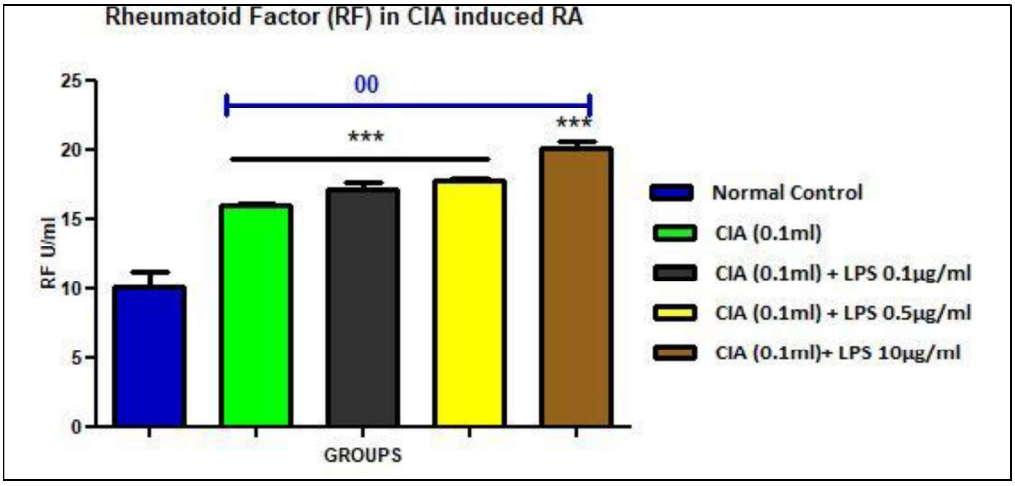
Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. ns is non-significant differences between normal and other groups and 0, 00, 000 is comparison between models

Anti-CCP *collagen induced groups showed an elevation in all the groups as compared to* ***normal control (2.3±0.15)*** *and in group* ***Vb, CIA 0.1ml + LPS 10µg/ml (9.6±0.26)*** *it is significantly high among all other groups;* ***group IIb collagen 0.1ml (7.4±0.36)*** *also showed the elevation in Anti-CCP confirming the RA in developed models.*

RF *for collagen induced groups showed an elevation in all the groups as compared to* ***normal control (10.2±1.08)*** *and in group* ***Vb, CIA 0.1ml + LPS 10µg/ml (20.1±0.49)*** *it is significantly high among all other groups;* ***group IIIb (17.0±0.52), group IVb (17.6±0.33)*** *and effective on increasing Rheumatoid factor in sensitized rats, which shows that collagen induced RA with extra LPS sensitization generates the immune responses earlier in disease progression.*

TLR-4 *suggests the initiation of crosslinking between inflammatory and immune responses in progression of CVD in RA. These results were strongly supported by higher expression of* ***TLR-4*** *in* ***model Vb, CIA 0.1ml + LPS 10µg/ml (0.93±0.7)*** *as compared to* ***normal control group (0.21±0.05)*** *which was significant in disease progression (fig.5-15).*





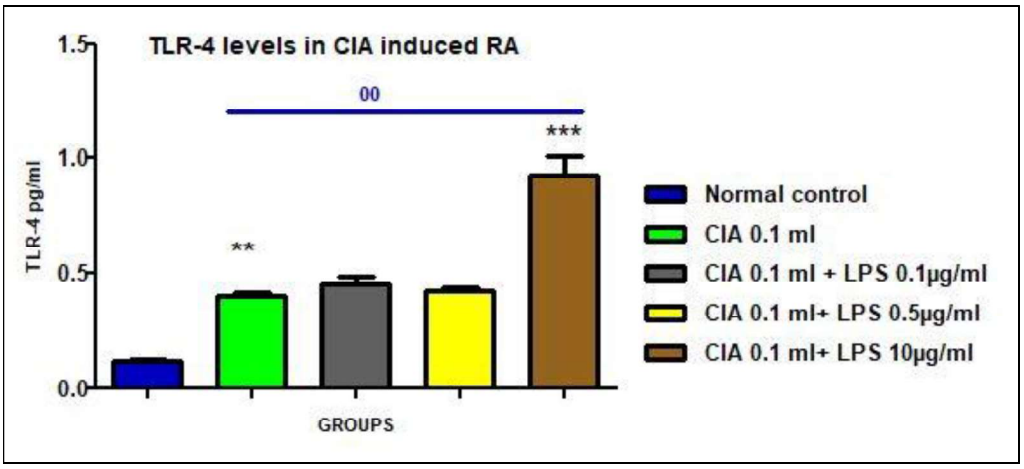


Fig. 5-15 (A) Effects of collagen and LPS on Anti-CCP (B) Effects of collagen and LPS on RF (C) Effects of collagen and LPS on TLR-4

Values are expressed as Mean ± SEM. Statistically evaluated using one way ANOVA analysis. *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. ns is non-significant differences between normal and other groups and 0, 00, 000 is comparison between models

C) Results of confirmatory indicators in collagen and collagen with LPS induced RA

X-ray and histopathology for collagen induced RA and collagen with LPS sensitization showed that there were a severe disease conditions in animals in these models as compared to CFA induced models. Moreover bone erosion, bone deformities as well as disability in movement and walking due to symmetric disease progression appeared much earlier. Some other body parts also showed the nodule formation and deformed structures due to disease which resembled with human conditions at some points. Results of this assessment were depicted in fig. 5-16 and gross comparison is done in succeeding part of the results to compare all the models and treatment groups with standard control.

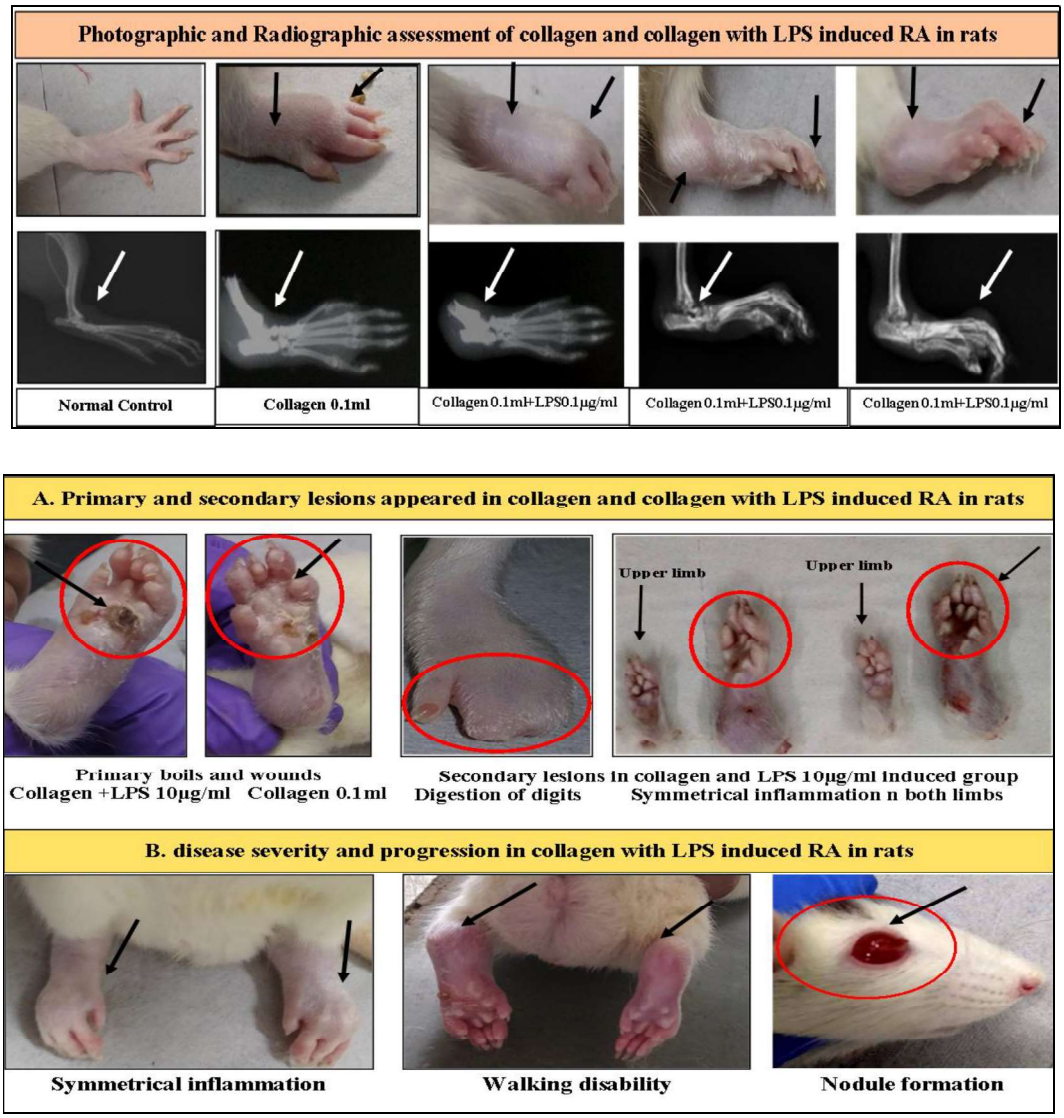


Fig. 5-16 Photographic and Radiographic assessment of collagen and collagen with LPS induced RA

Interpretation of Photographic and X-ray assessment

(A) Primary lesions in collagen induced RA in rats

Model control groups sensitized with collagen were compared with normal control groups on day 5 they showed the severe form of wound, scar and bone deformities, which suggest the severe effects of collagen and fast disease progression.

(B) Secondary lesions in collagen induced RA in rats

Photographs in this section shows severity in the form of secondary lesions on day 21 were comparable with the human RA like conditions, which includes deformities in both the paws (induced left paw/ non induced right paw) digestion of digits, symmetric patters of high grade edema and inflammation in both the paws. Some animals have deformities of eye which are appeared as nodes as depicted in above figure.

5.2.3 Result comparison of study I for selection of best RA model

As previously discussed, study design -I is for selecting best model for RA among-

CFA induced RA; group I (normal control), group IIa (CFA 0.1ml), group IIIa (CFA 0.1ml + LPS 0.1µg/ml), group IVa (CFA 0.1ml + LPS 0.5µg/ml) and group Va (CFA 0.1ml + LPS 10µg/ml) and

Collagen induced RA (CIA); group IIb (CIA 0.1ml), group IIIb (CIA 0.1ml + LPS 0.1µg/ml), group IVb (CIA 0.1ml + LPS 0.5µg/ml) and group Vb (CIA 0.1ml + LPS 10µg/ml).

5.2.3.1 Comparison on the basis of statistical data

All the data with graphical representation were discussed in previous sections. Here on the basis of above results we can conclude that-

RA associated physical markers showed the elevation in all created conditions- Only CFA, Only collagen, CFA + LPS (in different doses) and collagen + LPS (in different doses) but the intensity and duration of disease severity is different in all the conditions.

Model induced with CFA have disease modulation in all prospects but the low doses of LPS (0.1, 0.5 µg/ml) were unable to aggravate it. In some situations they have neutral effects on disease severity as well as on progression.

Collagen induced RA showed exponential disease severity with fast onset of disease. The groups sensitized with high dose of LPS with collagen showed the symptomatology of disease similar to human RA like conditions.

Biological/ biochemical estimation studies showed that the induction of CFA with and without LPS followed the inflammatory pathway for disease generation where in all the conditions, immunological markers were elevated in similar level.

On the other hand the collagen induced RA have two fold increase in inflammation as compare to CFA and when additional boost of LPS was provided the immune responses were visible in all groups.

Table 5-13 Mean±SEM values for study design 1

Parameters	**Normal**	**Group Ia (CFA 0.1 ml)**	**Group IIa (CFA 0.1ml + LPS 0.1µg/ml)**	**Group IIIa (CFA 0.1 ml + LPS 0.5 µg/ml)**	**Group IVa (CFA 0.1 ml + LPS 10 µg/ml)**	**Group Ib (CIA 0.1 ml)**	**Group IIb (CIA 0.1 ml + LPS 0.1 µg/ml)**	**Group IIIb (CIA 0.1 ml + LPS 0.5 µg/ml)**	**Group IVb (CIA 0.1 ml + LPS 10 µg/ml)**
Paw volume	0.24±0.01	0.82±0.14	0.33±0.04	0.31±0.07	0.9±0.01***	0.77±0.032	0.89±0.027	0.91±0.018	0.95±0.017***
Arthritic Index	0.0±00	15±1.25	13.1±.98	13.3±.51	14.5±1.81***	18±0.78	18±0.71	18.1±0.71	19±0.28***
Arthritic Score	0.00±.00 0.00±.00	5.5±0.36 7.1±1.08	5.8±0.44 6.1±1.08	4.8±0.28 5.8±0.22	5.8±0.44 8.6±1.37***	6±0.5 9.8±0.53	5.5±0.36 7.6±0.61	5±0.18 8±0.34	7.1±1.08 11±1.27***
CRP	2.2±0.30	6.05±0.29	3.0±0.25	2.7±0.15	7.36±0.24***	6±0.93	6±0.38	5.7±0.30	7.7±0.18***
ESR	0.66±0.22	9±0.25	3.66±0.66	3.9±0.56	10±0.61***	10±0.68	4.3±1.00	4.1±0.24	14.8±0.54***
HYC	4.8±0.36	6.7±0.30	4.8±0.49	5.2±0.08	7.3±0.29	7.5±0.29	4.7±0.20	4.7±0.29	8.3±0.14***
Anti- CCP	2.3±0.15	7.03±0.44	3.7±0.97	4.3±0.35	7.4±0.36	5.1±0.15	4.1±1.05	5.0±0.68	9.6±0.26***
RF	10.2±1.08	13.6±0.88	13.3±0.88	14.1±0.44	15.5±0.31***	15.9±0.15	17.0±0.52	17.6±0.33	20.1±0.49***
TNF-α	1.76±1.05	10.3±5.89	8.62±2.87	8.57±0.96	10.0±1.08***	12.8± 7.5	12.8± 7.5	13.0± 7.5	14.3±0.73***
IL-6	36.3±7.7	54.8±18.1	42.0±3.4	46.0±2.8	51.3±14.6	88.6±11.8	68.9±5.9	73.5±5.7	243.5±1.9***
NF kB	0.23±0.02	0.31±0.02	0.32±0.01	0.34±0.01	0.51±0.05***	0.63±0.06	0.57±0.08	0.53±0.02	0.98±0.01***
TLR-4	0.21±0.05	0.26±0.07	0.25±0.01	0.31±0.02	0.37±0.01	0.40±0.01	0.45±0.02	0.42±0.01	0.93±0.07***

5.2.3.2 Comparison on the basis of Photographic assessment

Photographic assessment is one of the basic perceptible data for evident records which are helpful in assessment of progression of disease with the days and duration of study period. In this study the progression of disease with increased severity and events occurred in different groups were recorded on alternate days. The photographs were taken with the help of normal mobile camera just to keep record and the photographs of all the groups were taken for-

Normal morphological characteristics of rat paw

Signs of inflammation and edema in injected paw

Primary and secondary lesions appeared during the experiment period

Any severity noticed during the disease generation and progression

Disability due to disease progression

Comparison between injected and non injected paws of rat

To assess effects of treatment in different groups

The secondary lesions in the collagen induced groups was very early within 7 days of induction and the digestion of digits occurred in 14 days of disease onset which was further increasing as symmetrical disease progression in LPS induced groups which is resembling situation with Stage IV of human RA where disability occurs with deformities and symmetric disease patterns. Confirmatory indicators such as radiography and histopathology were also supported the systemic level disease progression. In CFA and CFA with LPS induced groups bone deformity and bone erosion with edema was of low grade. On the other hand this was prominent and very severe in animals sensitized with CIA 0.1ml + LPS 10µg/ml.

In histopathology of bone section the CFA induced groups showed the tissue damage with changes in cell cytology but in collagen alone as well as in CIA 0.1ml + LPS 10µg/ml samples, cell infiltration and pannus formation was clearly observable which suggest the disease severity with multiple damaging factors

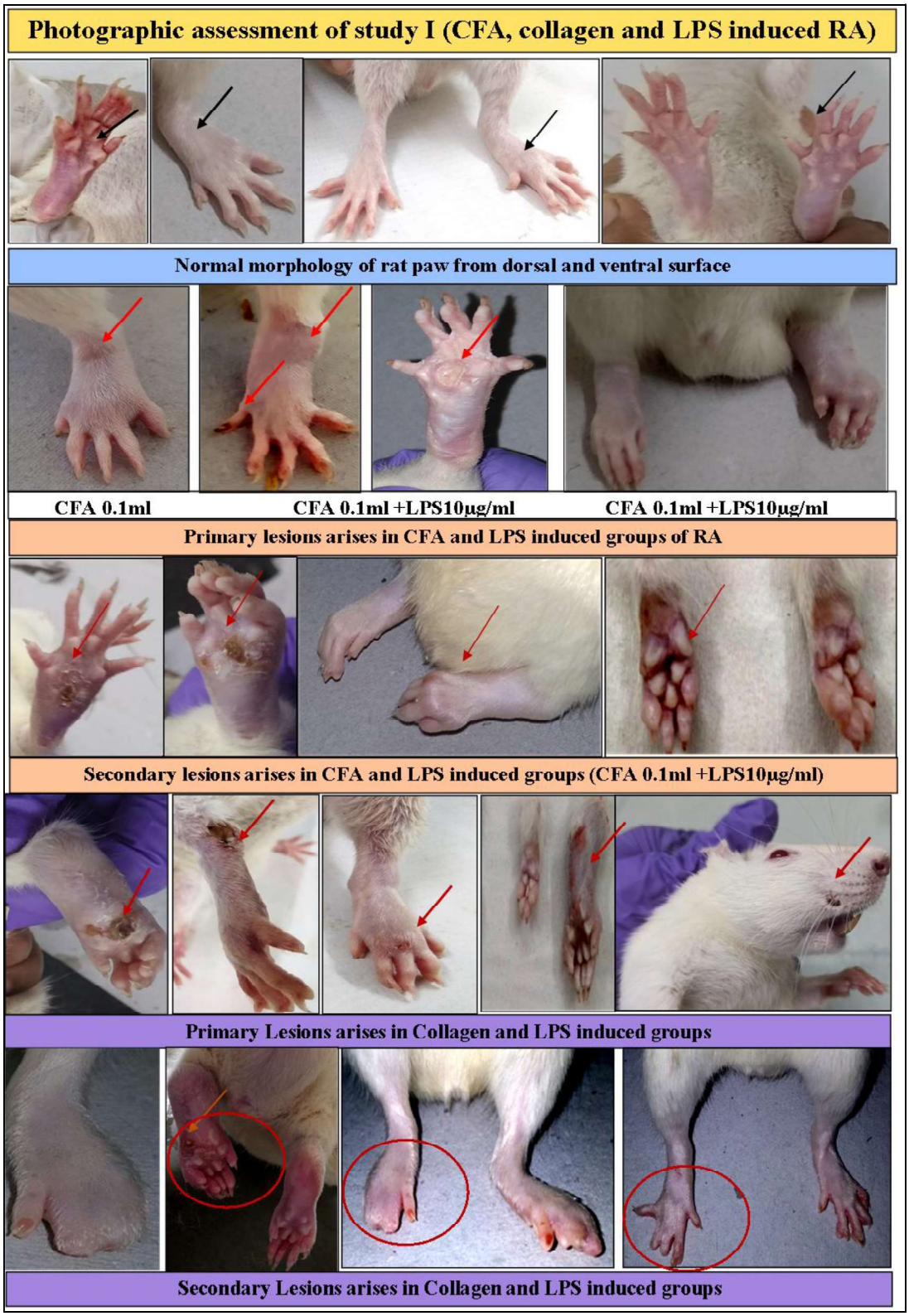


Fig. 5-17 Photographic assessment of study design I (CFA, Collagen and LPS induced RA in rats)

5.2.3.3 Radiographic Assessment of Disease development

Radiography is confirmatory estimation to evaluate the damage occurred in bones and skeletal system due to induction of RA. Different chemical inducers used in this study were compared to check the maximum effect to select best inducer for RA. In the following figures different images shows the progression of disease on different stages.

All the photographs were taken from digital x-ray images and they were evaluated against normal control groups and treatment groups. The images were evaluated according to-

Edema and bone erosion occurred in injected paw of animals
Edema and bone erosion seen in non injected paw of animals
Bone disruption and erosion in upper limb as deformity and secondary lesion
Deformities in the form of digestion of digits as severity of disease progression
Nodule formation in tail of animals as secondary lesions and highest severity of disease
Whole body x-ray was performed to confirm the bone deformities in model control groups to check the human like deformity symptoms in animals.

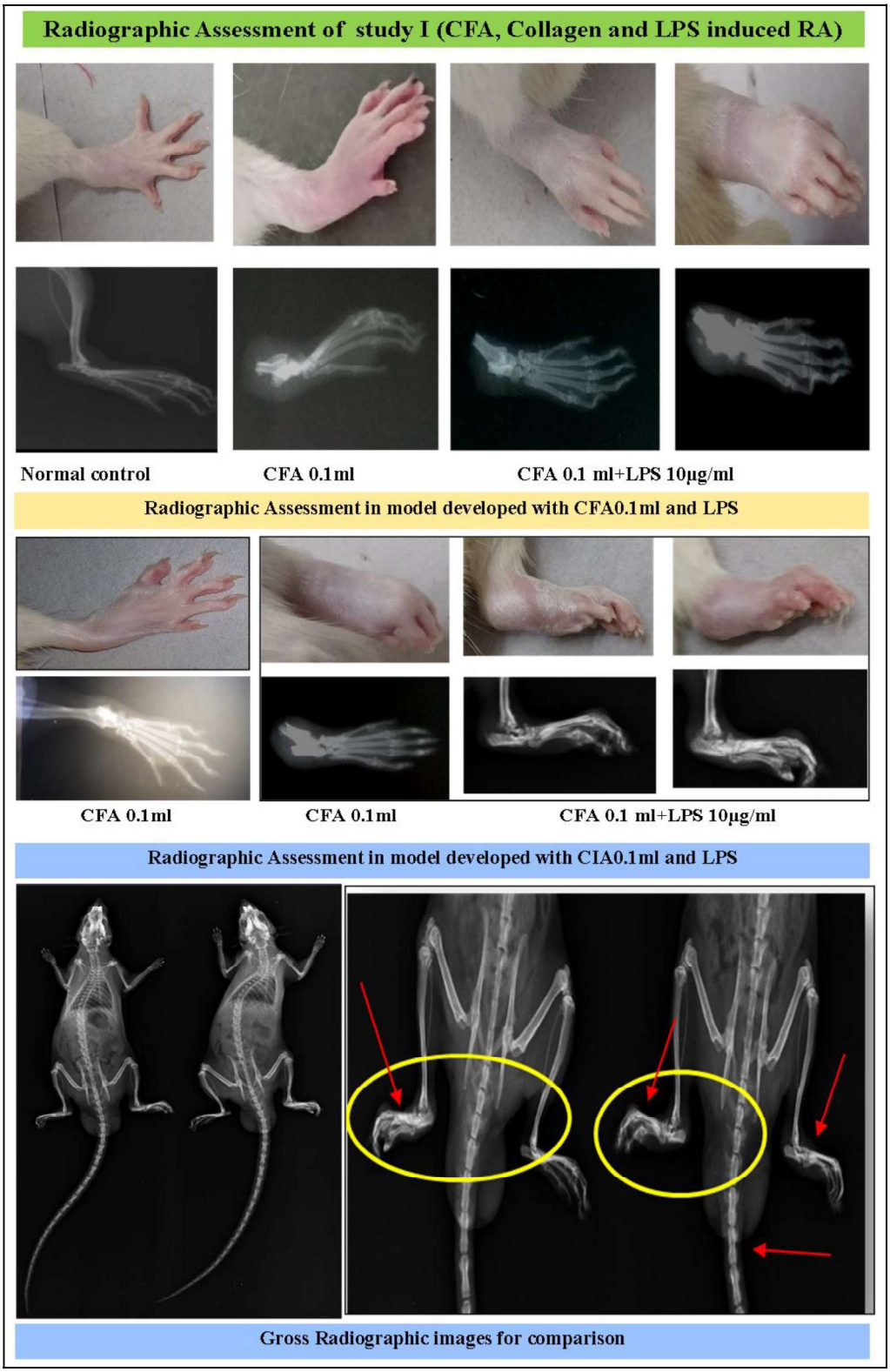


Fig. 5-18 Radiographic assessment of study I (CFA, collagen and LPS induced RA in rats)

Interpretations of Radiographic assessment

When X-ray was taken for confirmation of bone deformities in rats on 28 and 42 days of studies, various changes were observed in these animals.

(A) In this section picture 1 normal control x-ray when compared with picture 2 and 3 of model control group represents the severity in the form of bone deformities where, edema and inflammation with bone erosion with symmetric deformity is clearly visible in picture 2 where the spinal cord of rat is completely deformed and the animal was unable to walk properly which shows the human like severity and deformity in animal in collagen and LPS induced RA in rats.

(B) Here we can clearly seen that the x-ray of animals showed the bone deformity in left hind paw of animal in picture 1 and in picture 2 we can see the morphological as well as bone deformities of the paw of animals where upper and lower both limbs are affected which clearly indicates the symmetric patterns of diseases and in last images in picture 2 the deformed paw and its x-ray image is taken which confirms the human like deformity in the form of digestion of digits and bone deformity.

5.2.4 Comparative analysis for CFA and CIA induced RA with LPS:

To analyze the impact of CFA and CIA as primary inducers and LPS as secondary inducer for development of RA in rats major disease indicators were analyzed by giving them score on 0 -4 scale for disease severity. The graphical representation for the same is depicted in following figure where group Vb CIA 0.1ml + LPS 10µg/ml showed higher impact on all three disease indicators *viz;* physical, biochemical and confirmatory with this scoring collagen induced RA scored highest among all other groups which was further analyzed with scoring system adopted for prevalidation.

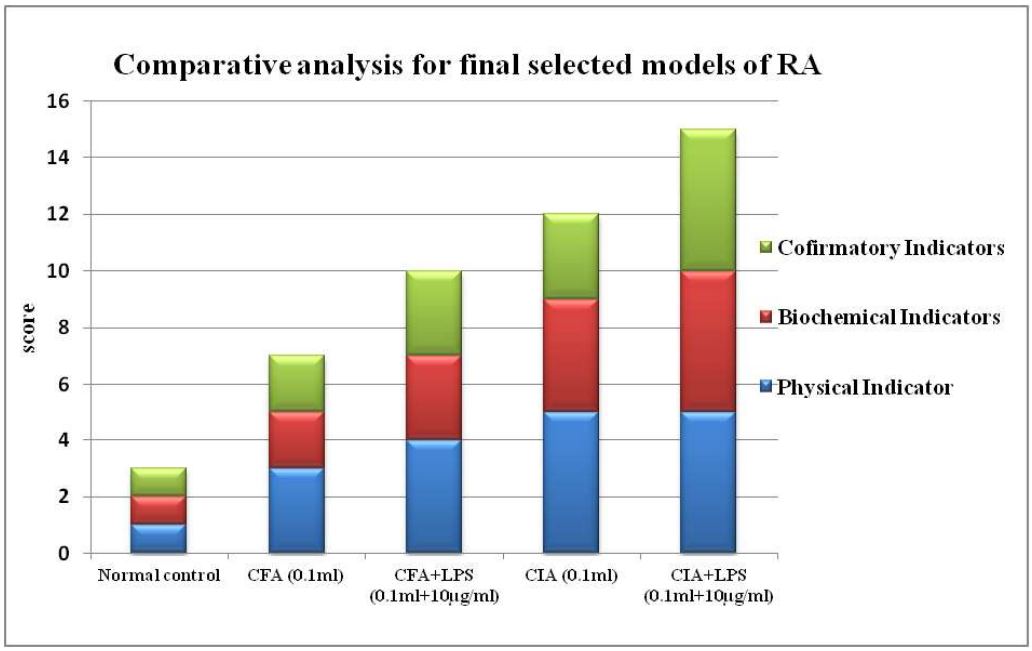


Fig. 5-19 Comparative analysis for CFA and CIA for disease severity indicators

On the basis of above accounts, two groups – group Va, CFA 0.1ml + LPS 10µg/ml form CFA and group Vb CIA 0.1ml + LPS 10µg/ml from CIA induced RA were carried forward for model validation as well as to give an additional biological condition i.e. cardiovascular complication in the form of atherosclerosis in existing RA.

5.2.5 Results of study design- II (Model Development studies for cardiovascular complications in Rheumatoid Arthritis)

The final selected models for developing cardiovascular complications were added in the form of high fat diet in this particular attempt with CFA, collagen and LPS selected models from previously developed model for RA(as discussed in methodology section II).

Dietary manipulation in the form of secondary inducer in this study was added to link the metabolic correlation of RA as well as CVD. Hypothesis is designed to incorporate atherogenic diet to induce metabolic dysbiosis to link generation of autoimmune responses. Here an animal when sensitized with primary adjuvant (CFA, collagen) for RA induction, their biological system is already compromised and body is in protective mode. In this situation, HFD works on two fronts; primarily it affects the metabolic system and on LPS addition it will lead leaky gut which stimulates due to alteration of small intestine gut flora, increased endotoxin release (steatosis, metabolic dysbiosis) as well as antibody generation against body's own enzymes and proteins (RF, Anti-CCP, Hyc) responsible for homeostasis. Due to this primary activation, in the form of secondary response body defense system activates multiple pathways (TLR-4) and releases markers (TNF-α, IL-6, NF-κB) easily connect multiple responses at the same time in body which can cause extra organ manifestations (CVD) in existing disease state (RA). On the basis of above discussion, the following groups from study design I and study design II were selected for development of model for cardiovascular complications in RA-

Va (CFA 0.1ml + LPS 10µg/ml) as Model I
Vb (CIA0.1ml+ LPS 10µg/ml) as Model II } From Study design-I

VII (CFA0.1ml+ HFD+ LPS 10µg/ml) as Model III
IX (CIA0.1ml+ HFD+ LPS 10µg/ml) as Model IV } From Study design-II

All the groups were statistically compared for parameters of RA as well as for parameters of cardiovascular complications in form of atherosclerosis.

Already mentioned parameters for RA as **paw volume, arthritic index, arthritic score, C-RP, ESR, RF, Anti-CCP** were estimated for RA generation. To check the initiation of auto immune responses via inflammatory stimulation, **IL-6, TNF-α, NF-κB** and **homocystein and TLR-4**[99] were evaluated.

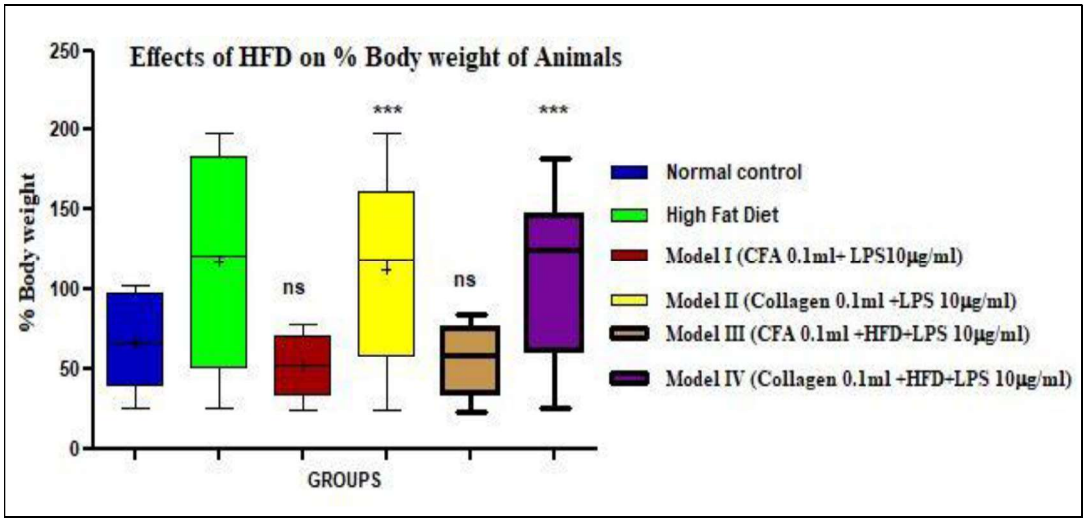
Specific parameters to check atherosclerosis were compared with high fat sensitized groups in these animals lipid profile was evaluated weekly to check the progression and impact of

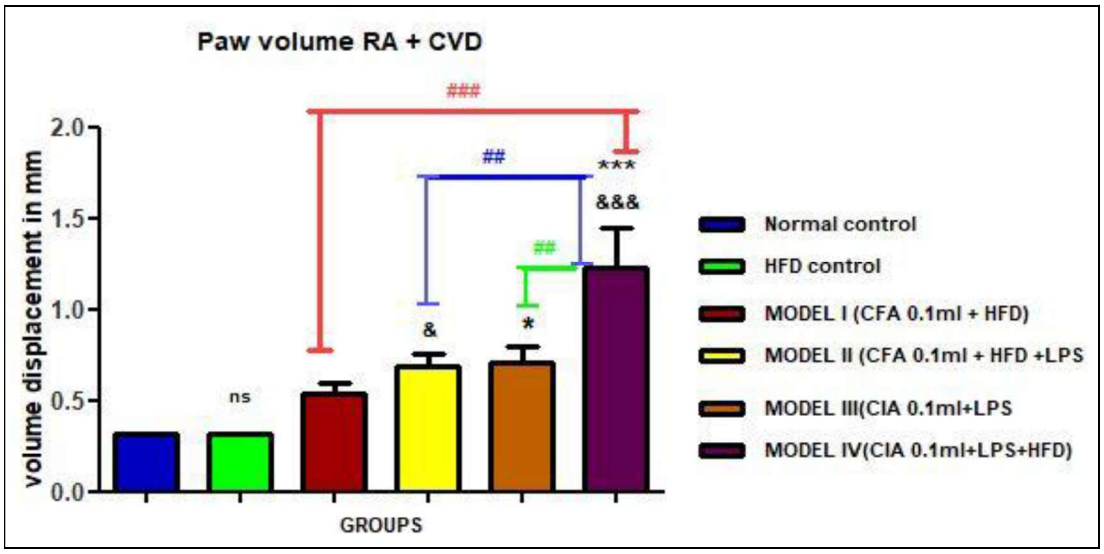
providing high fat diet and parameter such as **TG, TC, LDL, HDL TG, TC, LDL**, and **HDL** were assessed. As confirmatory indicators **atherogenic index** and **Cardiac Risk Ratio (CRR)** were selected to compare the progression of atherogenicity.

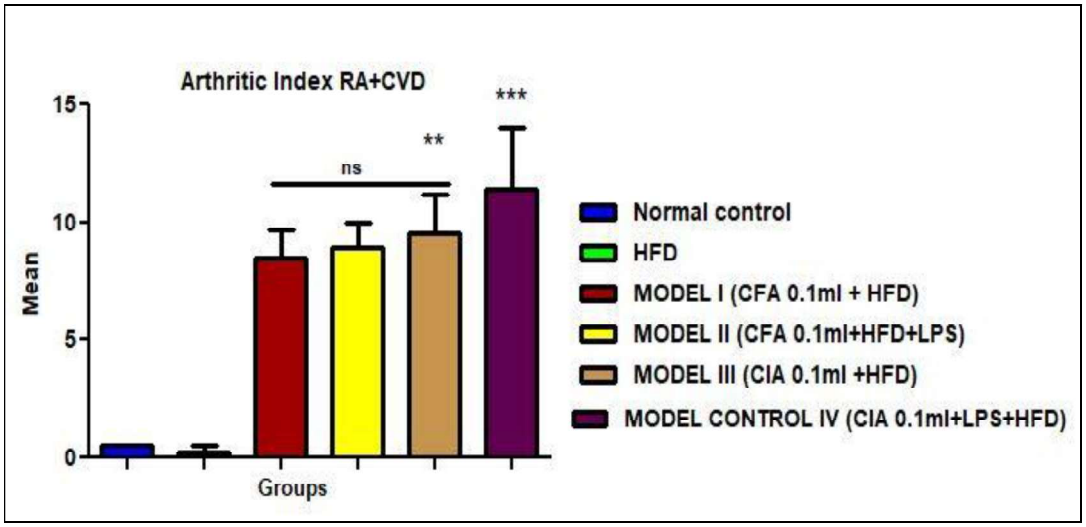
These groups were further taken for histopathological confirmation via cytological examination of **aorta, heart and thigh bone muscles; vastus medialis and biceps femoris** which plays an important role to give strength to skeletal muscles present in thigh and helps in coordination and movement. These skeletal muscles naturally get weaken as person aged but these muscles also found ruptured in some studies of osteoporosis as well as RA which participated in disability among the patients having long term disease onset. On the other hand fat deposition or metabolic dysbiosis in the form of hyperinsulinemia or adipose tissue deposition also plays important role in muscle atrophy[(103)]. On constant fat depositions these muscles loss their elasticity and start to break with pressure. This shortening of fibre like muscles when get torn by rupture they are unable to handle load which appeared as disability.

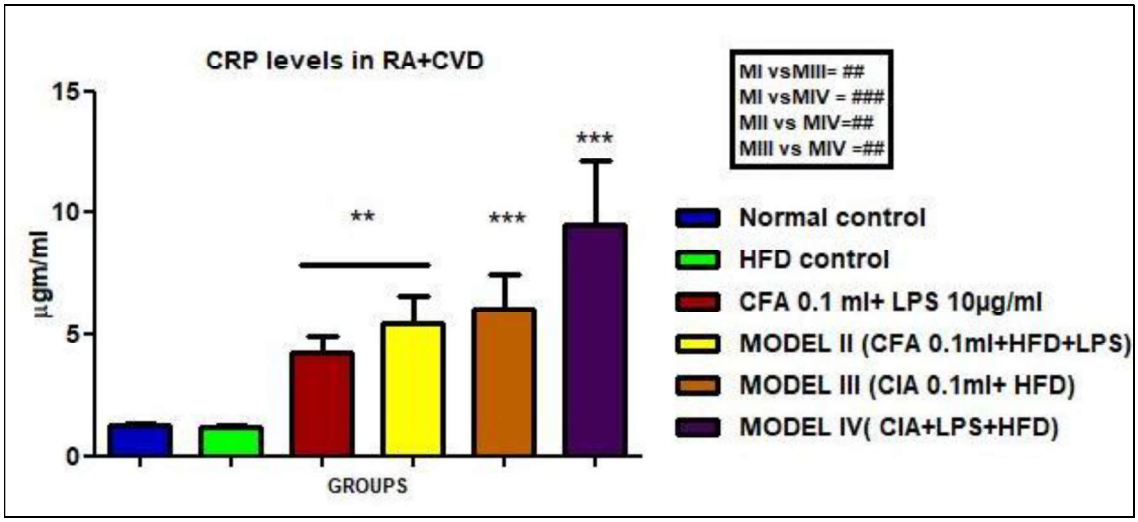
All the parameters for assessment of RA were performed on different time points for 28 days in CFA groups and 42 days in CIA groups respectively. These groups were further analyzed for the progression of Atherosclerosis on different time points using-

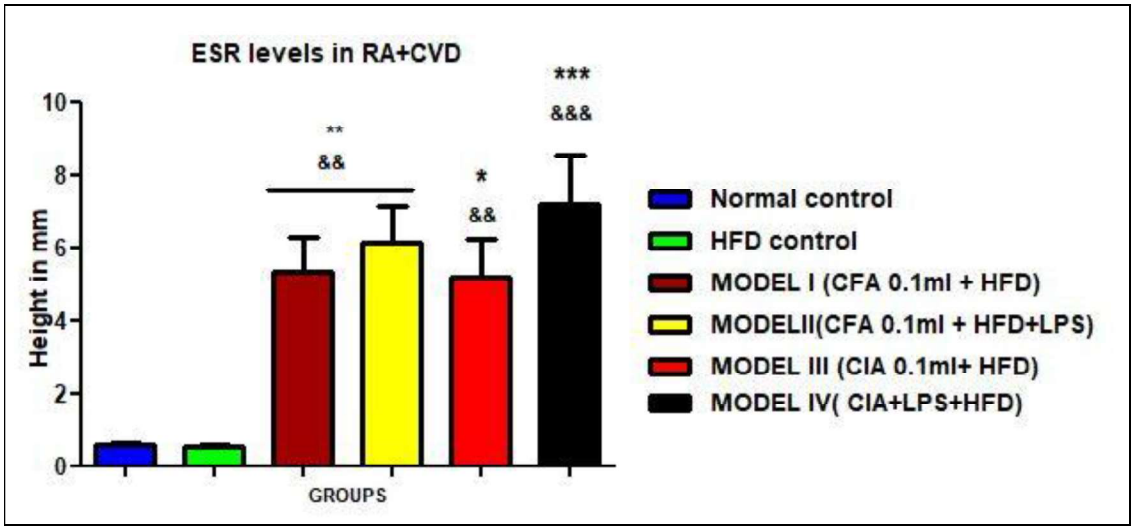
All the groups were compared statistically where graphical representation of data is depicted in fig. 5-20 and Mean±SEM data is given in succeeding table after comparing all the groups model IV (CIA 0.1ml + LPS 10µg/ml+ HFD) showed the higher development of atherogenic conditions in rats as compared to other groups which was significant in all the parameters evaluated for the developed model.

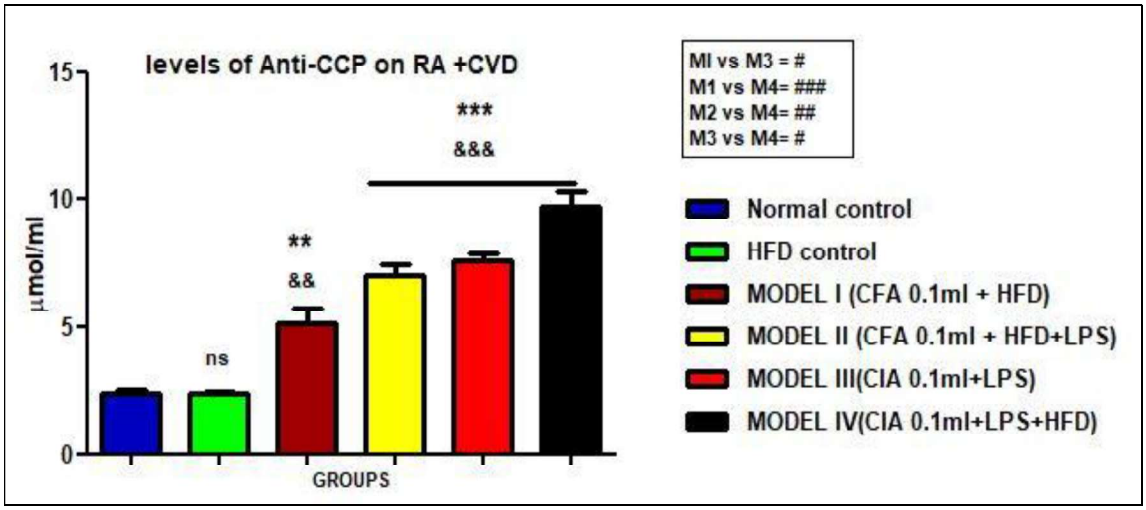

Effects of HFD on % Body weight of Animals
% Body weight
250
200
150
100
50
0
ns
***
ns
***
GROUPS
Normal control
High Fat Diet
Model I (CFA 0.1ml+ LPS10µg/ml)
Model II (Collagen 0.1ml +LPS 10µg/ml)
Model III (CFA 0.1ml +HFD+LPS 10µg/ml)
Model IV (Collagen 0.1ml +HFD+LPS 10µg/ml)

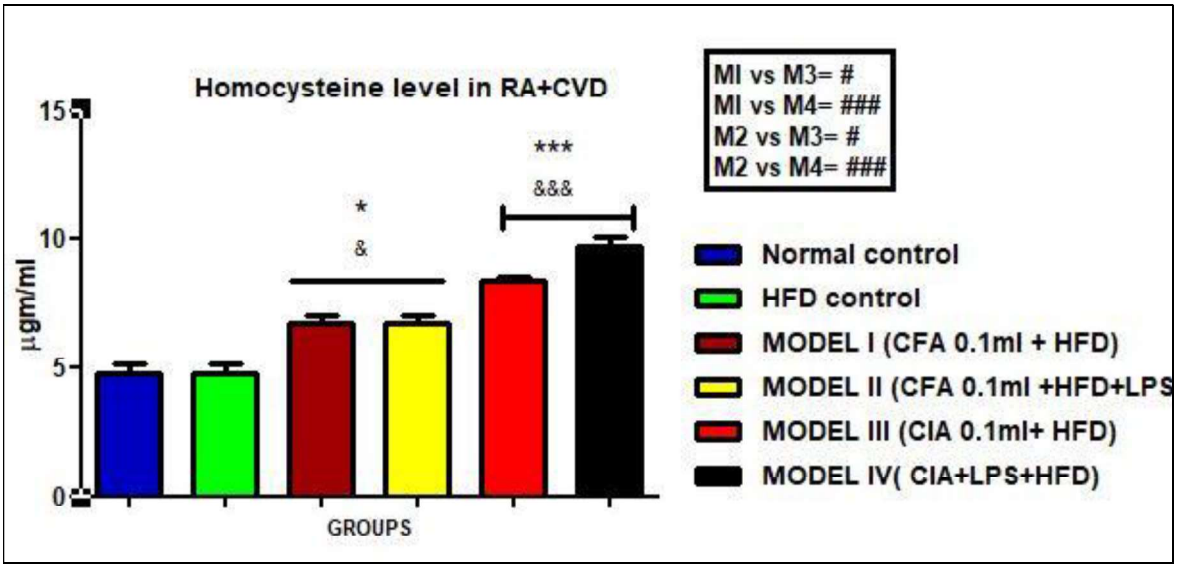

Paw volume RA + CVD
volume displacement in mm
2.0
1.5
1.0
0.5
0.0
###
##
***
&&&
##
&
*
ns
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml + HFD +LPS
MODEL III(CIA 0.1ml+LPS
MODEL IV(CIA 0.1ml+LPS+HFD)

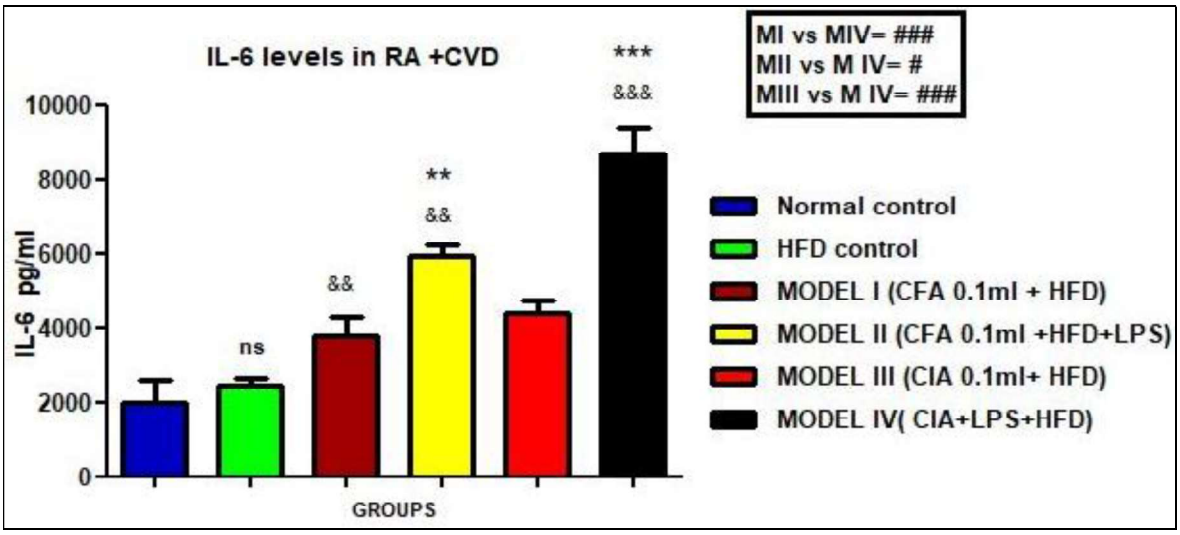

Arthritic Index RA+CVD
Mean
15
10
5
0
***
**
ns
Groups
Normal control
HFD
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml+HFD+LPS)
MODEL III (CIA 0.1ml +HFD)
MODEL CONTROL IV (CIA 0.1ml+LPS+HFD)

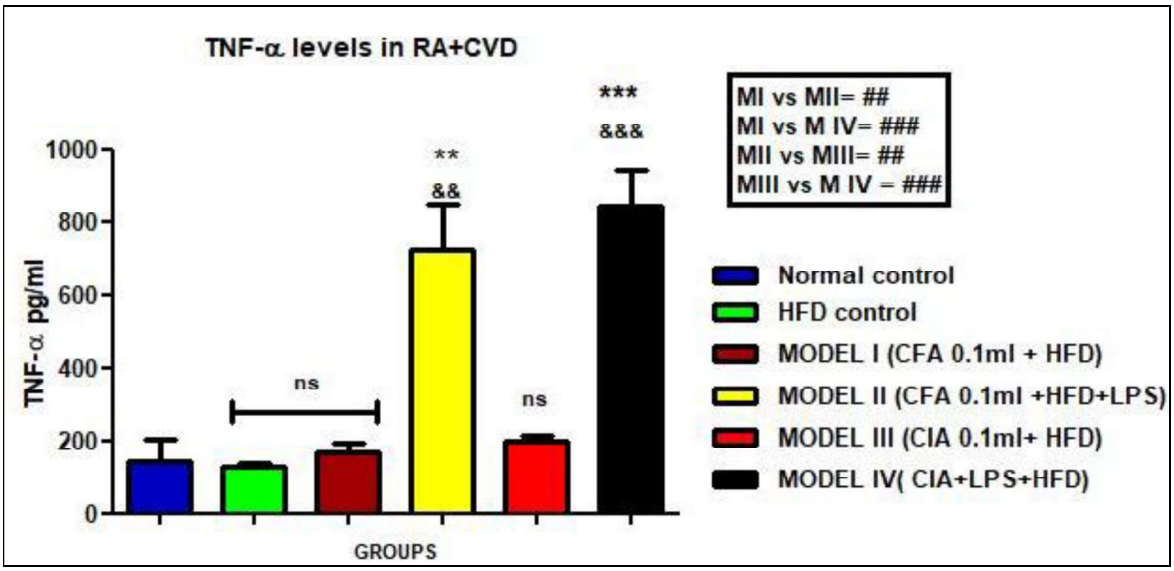

CRP levels in RA+CVD
µgm/ml
15
10
5
0
**
***
***
GROUPS
MI vsMIII= ##
MI vsMIV = ###
MII vs MIV=##
MIII vs MIV =##
Normal control
HFD control
CFA 0.1 ml+ LPS 10µg/ml
MODEL II (CFA 0.1ml+HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)

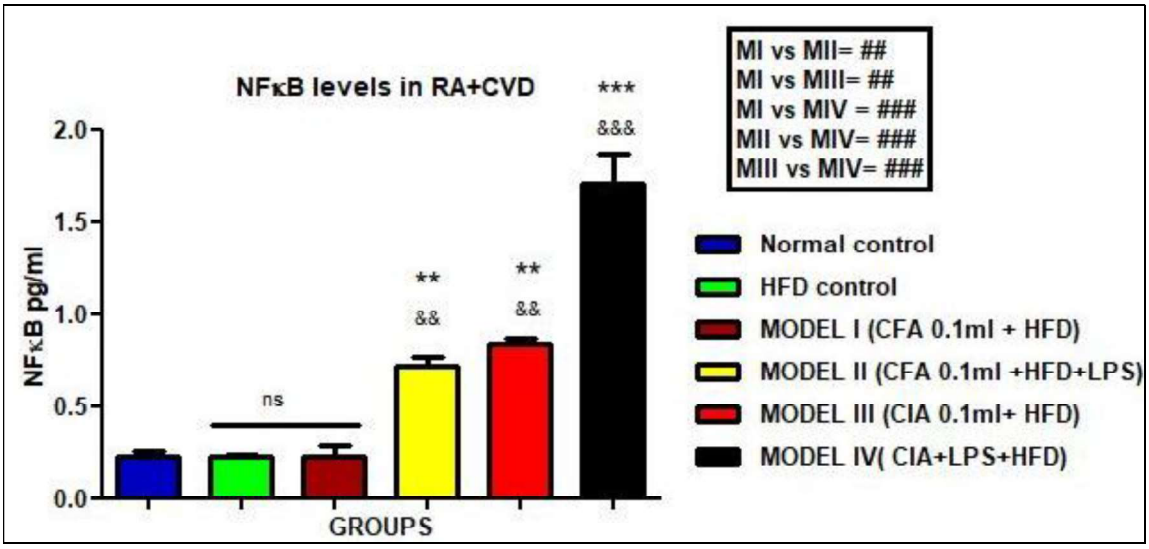

ESR levels in RA+CVD
Height in mm
10
8
6
4
2
0
**
&&
*
&&
***
&&&
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODELII(CFA 0.1ml + HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)

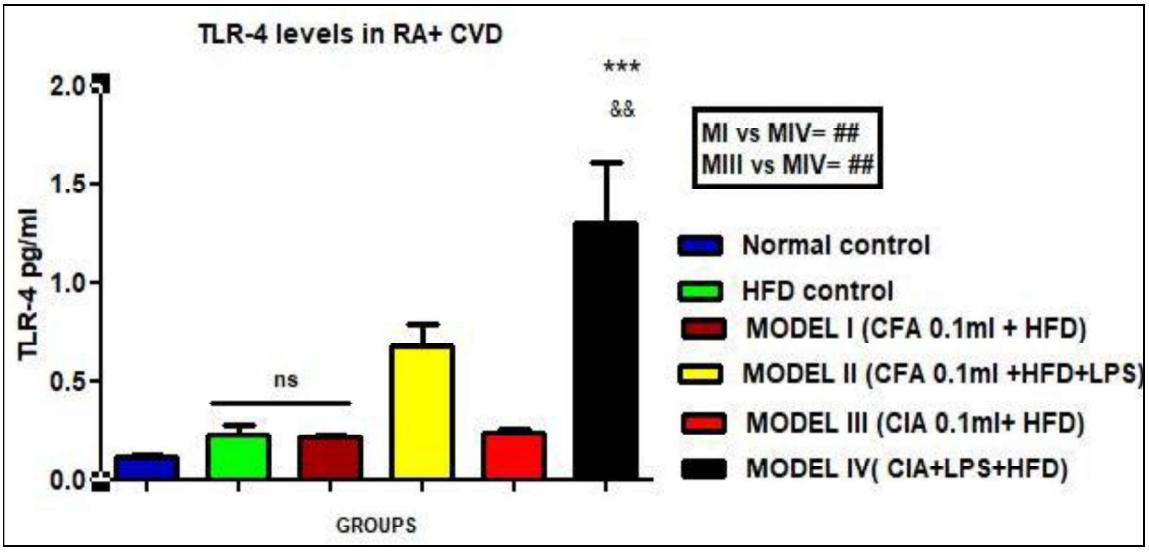

levels of Anti-CCP on RA +CVD
µmol/ml
15
10
5
0
ns
**
&&
***
&&&
GROUPS
MI vs M3 = #
M1 vs M4= ###
M2 vs M4= ##
M3 vs M4= #
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml + HFD+LPS)
MODEL III(CIA 0.1ml+LPS)
MODEL IV(CIA 0.1ml+LPS+HFD)

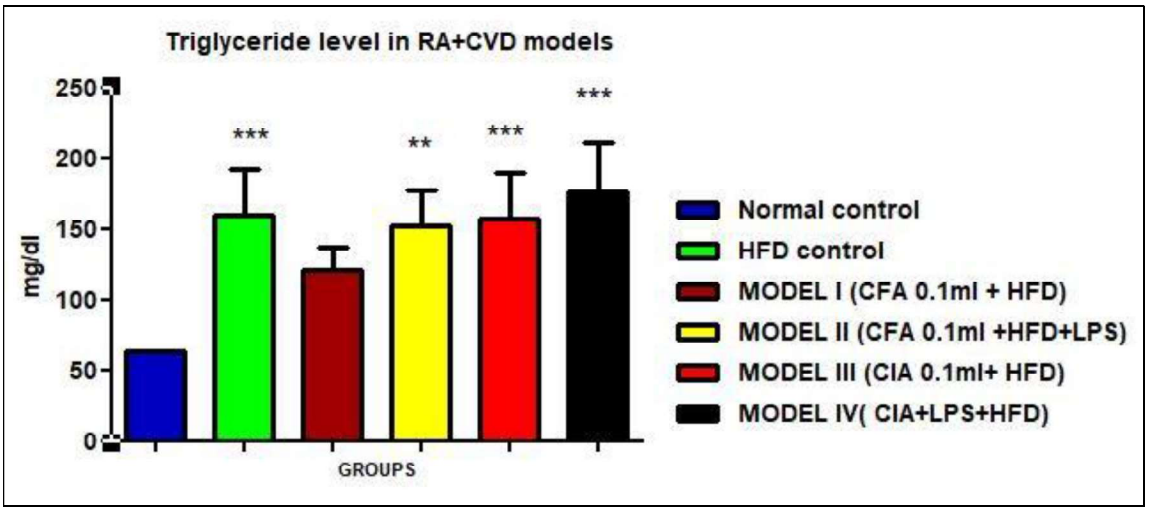

Homocysteine level in RA+CVD
MI vs M3= #
MI vs M4= ###
M2 vs M3= #
M2 vs M4= ###
µgm/ml
15
10
5
0
*
&
***
&&&
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)

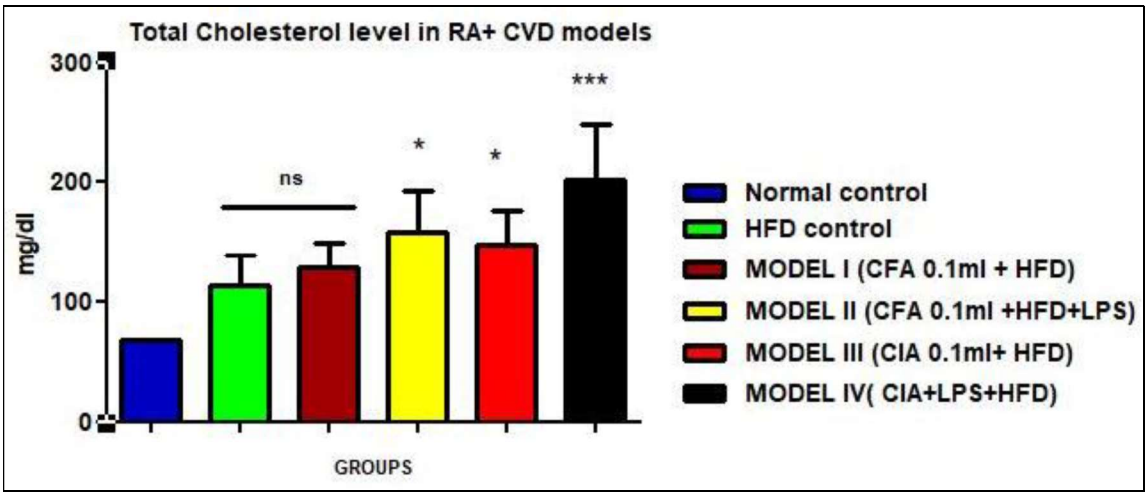

IL-6 levels in RA +CVD
MI vs MIV= ###
MII vs M IV= #
MIII vs M IV= ###
IL-6 pg/ml
10000
8000
6000
4000
2000
0
ns
&&
**
&&
***
&&&
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)

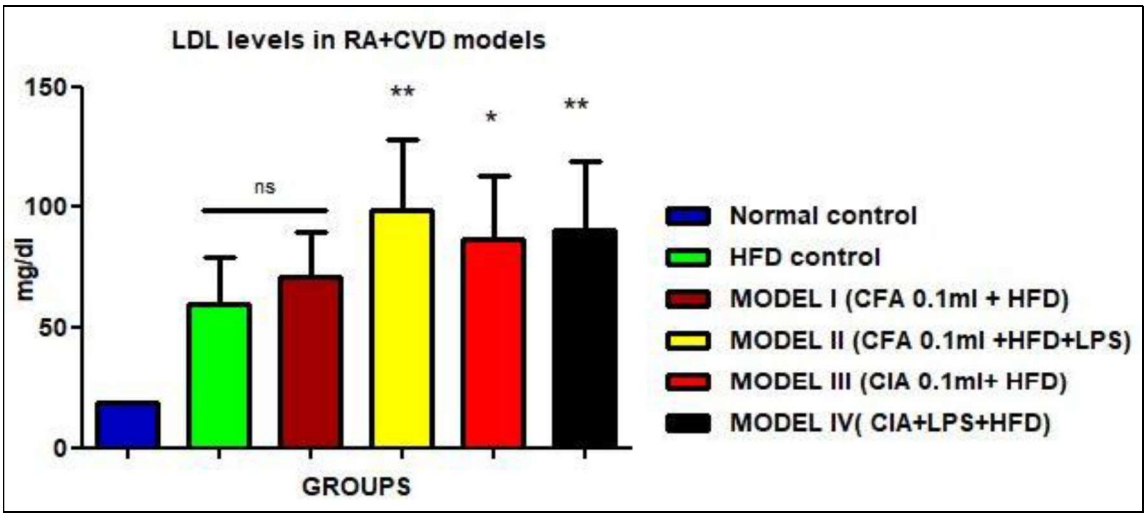

TNF-α levels in RA+CVD
MI vs MII= ##
MI vs M IV= ###
MII vs MIII= ##
MIII vs M IV – ###
TNF-α pg/ml
1000
800
600
400
200
0
ns
**
&&
ns
***
&&&
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)

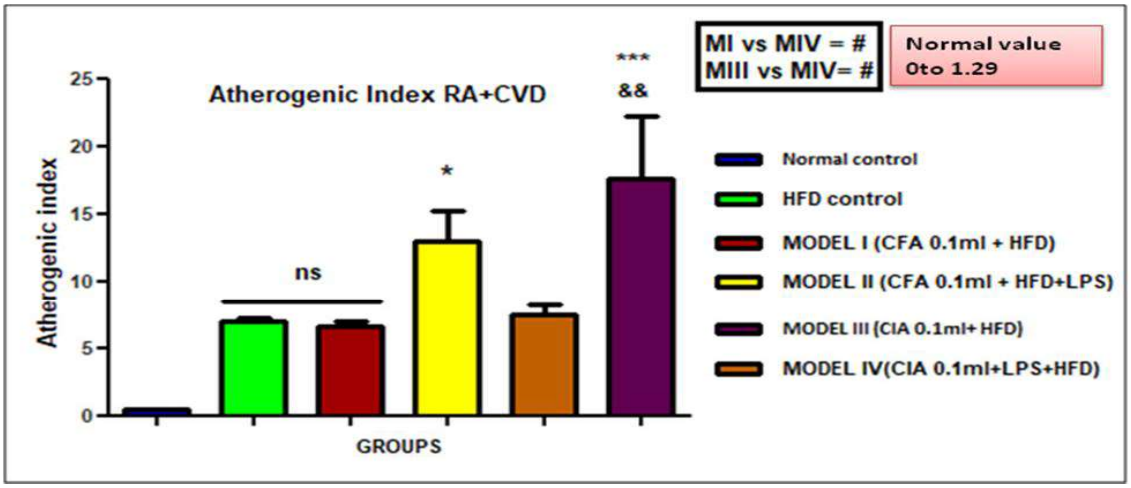

NFκB levels in RA+CVD
NFκB pg/ml
2.0
1.5
1.0
0.5
0.0
ns
**
&&
**
&&
***
&&&
GROUPS
MI vs MII= ##
MI vs MIII= ##
MI vs MIV = ###
MII vs MIV= ###
MIII vs MIV= ###
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)


TLR-4 levels in RA+ CVD
TLR-4 pg/ml
2.0
1.5
1.0
0.5
0.0
ns
***
&&
GROUPS
MI vs MIV= ##
MIII vs MIV= ##
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)


Triglyceride level in RA+CVD models
mg/dl
250
200
150
100
50
0
***
**
***
***
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)


Total Cholesterol level in RA+ CVD models
300
200
100
0
mg/dl
ns
*
*
***
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)


LDL levels in RA+CVD models
150
100
50
0
mg/dl
ns
**
*
**
GROUPS
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml +HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA+LPS+HFD)


Atherogenic Index RA+CVD
25
20
15
10
5
0
Atherogenic index
ns
*
***
&&
GROUPS
MI vs MIV = #
MIII vs MIV= #
Normal value
0to 1.29
Normal control
HFD control
MODEL I (CFA 0.1ml + HFD)
MODEL II (CFA 0.1ml + HFD+LPS)
MODEL III (CIA 0.1ml+ HFD)
MODEL IV(CIA 0.1ml+LPS+HFD)

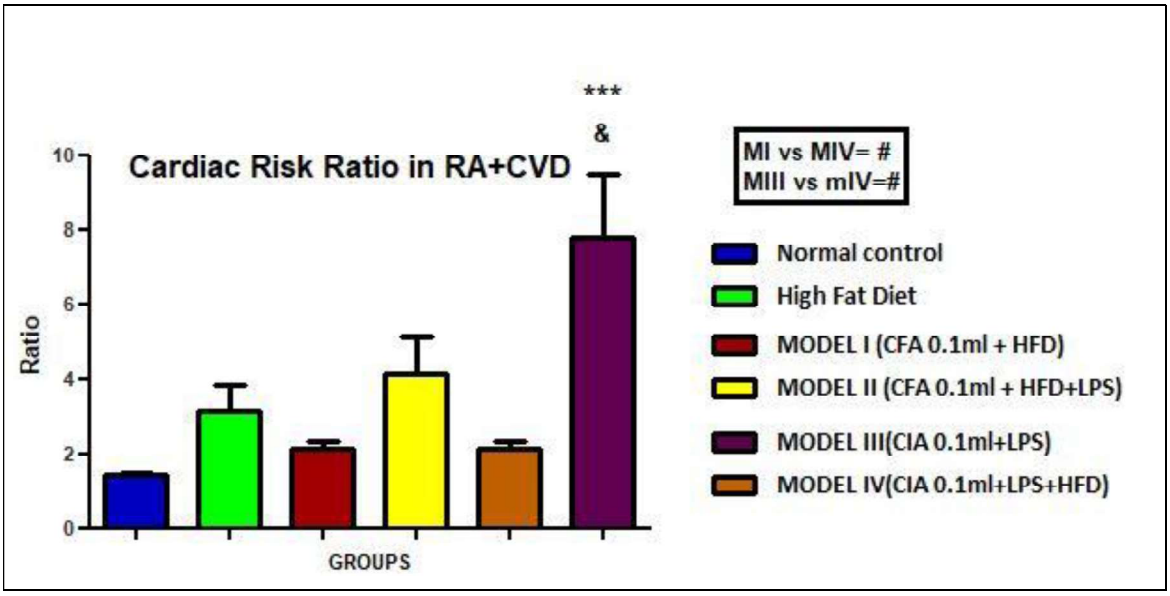


Fig. 5-20 Graphical representation of statistical evaluation for induction of RA with CVD

Values are expressed as Mean ± SEM. Values are statistically evaluated using repeated measure ANOVA (Paw volume) and one way ANOVA analysis followed by suitable post hoc test (Bonferroni's, Dunnett's and Tukey's). *, **, *** represent significant difference (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control groups with model controls. ns is non-significant differences between normal and groups,&, &&, &&& is comparison between high fat diet vs. other groups. @,@@,@@@ comparison between model control vs. other groups. #,##,### comparison between different models.

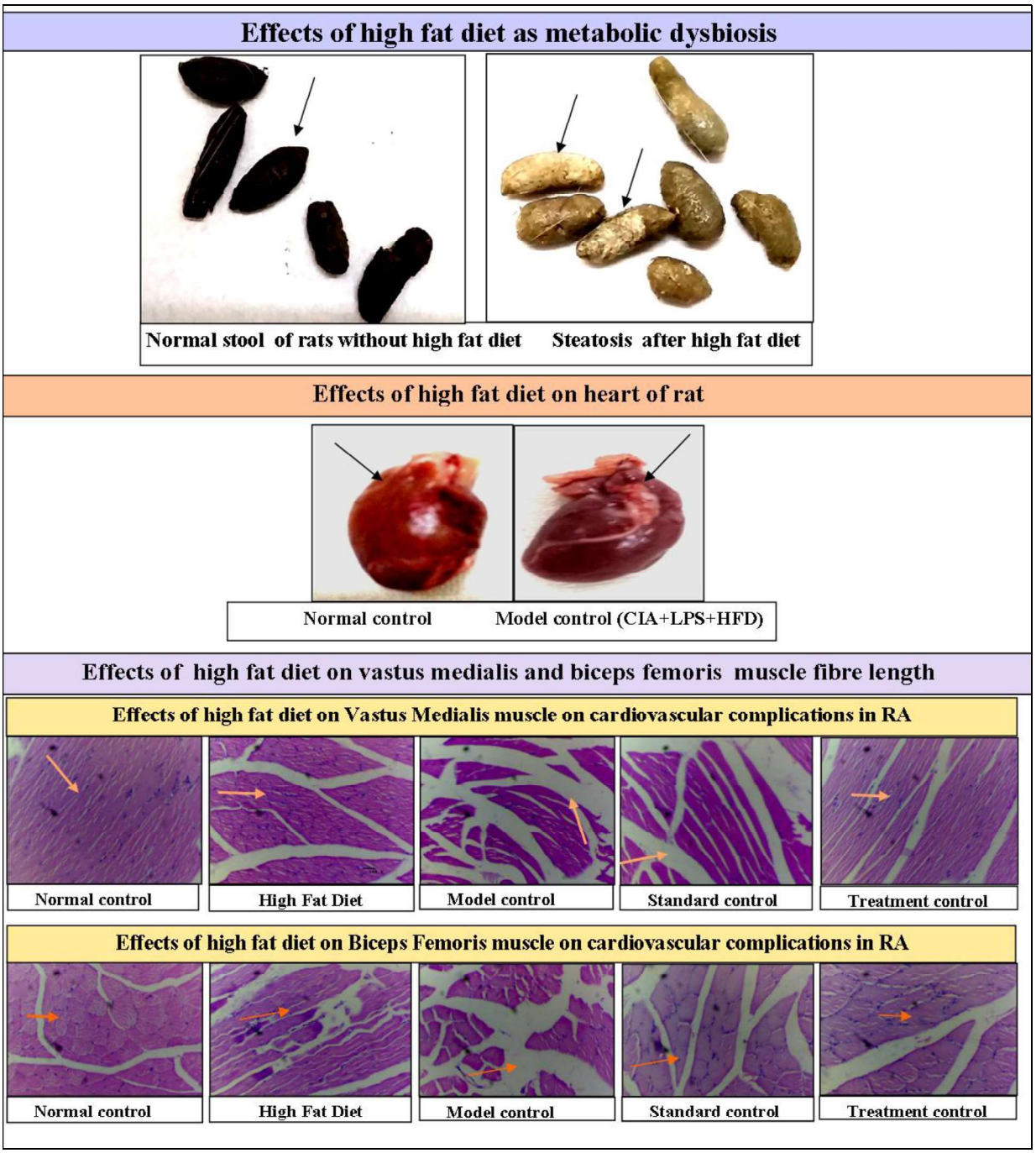


Fig. 5-21 Effects of high fat diet on initiation of atherosclerosis

Image 1- Shows the images of stool collection where steatosis in picture b which clearly shows the change in stool color due to excretion of fat in stool which indicates the metabolic dysbiosis.

Image 2- Shows the changes in heart morphology where in picture b model control group animal showed the fatty depositions on heart

Image 3- Shows the histopathological changes in fibre length of vastus medialis and biceps femoris muscles where normal control shows the intact muscle sections and in model and high fat diet groups there was some tear and shortness of muscle fibres.

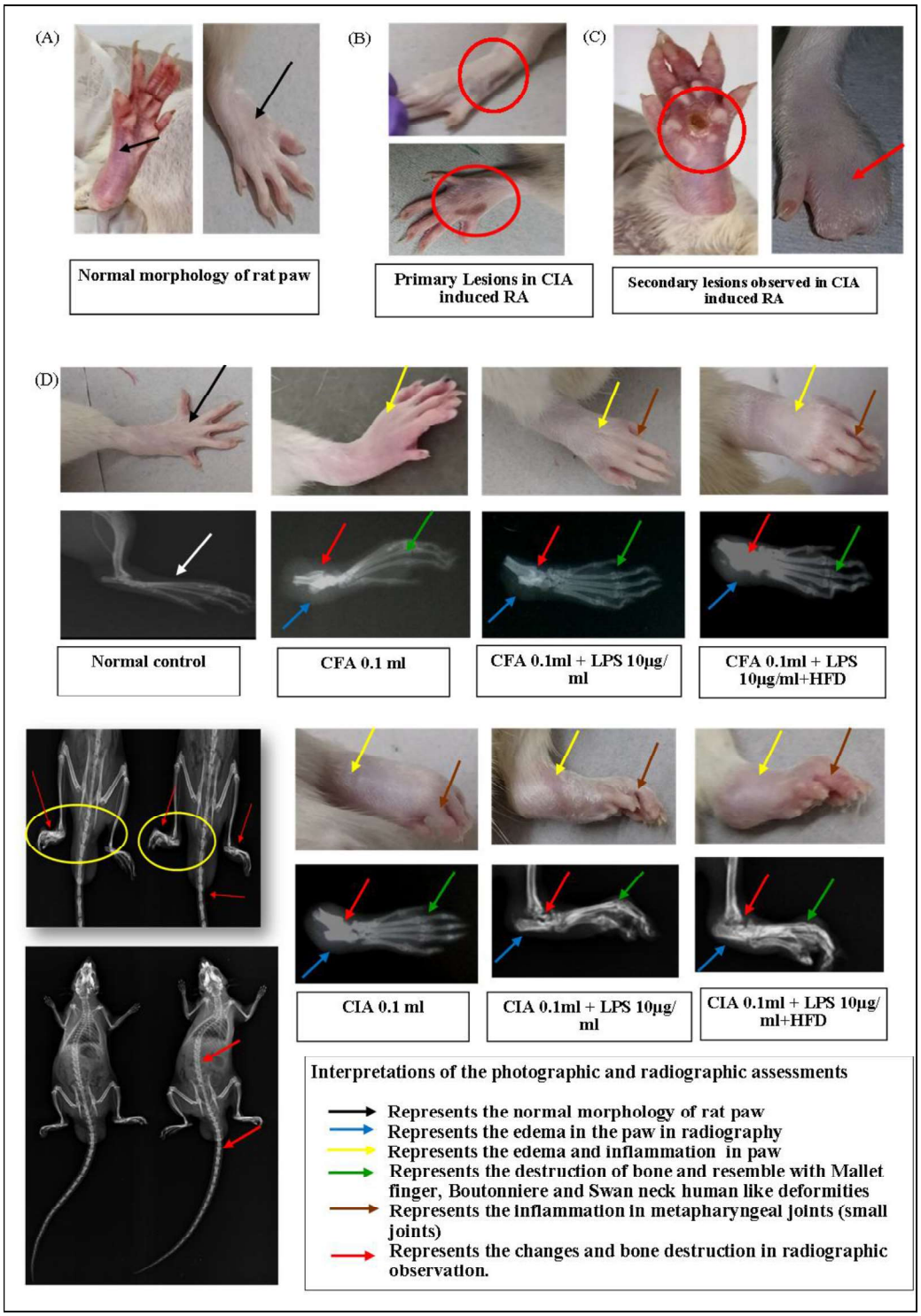


Fig. 5-22 Photographic and radiographic changes in inflammation, edema and bone erosion in RA+CVD groups
Image A shows the normal morphology of paw, B shows primary lesions, C shows secondary lesions in models
Image B shows inflammatory and bone erosions as photographic and radiographic sections of paw of different developed models

5.3 Results of Section III (Results of adopted model validation steps Model)

5.3.1 Results of Model validation on the basis of face validity constrict validity and predictive validity measures)

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 are used in preclinical *in- vivo* model designing in the current study replicates external and internal validity parameters. After designing models on adopted standardized methods the points of preclinical and clinical gaps were analyzed and rectified as effectively as possible.

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 statistic method.[124, 126]

5.3.2 Results of Primary validation methods to optimize external and predictive validity

The final selected Models from study 1 model I (CFA+ LPS10µg/ml) model II (CIA0.1ml+ LPS 10µg/ml), model III (CFA0.1ml+ HFD+ LPS) and model IV (CIA0.1ml+ HFD+ LPS) were carried out for optimization on the validity score given in Table 5-14.

The models were compared for all the criteria given in the scoring system. Each and every model was evaluated on these parameters for individual parameter and the final value was obtained as summation of score gained for each parameter. The final model were compared here with the normal control group to check the disease induction as compared to normal on different criteria where disease specific criteria were scored lowest as per the scoring system adopted. The following table represents the basic validity score obtained using this method.

Table 5-14 Validity scores for primary validation

Sr. no.	Groups	Parameter	Score	Criteria	Total score
1	Normal Control	All		All five criteria	66
2.	Model I	Paw volume	13	Species	142
		Arthritic score	12		
		Arthritic Index	13	Disease stimulation	
		ESR	14		
		CBC	14	Face Validity	
		CRP	13		
		Anti-CCP	13	Complexity	
		Homocysteine	13		
		IL-6	14	Predictivity	
		TNF-α	13		
		Lipid profile	09		
		RF-Factor	13		
3	Model II	All above	-	All above	148
4	Model III	All above	-	All above	**162**
5	Model IV	All above	-	All above	**181**

On the basis of above results the predictive values were obtained which are insufficient to give the conclusion which we were expecting in the present study. To get better insight these final selected models were again compared with FIMD.

5.3.3 Outcomes of FIMD for optimization and comparison for developed models for RA

Model I (CFA 0.1ml+LPS10µg/ml) and Model II (CIA 0.1ml+LPS10µg/ml) which were developed for RA, were compared here to select the best model with higher validity score which can fit in to clinical relevance. On the basis of these results, ***Model I (CFA 0.1ml+LPS 10µg/ml)*** secured ***moderately validated score 64%*** with ***highest uncertainty factor 36%*** and ***Model II (CIA 0.1ml+LPS 10µg/ml)*** secured ***highly validated score with 82%*** which shows the maximum clinical resemblance for RA with clinical situations. Moreover the ***uncertainty factor*** for this model was ***18%*** which gives the accounts of the domain which are not common. The **similarity factors** was also calculated between these two models which shows the points in domains where both the models having ***29% similar representations of RA.***

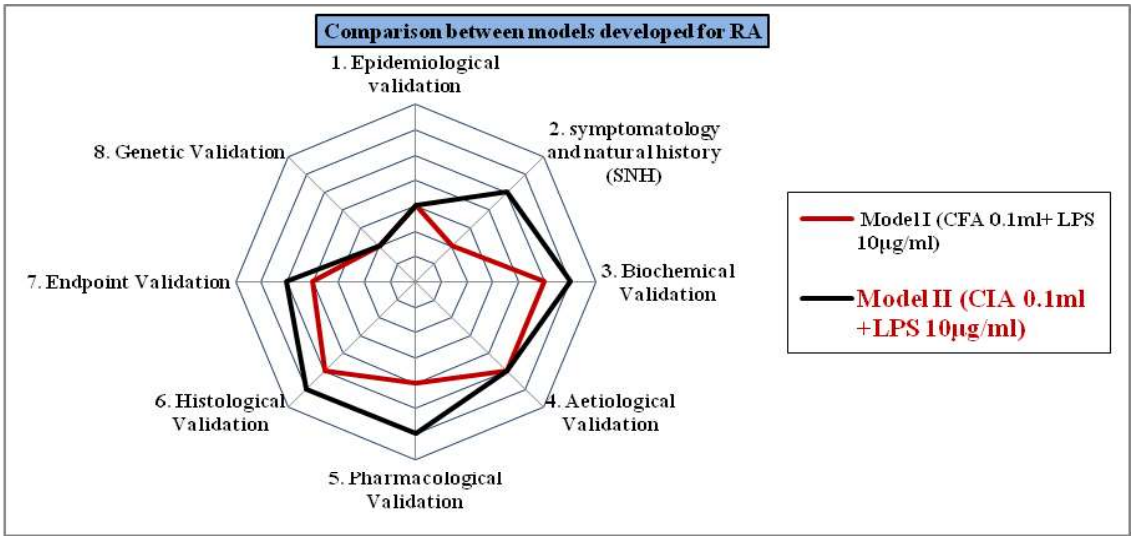


Fig. 5-23 Radar plot for comparison between models developed for RA

Table 5-15 Final scores of FIMD questionnaire for RA model

Validation	Model I CFA 0.1ml+LPS 10µg/ml	Model II CIA 0.1ml +LPS 10µg/ml+HFD
1. Epidemiological validation	0.3	0.3
2. symptomatology and natural history (SNH)	0.2	0.5
3. Biochemical Validation	0.5	0.6
4. Aetiological Validation	0.5	0.5
5. Pharmacological Validation	0.3	0.6
6. Histological Validation	0.5	0.6
7. Endpoint Validation	0.4	0.5
8. Genetic Validation	0.2	0.2
Similarity Factor	29%	
Uncertainty factor	36%	18%
Validation Score	64%	82%

5.3.3.1 Results of FIMD for optimization and comparison for developed models for RA alone model developed for cardiovascular complications in RA

The statistical data showed that there is a potential of developing cardiovascular complication in comparative groups; ***model I (CFA 0.1ml+LPS 10µg/ml), model II (CIA 0.1ml+LPS 10µg/ml) model III (CFA 0.1 ml+LPS 10µg/ml +HFD)*** and ***model IV (CIA 0.1 ml+LPS 10µg/ml +HFD)*** which were compared for optimizing the interconnecting parameters which are responsible for progression of cardiovascular complications in existing RA. In final comparison, ***model IV (CIA 0.1 ml+LPS 10µg/ml +HFD)*** secured ***higher validation score of 95%*** and ***uncertainty factor*** was ***5%*** which proves that, ***model IV*** has ***maximum resemblance with clinical situations.*** All these four models were having some similar domains as similarity factor among all the groups is ***18%.***

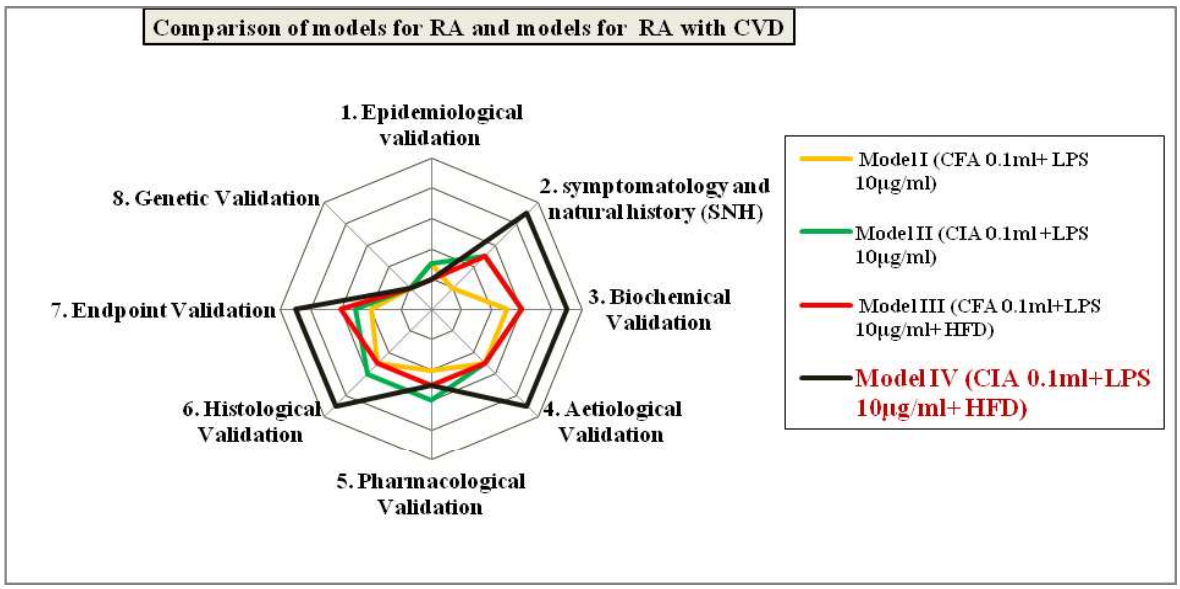


Fig. 5-24 Radar plot for comparison between models developed for RA and RA with CVD

Table 5-16 Final scores of FIMD questionnaire for RA models and RA with CVD models

Validation	Model I (CFA 0.1ml+ LPS 10µg/ml)	Model II (CIA 0.1ml +LPS 10µg/ml)	Model III (CFA 0.1ml+LPS 10µg/ml+ HFD)	Model IV (CIA 0.1ml+LPS 10µg/ml+ HFD)
1. Epidemiological validation	**0.3**	**0.3**	**0.2**	**0.2**
2. symptomatology and natural history (SNH)	**0.2**	**0.5**	**0.5**	**0.9**
3. Biochemical Validation	**0.5**	**0.6**	**0.6**	**0.9**
4. Aetiological Validation	**0.5**	**0.5**	**0.5**	**0.9**
5. Pharmacological Validation	**0.4**	**0.6**	**0.5**	**0.5**
6. Histological Validation	**0.5**	**0.6**	**0.5**	**0.9**
7. Endpoint Validation	**0.4**	**0.5**	**0.6**	**0.9**
8. Genetic Validation	**0.2**	**0.2**	**0.2**	**0.2**
Interpretations from radar values				
Uncertainty Factor	**36%**	**18%**	**12%**	**5%**
Similarity Factor			**18%**	
Validation Score	**64%**	**82%**	**88%**	**95%**

5.3.3.2 Final Comparison for novel, optimized and validated model of RA with associated co- morbid cardiovascular complications with maximum human resemblance

The final comparison was done for optimizing a single model which can mimic the clinical symptoms of cardiovascular complications in patients suffering with RA. The objective was completed by comparison between the representative ***model of RA (CIA 0.1ml+LPS 10µg/ml)*** with representative model of RA with CV complications ***(CIA 0.1 ml+LPS 10µg/ml +HFD)*** and the models represents ***5% of similarities*** which show the interconnection between both the diseases in terms of disease progression.

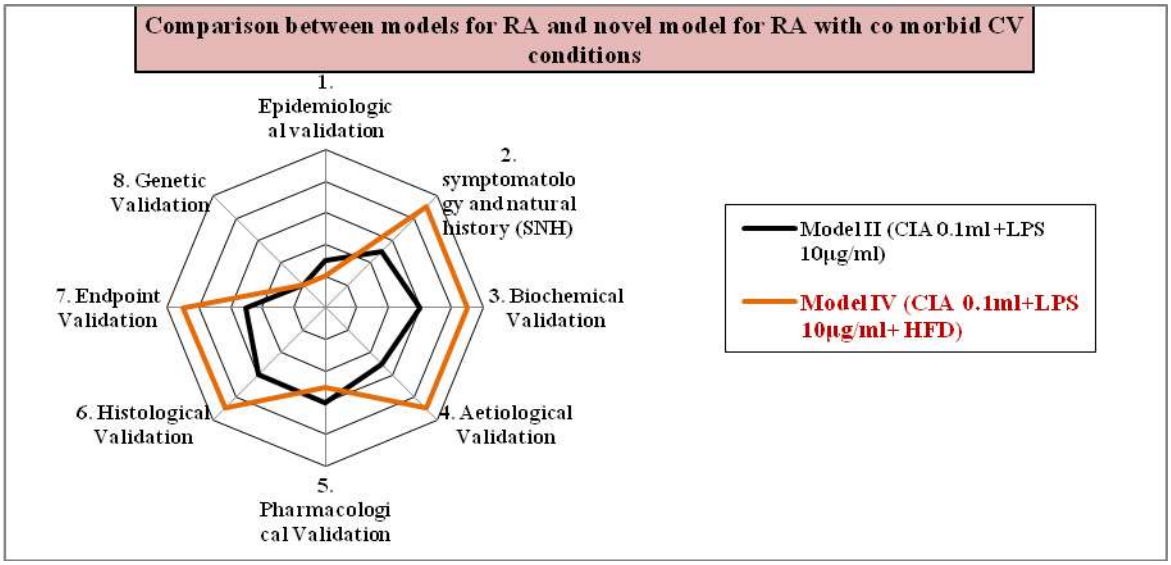


Fig. 5-25 Radar plot for comparison between final models developed for RA and RA with CVD

Table 5-17 Final comparison for RA models and RA with CVD models for model validation

Validation	Model II (CIA 0.1ml +LPS 10µg/ml)	Model IV (CIA 0.1ml+LPS 10µg/ml+ HFD)
1. Epidemiological validation	0.3	0.2
2. symptomatology and natural history (SNH)	0.5	0.9
3. Biochemical Validation	0.6	0.9
4. Aetiological Validation	0.5	0.9
5. Pharmacological Validation	0.6	0.5
6. Histological Validation	0.6	0.9
7. Endpoint Validation	0.5	0.9
8. Genetic Validation	0.2	0.2
Uncertainty Factor	18%	5%
Similarity Factor	14%	
Validation Score	82%	95%

5.4 Results of section IV

5.4.1 Comparison of NSAE, CPAE and MCAE in validated model of Rheumatoid Arthritis:

Model developed for RA were developed with two different inducing agents; CFA and Collagen with LPS as a secondary inducing agent. The findings of the study suggested that, Model II (CIA 0.1ml +LPS 10µg/ml) represents the highest disease induction in physical, perceptive and biochemical assessments. As per the above observation the selected tests drugs; aqueous extract of NSAE, CPAE and MCAE were evaluated for three different doses in this validated models. The results are depicted graphically in following fig. and after analysis of the best suitable doses for all three test components the final doses are evaluated for final selected model of cardiovascular complications in RA.

The data were compared for Normal control group, Model control group, standard control group and nine groups for tests (100, 200 and 400mg/kg doses) for NSAE, CPAE and MCAE. These results were compared using one way ANOVA and repeated measure ANOVA using GraphPad Prism.

5.4.1.1 Effects of NSAE, CPAE and MCAE on Paw volume in Model of Rheumatoid Arthritis

Paw volume is one of the major criteria for assessment of inflammation and edema in the animals for evaluation of RA. The Paw edema of animals of ***Model control (2.11±0.45)*** was constantly increased throughout the study period. ***Standard control*** animals were treated with MTX 0.6mg/kg doses weekly and they also showed gradual increase in paw volume in study period which was significantly different ***(1.75±0.45)*** as compared to treatment groups. NSAE treated groups, (Test I, II and III) showed the decrease in Paw volume where ***Test II (NSAE 200mg/kg)*** was significantly effective on paw volume ***(0.32±0.002)*** as compared to other two doses. Similarly in CPAE treated groups Test IV, V and VI were analyzed for 100, 200 and 400mg/kg doses. In this comparison ***Test IV (CPAE 100mg/kg)*** was significant among all other groups ***(0.32±0.01)*** for lowering the inflammation and edema as paw volume. MCAE treated

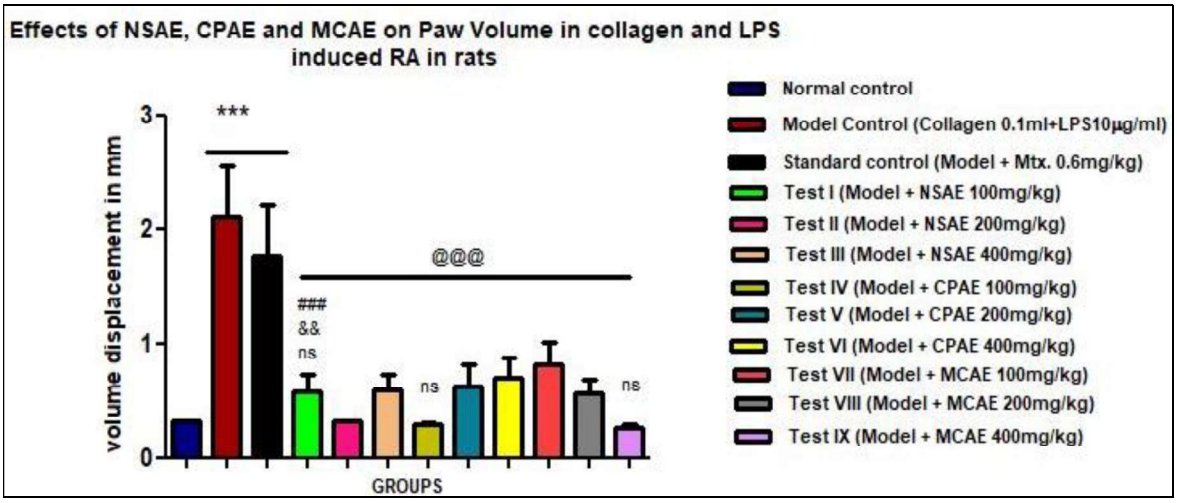
groups were designated as Test VII, VIII and IX, among which ***Test IX (MCAE 400mg/kg)*** group proved to decrease paw edema more effectively ***(0.31±0.01)*** than other doses.

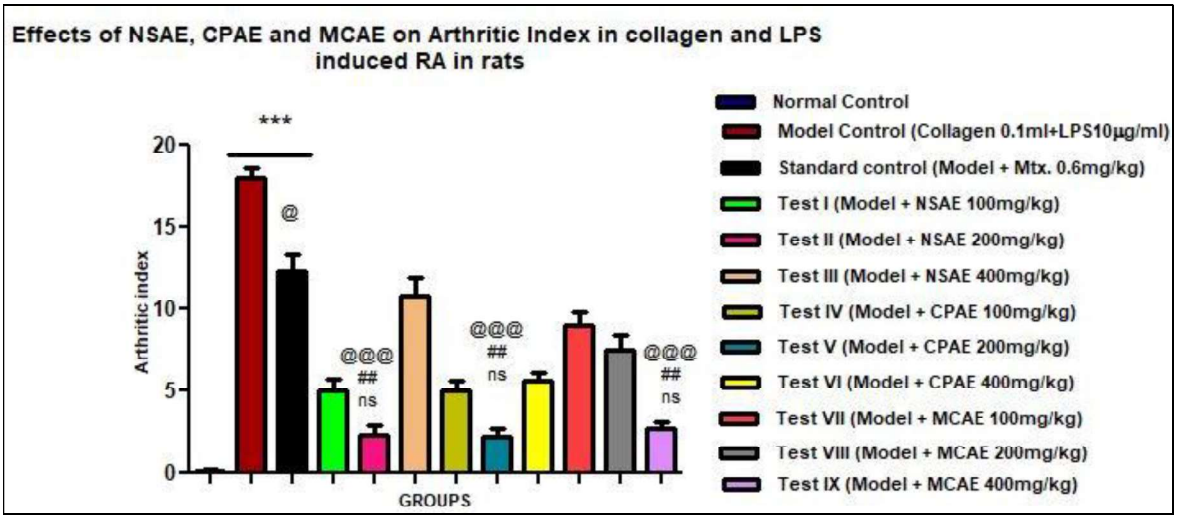
5.4.1.2 Effects of NSAE, CPAE and MCAE on Arthritic index in Model of Rheumatoid Arthritis

Arthritic index in ***model control*** group was high ***(18±0.57)*** as compared to ***standard control (12.3±0.95)*** and test groups. The arthritic score of preventive treatment of ***NSAE 200mg/kg (2.33±0.55), CPAE 100mg/kg (05±0.57) and MCAE 400mg/kg (2.6±0.42)*** group was significantly lower than the other doses and it was found significant from model control and standard group.

5.4.1.3 Effects of NSAE, CPAE and MCAE on Arthritic Score in Model of Rheumatoid Arthritis

Preventive treatment of NSAE CPAE and MCAE against collagen and LPS induced RA showed the significant difference from day 5 and 21. On day 5 the ***model control group (7.1±0.6)*** showed the primary lesions and secondary lesions, wounds and severe inflammation were appeared on day 21***(9±0.46)***. Primary lesions of ***standard control*** were disappeared on day 21 but the inflammation was significantly high ***(7.3±0.34)***. In treatment control groups primary lesions were seen which were less severe than model control groups and these lesions were healed on day 21. When comparison was done between three doses for all three ***test drugs, NSAE 200mg/kg (01±0.40), CPAE 100mg/kg (1.3±0.61) and MCAE 400mg/kg (0.83±0.40)*** showed significant decrease in arthritic score as compared to other test groups in dose dependent manner.





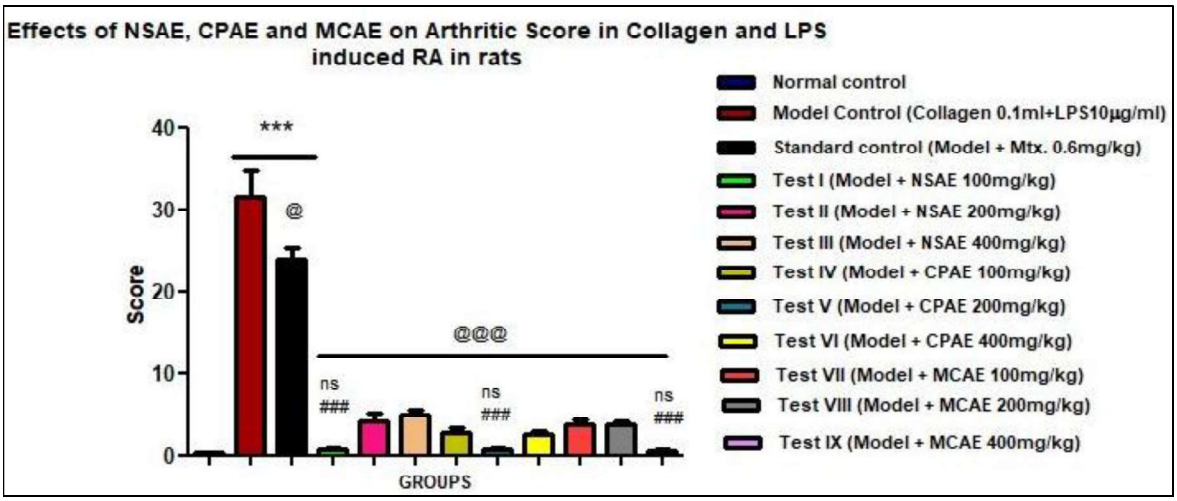


Fig. 5-26 Effects of NSAE, CPAE and MCAE on (A) Paw volume (B) Arthritic index (C) Arthritic score in collagen and LPS induced RA in rats

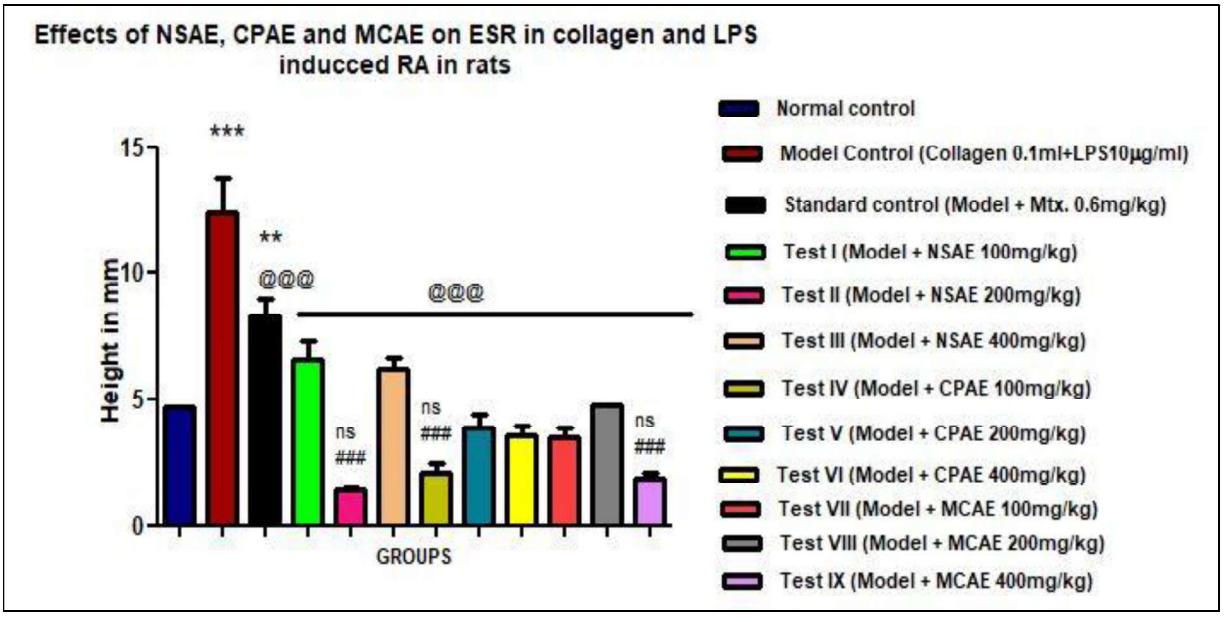
Values are expressed as Mean ± SEM. Values are statistically evaluated using one way ANOVA analysis *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) comparison between normal control and other groups. @, @@, @@@ is comparison between model and other groups, #,##,### comparison between standard and test groups, ns is no significance between normal and other groups.

5.4.1.4 Effects of NSAE, CPAE and MCAE on ESR in collagen and LPS induced RA in rats

Erythrocyte Sedimentation Rate is again one of the factors for inflammatory responses. In this study ***NSAE 200mg/kg (1.42±0.06), CPAE 100mg/kg (2.09±0.32), and MCAE 400mg/kg (1.88±0.19)*** showed the highest effect on ESR levels as compared to other treatment doses. ESR was in decreasing trends after day 7 in all the groups except ***model control group*** (**12.3±1.35**) which showed highest rates of ESR throughout the study period which was significant from normal control and test groups. The ***standard treatment group (8.3±0.68)*** also showed the increase in ESR levels when compared with ***normal control (4.6±0.01)*** and test groups.

5.4.1.5 Effects of NSAE, CPAE and MCAE on CRP levels in Collagen and LPS induced RA in rats

CRP is major marker for progression of inflammatory cytokine in body. Here ***model control*** groups sanitized with collagen and LPS showed the higher levels of CRP ***(7.9±0.99)*** as compared to ***normal control (2.2±0.02)*** and test control groups. The preventive treatment of ***NSAE 200mg/kg (2.2±0.01), CPAE 100mg/kg (2.2±0.02) and MCAE 400mg/kg (2.2±0.07)*** showed the highest decrease in CRP levels as compared to other test doses as well as ***standard control*** group ***(6.3±0.75)***.



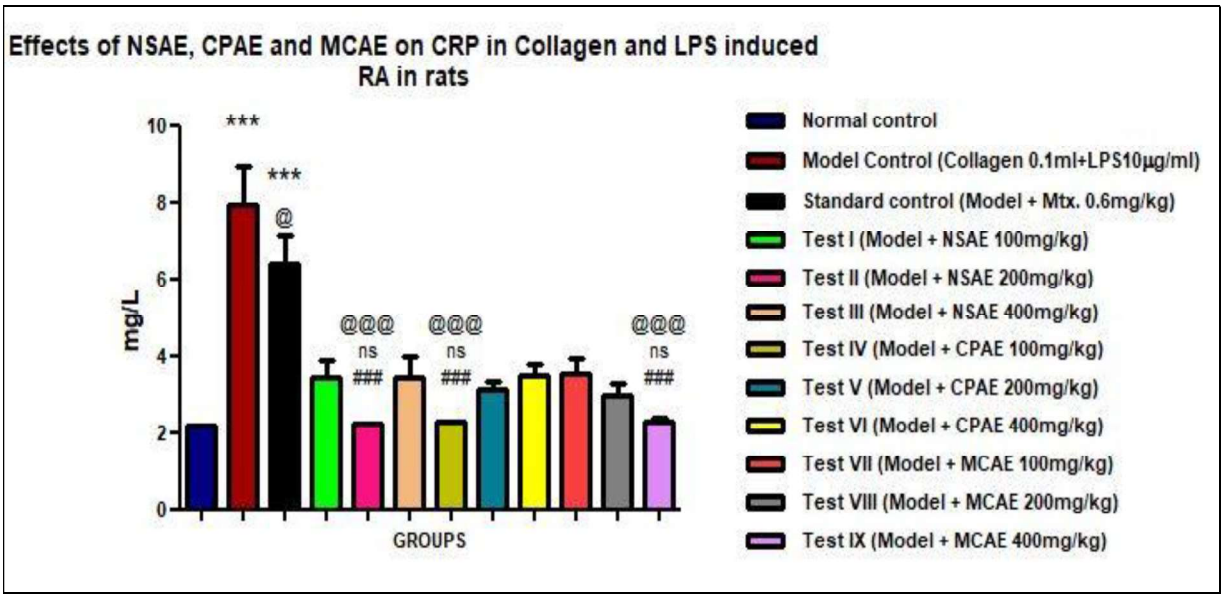


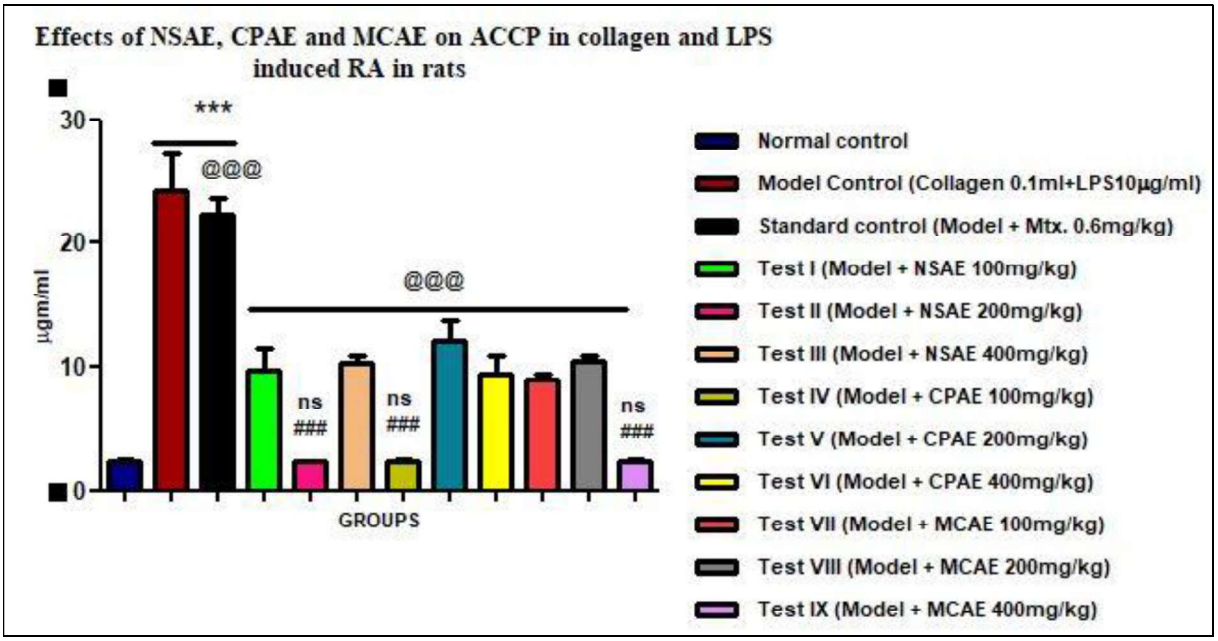
Fig. 5-27 Effects of NSAE, CPAE and MCAE on (A) ESR levels (B) CRP levels in collagen and LPS induced RA in rats Values are expressed as Mean ± SEM. Values are statistically evaluated using one way ANOVA analysis *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) comparison between normal control and other groups. @, @@, @@@ is comparison between model and other groups, #, ##,### comparison between standard and test groups, ns is no significance between normal and other groups.

5.4.1.6 Effects of NSAE, CPAE and MCAE on ACCP in collagen and LPS induced RA in rats

Anti-CCP is one of the markers for differentiating between RA and osteoarthritis. The levels of ACPA in ***model control (24.2±3.01)*** and ***standard control (22.2±1.48)*** were significantly high as compared to ***normal (2.38±0.15)*** and treatment groups. The elevation in ACPA showed that RA was generated in the animals and the preventive treatments of ***NSAE (2.38±0.02), CPAE (2.38±0.13) and MCAE (2.34±0.12)*** were effective to prevent the generation of ACPA in animals as the results were significant as compared to model and standard treatment.

5.4.1.7 Effects of NSAE, CPAE and MCAE on RF in collagen and LPS induced RA in rats

Rheumatoid factor is one of the markers which represent the immunological intervention in progression of RA. Here the ***NSAE 200mg/kg (2.73±0.37), CPAE 100mg/kg (4.93±0.40), MCAE 400mg/kg (2.5±0.20)*** treated groups showed the significant decrease in RF as compared to ***model control (13.6±0.88)*** and ***standard control*** group ***(7.43±0.33)*** when compared with ***normal control*** group ***(1.13±0.06).***



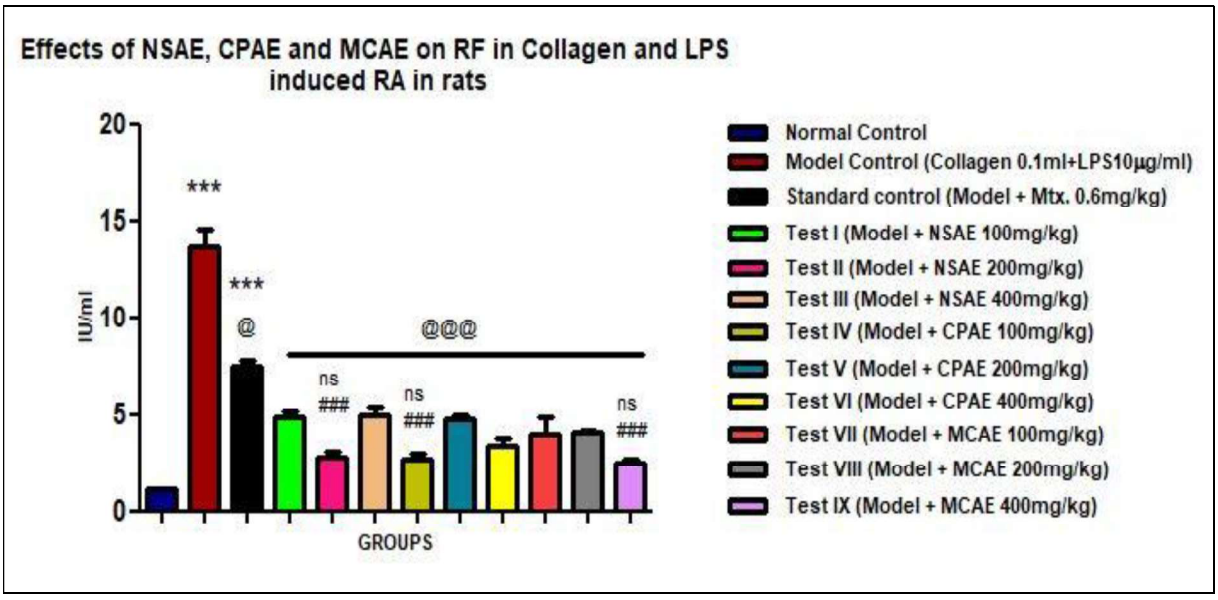


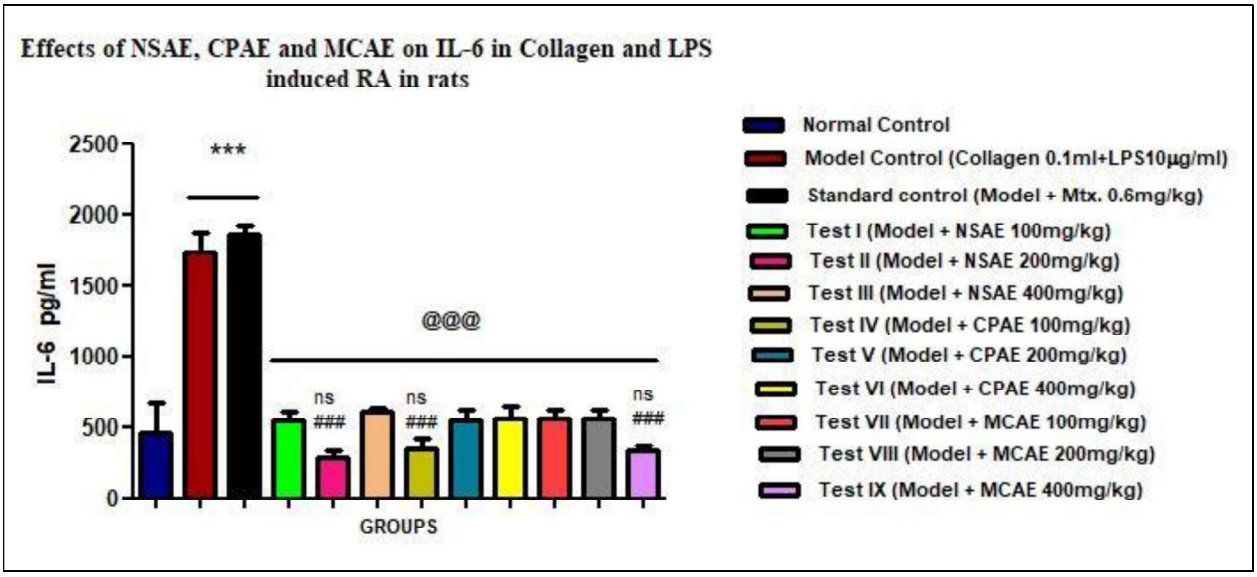
Fig. 5-28 Effects of NSAE, CPAE and MCAE on (A) ACCP levels (B) RF levels in collagen and LPS induced RA in rats Values are expressed as Mean ± SEM. Values are statistically evaluated using one way ANOVA analysis *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) comparison between normal control and other groups. @, @@, @@@ is comparison between model and other groups, #,##,### comparison between standard and test groups, ns is no significance between normal and other groups.

5.4.1.8 Effects of CPAE on Interleukin-6 in collagen and LPS induced RA in rats

IL-6 is immunological markers which are highly expressed in synovial membrane in response to immunological insult. Here IL-6 highly activated in the ***model control*** group ***(1733±140)***. Treatment groups of ***NSAE 200mg/kg (287±40), CPAE 100mg/kg (350±72) and MCAE 400mg/kg (328±36)*** and ***standard treatment*** group Mtx. 0.6mg/kg showed ***(1858±65)*** the elevation in IL6 and among all the treatment groups, CPAE 100mg/kg treated animals showed the maximum effect on IL-6 levels which were found to be decreased after preventive treatment when compared to ***normal control group (462±207)***.

5.4.1.9 Effects of CPAE on TNF-α in collagen and LPS induced RA in rats

Tumor Necrosis Factor alpha was found to be expressed in higher levels in ***model control*** group ***(725±125)*** as compared to ***normal control*** groups ***(175±28.8)*** and the effects of treatment group were high on TNF-α level. The maximum effect was seen in ***NSAE 200mg/kg (180±39), CPAE 100mg/kg (175±28.14) and MCAE 400mg/kg (153±19)*** treated group as compared to model control and ***standard control*** groups ***(526±85)***.



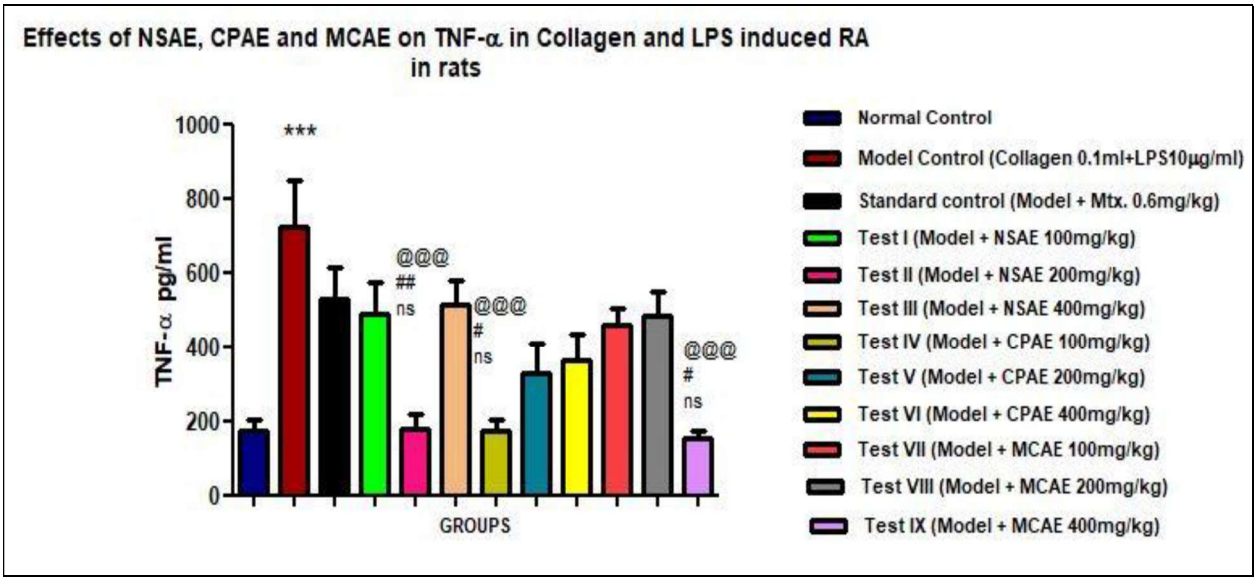


Fig. 5-29 Effects of NSAE, CPAE and MCAE on (A) IL-6 levels (B) TNF-α levels in collagen and LPS induced RA in rats
Values are expressed as Mean ± SEM. Values are statistically evaluated using one way ANOVA analysis *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) comparison between normal control and other groups. @, @@, @@@ is comparison between model and other groups, #,##,### comparison between standard and test groups, ns is no significance between normal and other groups.

Table 5-18 Statistical values of evaluations (Mean±SEM)

	Normal		**HFD**		**Model**		**Std**		**T1 (NSAE)**		**T2 (CPAE)**		**T3(MCAE)**	
ACPA	2.38±0.15		2.38±0.13		24.2±3.01		22.2±1.48		2.38±0.02		2.38±0.13		2.34±0.12	
AI	00±00		00±00		18 ±0.57		12.3±0.95		2.33±0.55		5±0.57		2.6±0.42	
AS	Day 5	Day 21	Day 5	Day 21	Day 5	Day 21	Day 5	Day 21	Day 5	Day 21	Day 5	Day 21	Day 5	Day 21
	00±00	00±00	00±00	00±00	7.1±0.6	9±0.46	7.1±0.57	5.8±0.36	4.1±0.18	3.3±0.36	4.1±0.18	3.3±0.36	4.1±0.18	3.3±0.36
RF	1.13±0.06		1.16±1.20		13.6±0.88		7.4±0.33		2.73±0.37		4.93±0.40		2.5±0.20	
CRP	2.08±0.33		2.23±0.24		7.9±0.99		6.3±0.75		2.2±0.01		2.2±0.02		2.2±0.07	
ESR	4.6±0.01		1.38±0.24		12.3±1.35		8.3±0.68		1.42±0.06		2.09±0.32		1.88±0.19	
Paw volume	0.32±0.002		0.32±0.006		2.11±0.45		1.75±0.45		0.32±0.02		0.32±0.01		0.31±0.01	
Hyc	4.8±0.36		4.8±0.26		8.3±0.14		7.2±0.80		4.9±0.51		4.9±0.23		4.8±0.14	
Atherogenic index	67.3±0.08		73.3±4.97		151.1±32.3		129.6±44.0		67.3±0.11		66.7±1.64		69.7±2.25	
HDL	44.3±0.18		39.2±1.83		36.7±2.57		39.3±1.75		44.4±0.29		44.6±0.43		44.4±0.25	
LDL	18.4±0.08		90.8±28.5		98.6±29.6		90.8±28.5		18.6±0.11		18.4±0.05		18.6±0.11	
TG	47.9±0.25		117.4±23.5		123.6±25.3		108.8±20.8		47.9±0.20		47.6±0.61		47.6±0.50	
TC	68.2±0.029		148.0±29.6		.1151.9±32		130.4±43.7		68.1±0.04		66.3±2.45		68.4±0.31	
IL-6	462±207		4354±248		1733±140		1858±65		287±40		350±72		328±36	
TNF-α	175±28.8		282±47.0		752±125		526±85		180±39		175±28.14		153±19	

5.4.2 Effects of combination of NSAE, CPAE and MCAE on Paw volume in cardiovascular complications in RA

The assessment of suitable dose for further models was become complicated in this situation (as discussed in 4.11). To overcome this problem 3^2 factorial design was applied and total nine combinations were selected the dose of all three drugs were selected as 50mg/kg, 100mg/kg and 200mg/kg as there was dose dependency in individual screening of drugs as well as placebo effects of drugs were better on the lower side.

Here to evaluate different combinations NSAE 200mg/kg, CPAE 50mg/kg and MCAE 400mg/kg combination dose was proved to give the preventive treatment in final validated model of cardiovascular complications in RA (CIA 0.1ml+LPS 10µg/ml+ HFD). On this basis the following statistical data were obtained for different evaluation parameters.

5.4.2.1 Effects of combination of NSAE, CPAE and MCAE on paw volume in cardiovascular complications in RA

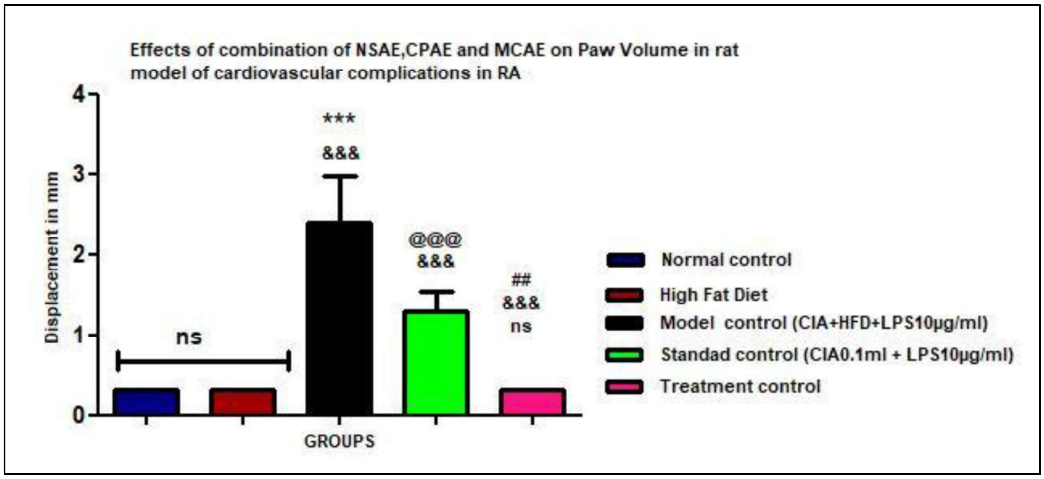
Paw volume in all groups except normal control group (0.32±0.001) was increased gradually. Model control group (2.11±0.45) showed the increased inflammation, primary and secondary lesions which is indicator of disease severity and inflammation. After day 7 the treatment control groups showed the significant decrease in paw volume where T2 combination dose showed the significant decrease in paw volume as compare to other doses (0.32±0.002). A significant difference between standard control (1.75±0.45), model control and standard doses also observed as per the values (Mean ± SEM)

5.4.2.2 Effects of combination of NSAE, CPAE and MCAE on Arthritic Score in cardiovascular complications in RA

Preventive treatment of combination of all three extracts against CVD in RA showed the significant difference from day 5 and 21. On day 5 the model control group showed the primary and secondary lesions, wounds and severe inflammation (6.1±0.57). Primary lesions of standard control were disappeared on day 21 but the inflammation was significantly high (10±0.28). In treatment control groups primary lesions (4.1±0.18) were seen which were less severe than model control groups and these lesions were healed on day 21. When comparison was done between three doses; T2 combination dose showed significant decrease in arthritic score (01±0.40).

5.4.2.3 Effects of combination of NSAE, CPAE and MCAE on Arthritic Index in cardiovascular complications in RA

Arthritic index in model control group (18±0.57) was high as compared to standard control (15±0.33) and test groups. The arthritic score of T2 combination group was significantly lower (2.33±0.55) than the other doses and it was found significant from model control group.



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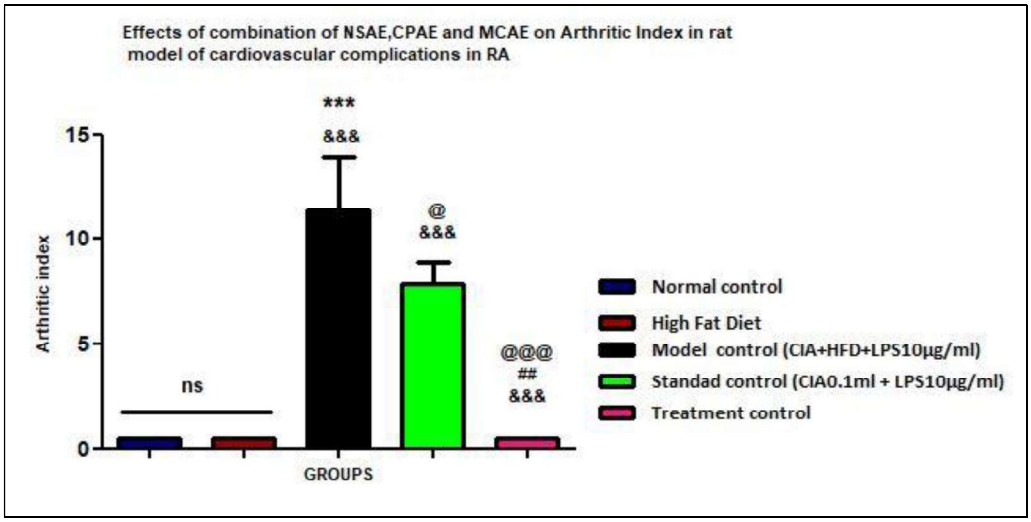


Fig. 5-30 Effects of combination of NSAE, CPAE and MCAE on Paw volume in cardiovascular complications in RA
Values are statistically evaluated using and one way ANOVA analysis followed by suitable post hoc test. *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control with other groups. &, &&, &&& comparison between high fat diet vs. other groups. @, @@, @@@ comparison between model vs. other groups. #, ##, ### comparison between standard vs. test groups. ns is non-significant differences between groups.

5.4.2.4 Effects of combination of NSAE, CPAE and MCAE on CRP in cardiovascular complications in RA

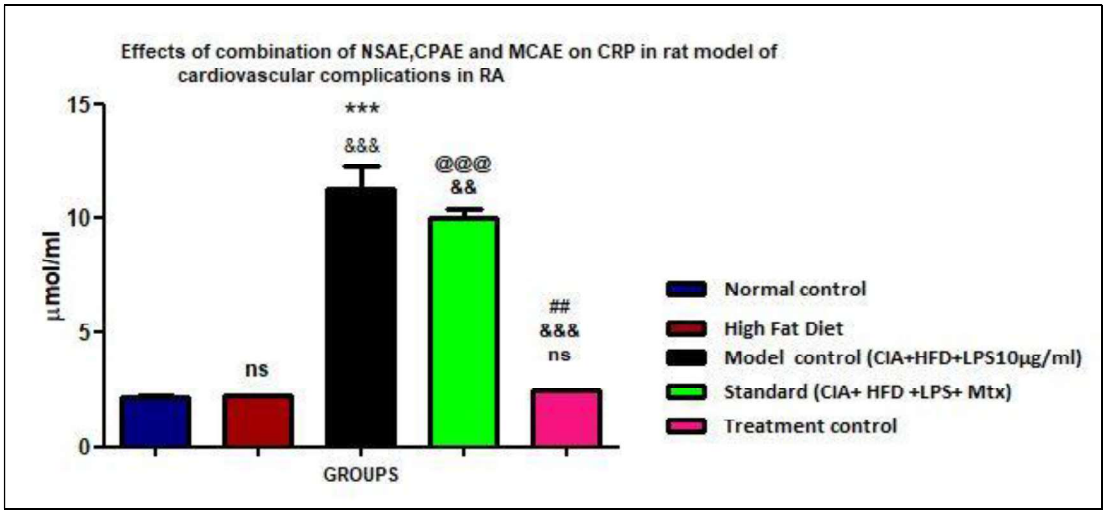
CRP is major marker for progression of inflammatory cytokine in body. Here model control groups sanitized with Collagen, LPS and HFD showed the higher levels of CRP (7.9±0.99) as compared to normal control (2.2±0.02) and test control groups. The treatment group T2 showed the highest decrease (2.2±0.01) in CRP levels as compared to other combinations.

5.4.2.5 Effects of combination of NSAE, CPAE and MCAE on ESR in cardiovascular complications in RA

Erythrocyte Sedimentation Rate is again one of the factors for inflammatory responses. In this study T2 combination dose showed the highest effect on ESR levels (1.42±0.06) as compared to other doses. ESR was in decreasing trends after day 7in all the groups except model control group (12.3±1.35) which showed highest rates of ESR after disease induction.

5.4.2.6 Effects of combination of NSAE, CPAE and MCAE on RF in cardiovascular complications in RA

Rheumatoid factor is one of the significant markers of rheumatoid Arthritis. In this comparison test drug combination T2 (2.73±0.37) was proved to give the maximum effects on RF as compared to model control (13.6±0.88) and standard control (7.4±0.33). The RF value of other test groups was also on the decreasing trend which shows the effectiveness of chosen herbs in CVD in RA.



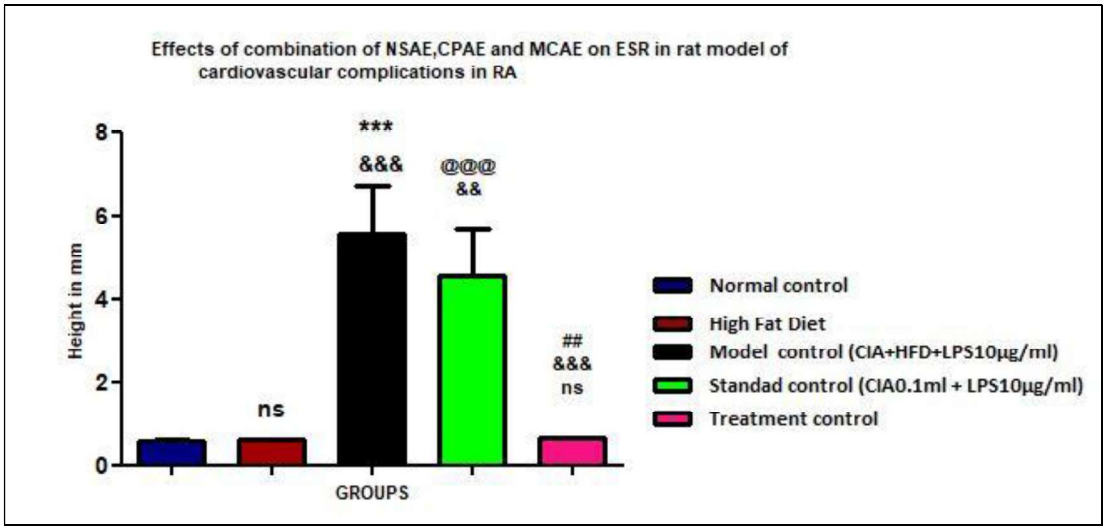


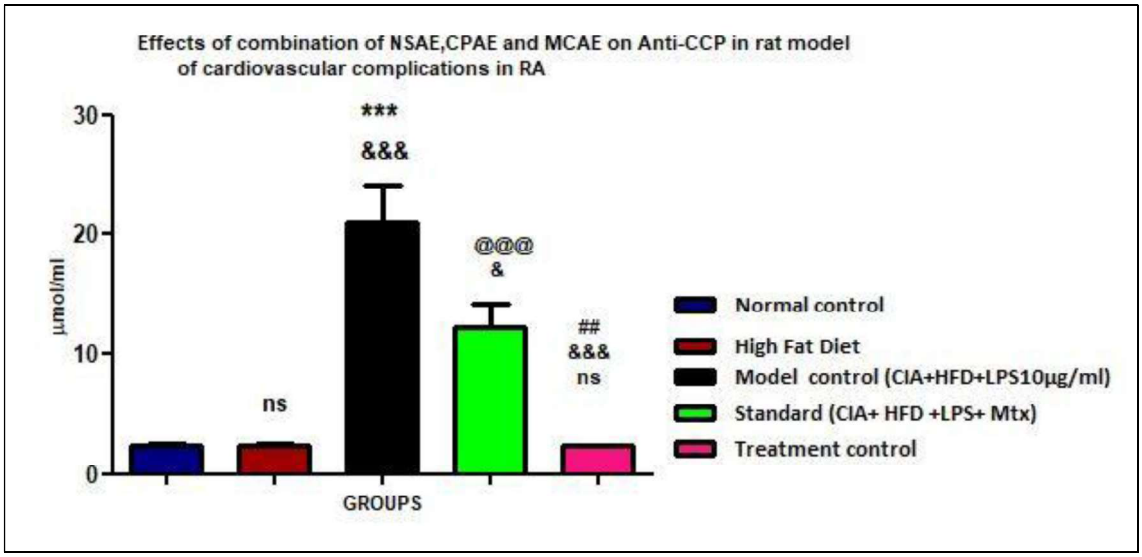
Fig. 5-31 Effects of combination of NSAE, CPAE and MCAE on (A) CRP (B) ESR in cardiovascular complications in RA Values are statistically evaluated using and one way ANOVA analysis followed by suitable post hoc test. *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control with other groups. &, &&, &&& comparison between high fat diet vs. other groups. @, @@, @@@ is comparison between model vs. other groups. #, ##, ### comparison between standard vs. test groups. ns is non-significant differences between groups.

5.4.2.7 Effects of combination of NSAE, CPAE and MCAE on Anti-CCP in cardiovascular complications in RA

Anti-CCP or ACCA is another marker for identification and confirmation of RA. In this study the values of ACCP in test groups were significantly lower than the model (24.2±3.01) and standard control groups (22.2±1.48). The maximum effects of T2 combination dose was seen on ACCP (2.38±0.02) in the study as compared to model and standard groups.

5.4.2.8 Effects of combination of NSAE, CPAE and MCAE on Homocysteine in cardiovascular complications in RA

Homocysteine (Hyc) is one of the major factors for initiation of extra articular manifestations in RA and it is important marker for vascular inflammation. In this study the model developed for cardiovascular complications in RA showed the increased levels of Hyc (19±1.29) which suggests the initiation of vascular inflammation in the rats. The expression of Hyc was higher in model control group as compared to T2 treatment control groups (5.3±0.22) and standard control groups (6.1±0.66).



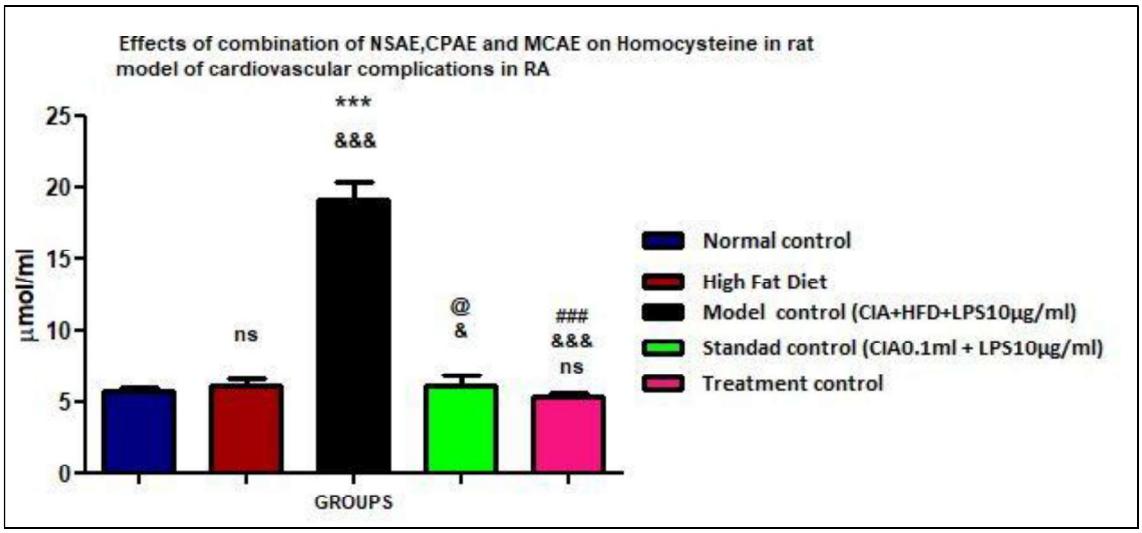


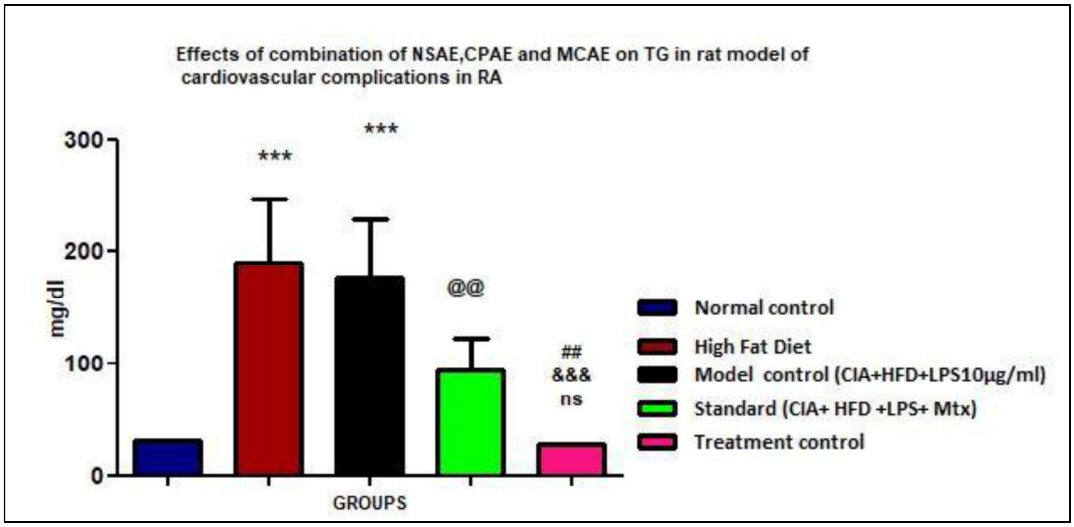
Fig. 5-32 Effects of combination of NSAE, CPAE and MCAE on (A) Anti-CCP (B) RF in cardiovascular complications in RA Values are statistically evaluated using and one way ANOVA analysis followed by suitable post hoc test. *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control with other groups. &, &&, &&& comparison between high fat diet vs. other groups. @, @@, @@@ is comparison between model vs. other groups. #, ##, ### is comparison between standard vs. test groups. ns is non-significant differences between groups.

5.4.2.9 Effects of combination of NSAE, CPAE and MCAE on TG in cardiovascular complications in RA

Triglyceride is important lipid content for initiation of higher fatty contents in blood. The TG levels were significantly high in model control group (128±15) which shows the effectiveness of diet as well as activation of lipid accumulation as cardiovascular complications in already compromised rats with RA. The levels of TG were significantly at lower side in treatment groups which shows the effects of selected drugs on TG. Among all the combinations, T2 combination group (28±0.17) showed the maximum effects on lowering the TG levels.

5.4.2.10 Effects of combination of NSAE, CPAE and MCAE on TC in cardiovascular complications in RA

Total cholesterol is another marker which signifies the activation of fatty depositions in the arteries. The values of TC levels in treatment control groups were significantly lower as compared to model (179±22) and standard control groups (189±20). The maximum effect was seen in T2 combination groups (68±0.06).



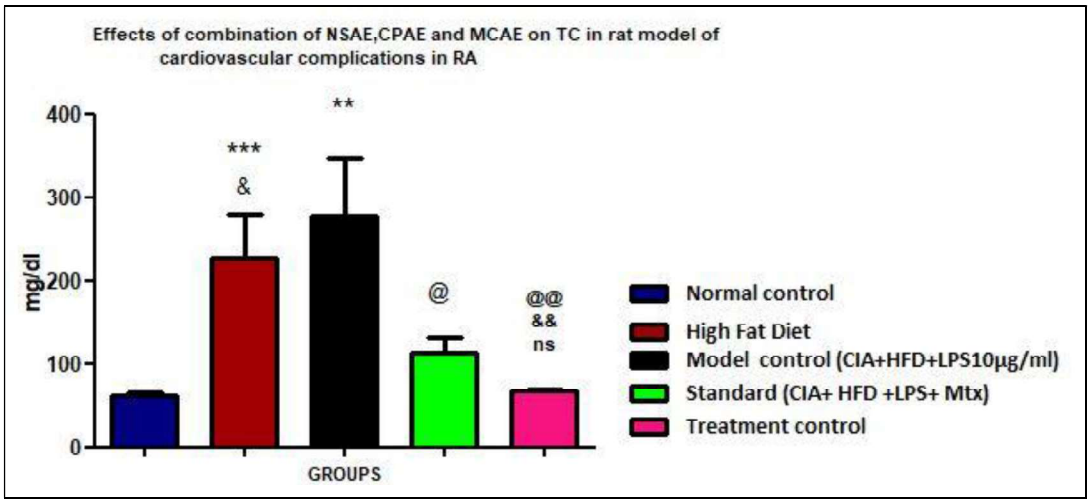


Fig. 5-33 Effects of combination of NSAE, CPAE and MCAE on (A) TG (B) TC in cardiovascular complications in RA Values are statistically evaluated using and one way ANOVA analysis followed by suitable post hoc test. *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control with other groups. &, &&, &&& comparison between high fat diet vs. other groups. @, @@, @@@ is comparison between model vs. other groups. #, ##, ### is comparison between standard vs. test groups. ns is non-significant differences between groups.

5.4.2.11 Effects of combination of NSAE, CPAE and MCAE on Atherogenic Index in cardiovascular complications in RA

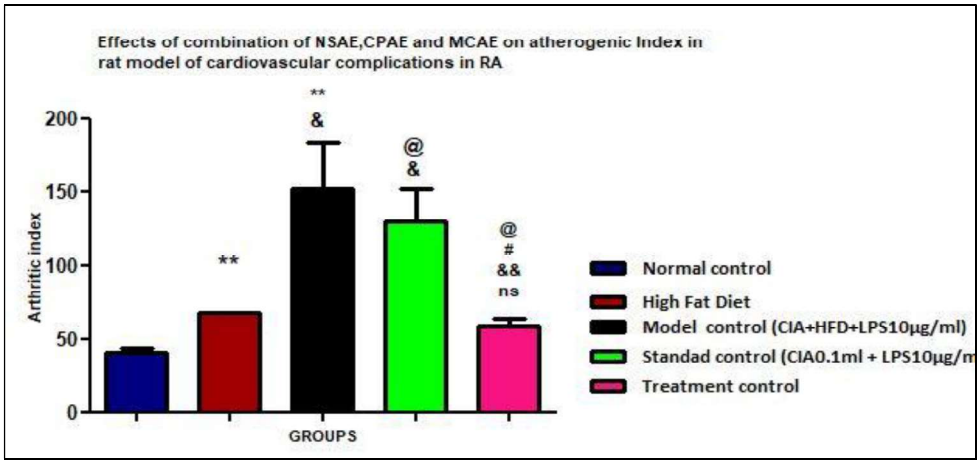
Atherogenic index is other significant parameters for identification of risk for deposition of fatty contents in the vascular system. The atherogenic index of model control group (179±22) was significantly higher than the standard (150±10) and treatment control groups; however maximum effect was seen in T2 group (56±5.6).

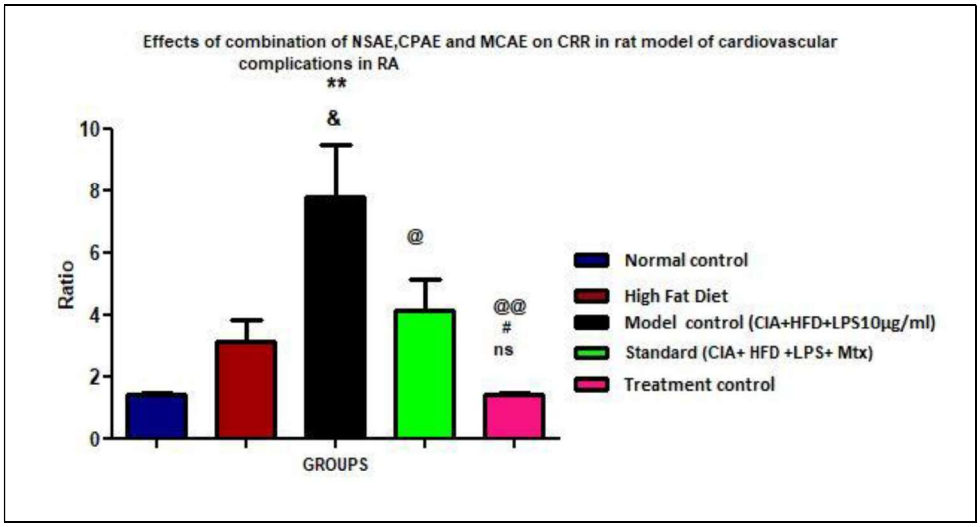
5.4.2.12 Effects of combination of NSAE, CPAE and MCAE on Cardiac risk ratio in cardiovascular complications in RA

Cardiac risk ratio is one of the mathematically derived ratios using lipid profile in models to identify the risk related to initiation of cardiovascular complications in model control animals. The cardiac risk ratio was found to be high in model (7.77±1.37) and standard (4.13±0.81) which indicates the initiation of cardiovascular complications in these groups. The test control groups were showed the significantly lower values of CRR (1.44±0.02) as compared to model and standard which shows the effectiveness of this combination on RA as well as CVD.

5.4.2.13 Effects of combination of NSAE, CPAE and MCAE on TLR-4 expression in cardiovascular complication in RA

TLR-4 was selected as one of the confirmatory markers for evaluation of CVD progression via interconnection between inflammatory and immune responses against HFD and LPS stimulated receptor expressions. The expression of TLR-4 was increased in model control (930±0.7) and standard control groups (456±0.27). The levels of this marker were found decreased in treatment control groups which show the effectiveness of the treatment. The maximum effect was seen in T2 treatment group (215±0.13).





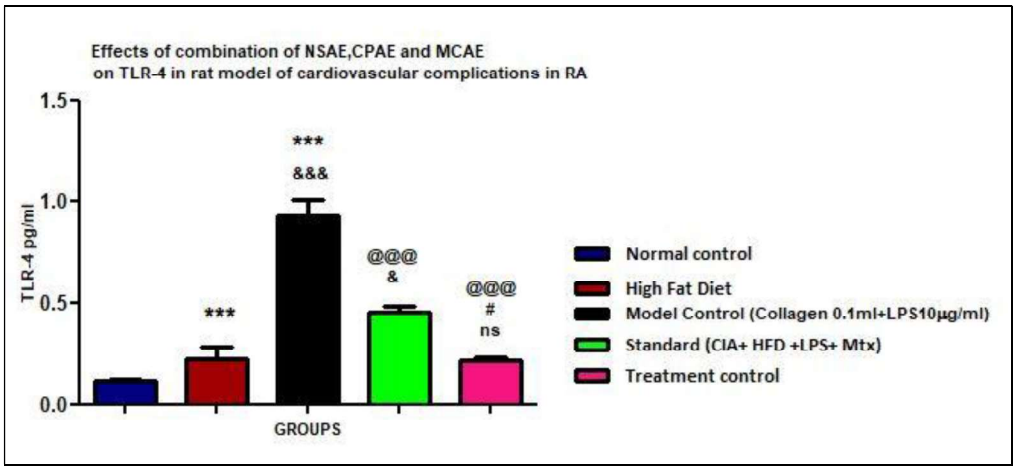


Fig. 5-34 Effects of combination of NSAE, CPAE and MCAE on (A) Atherogenic index (B) Cardiac risk ratio (C) TLR-4 expression in cardiovascular complications in RA

Values are statistically evaluated using and one way ANOVA analysis followed by suitable post hoc test. *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control with other groups. &, &&, &&& comparison between high fat diet vs. other groups. @, @@, @@@ is comparison between model vs. other groups. #, ##, ### is comparison between standard vs. test groups. ns is non-significant differences between groups.

5.4.2.14 Effects of combination of NSAE, CPAE and MCAE on neutrophil count in cardiovascular complications in RA

Neutrophil count is another important marker which leads inflammation to immunological intervention. The maximum increase in neutrophil was observed on day 21 in model control group (60-65) as compared to treatment control (18-20) with preventive treatment of combination of NSAE, CPAE and MCAE.

5.4.2.15 Effects of combination of NSAE, CPAE and MCAE on IL-6 in cardiovascular complications in RA

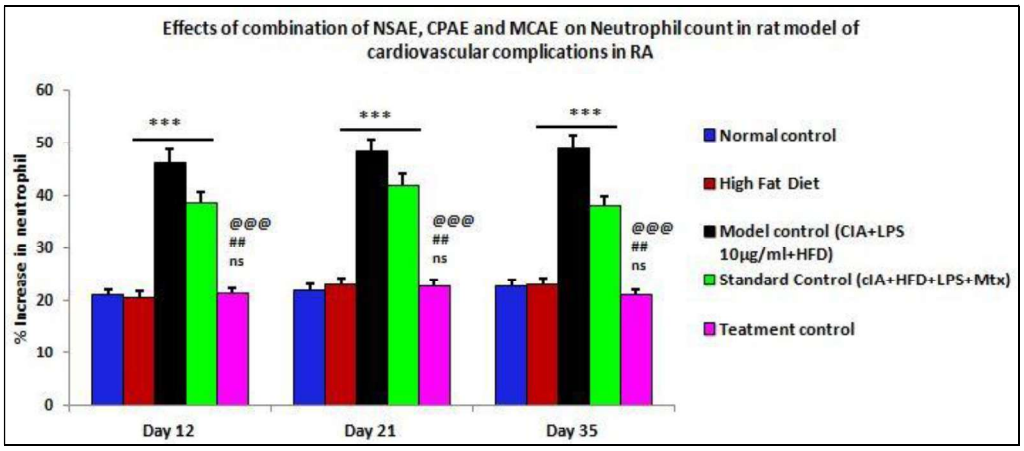
IL-6 is immunological markers which are highly expressed in synovial membrane as well as in vascular system in response to immunological insult. Here IL-6 highly activated in the model control group (1733±140). The expression of IL-6 was significantly low in treatment groups. Standard treatment group (Mtx. 0.6mg/kg) showed the elevation in IL-6 (1858±65) and among all the treatment groups, T2 combination dose treated animals showed the maximum effect on IL-6 levels which were found to be decreased (287±40) after preventive treatment.

5.4.2.16 Effects of combination of NSAE, CPAE and MCAE on TNF-α in cardiovascular complication in RA

Tumor Necrosis Factor alpha was found to be expressed in higher levels in model control group (725±125) and the effects of treatment group were high on TNF-α level. The maximum effect was seen in T2 combination treated group (180±39) as compared to other groups.

5.4.2.17 Effects of combination of NSAE, CPAE and MCAE on NF-κB in cardiovascular complication in RA

NF-κB is one of the major markers for the immunological activation of chronic inflammatory conditions in diseases. The expression of NF-κB was increased in model control (170±0.16) and standard control groups (573±0.08). The levels of this marker were found decreased in treatment control groups which show the effectiveness of the treatment. The maximum effect was seen in T2 treatment group (216±0.08).


Effects of combination of NSAE, CPAE and MCAE on Neutrophil count in rat model of cardiovascular complications in RA
% Increase in neutrophil
60
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Day 12
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Normal control
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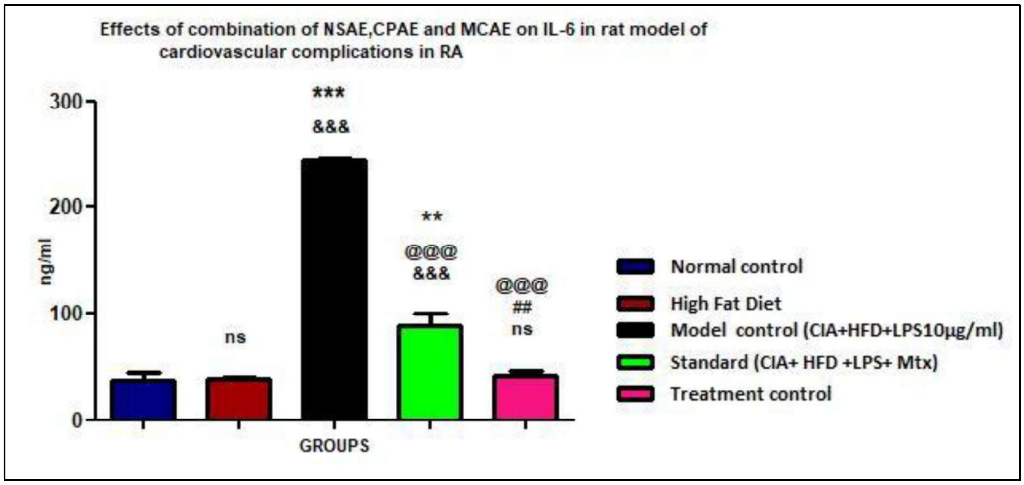
Model control (CIA+LPS 10µg/ml+HFD)
Standard Control (cIA+HFD+LPS+Mtx)
Teatment control


Effects of combination of NSAE,CPAE and MCAE on IL-6 in rat model of cardiovascular complications in RA
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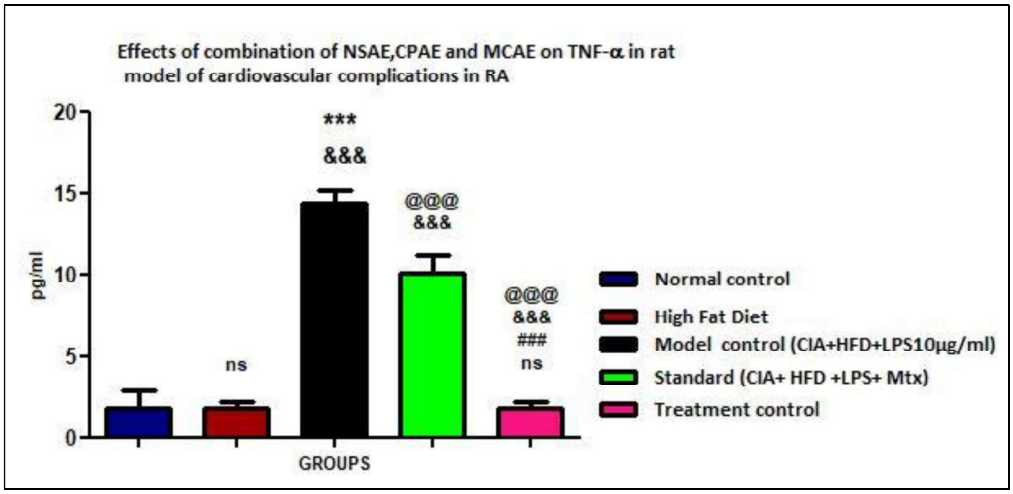
High Fat Diet
Model control (CIA+HFD+LPS10µg/ml)
Standard (CIA+ HFD +LPS+ Mtx)
Treatment control



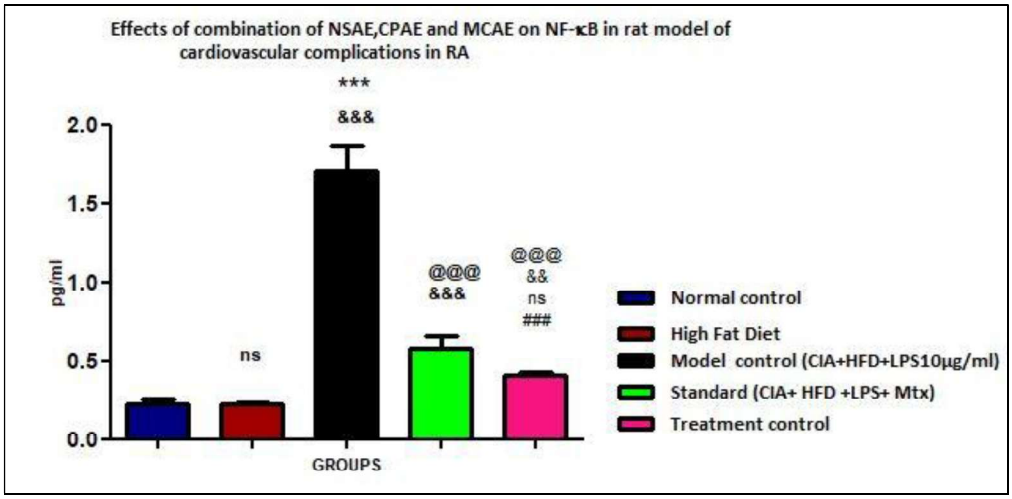


Fig. 5-35 Effects of combination of NSAE, CPAE and MCAE on (A) Neutrophil count (B) Interlukin-6 (C) TNF-α (D) NF-kB in cardiovascular complications in RA

Values are statistically evaluated using and one way ANOVA analysis followed by suitable post hoc test. *, **, *** (*P<0.05, **p<0.01, *** P<0.001 respectively) when compared normal control with other groups. &, &&, &&& comparison between high fat diet vs. other groups. @, @@, @@@ is comparison between model vs. other groups. #, ##, ### is comparison between standard vs. test groups. ns is non-significant differences between groups

Table 5-19 Final result compilation of combination of herbs in final validated model for RA with cardiovascular complications (values expressed I Mean±SEM)

	Normal		HFD		Model		Std		T2	
ACPA	**2.38±0.15**		**2.38±0.13**		**24.2±3.01**		**22.2±1.48**		**2.38±0.02**	
AI	**00±00**		**00±00**		**18±0.57**		**15±0.33**		**2.33±0.55**	
AS	**Day 5**	**Day 21**	**Day 5**	**Day 21**	**Day 5**	**Day 21**	**Day 5**	**Day 21**	**Day 5**	**Day 21**
	00±00	**00±00**	**00±00**	**00±00**	**6.1±0.57**	**10±0.28**	**7.1±0.57**	**5.8±0.36**	**4.1±0.18**	**01±0.40**
RF	**1.13±0.06**		**1.16±1.20**		**13.6±0.88**		**7.4±0.33**		**2.73±0.37**	
CRP	**2.2±0.02**		**2.23±0.24**		**7.9±0.99**		**6.3±0.75**		**2.2±0.01**	
ESR	**4.6±0.01**		**1.38±0.24**		**12.3±1.35**		**8.3±0.68**		**1.42±0.06**	
Paw volume	**0.32±0.001**		**0.32±0.006**		**2.11±0.42**		**1.75±0.42**		**0.32±0.002**	
Hyc	**5.7±0.2**		**6.08±0.46**		**19±1.29**		**6.1±0.66**		**5.3±0.22**	
Atherogenic index	**39±2.8**		**67±0.58**		**179±22**		**150±10**		**56±5.6**	
HDL	**44.3±0.18**		**39.2±1.83**		**36.7±2.57**		**39.3±1.75**		**31±2.5**	
TG	**30±0.72**		**140±15**		**128±15**		**119±23**		**28±0.17**	
TC	**67±0.33**		**128±28**		**179±22**		**189±20**		**68±0.06**	
CRR	**1.43±0.04**		**3.14±0.56**		**7.77±1.37**		**4.13±0.81**		**1.44±0.02**	
TLR-4	**218±0.0**		**223±0.8**		**930±0.7**		**456±0.27**		**215±0.13**	
IL-6	**462±207**		**435±248**		**1733±140**		**1858±65**		**287±40**	
TNF-α	**175±28.8**		**282±47.0**		**725±125**		**526±85**		**180±39**	
NF-κB	**230±0.02**		**230±0.05**		**170±0.16**		**573±0.08**		**216±0.08**	

5.4.3 Photographic assessment of disease severity at different phases for human resemblance

Photographic assessment is one of the basic perceptible data for evident records which are helpful in assessment of progression of disease with the days and duration of study period. In this study the progression of disease with increased severity and events occurred in different groups which were compared with human disease like progression and disease severity. The photographs were taken with the help of normal mobile camera just to keep record and the photographs of all the groups were taken for-

- **Signs of inflammation and edema in injected paw**
- **Primary and secondary lesions appeared during the experiment period**
- **Any severity noticed during the disease generation and progression**
- **Disability due to disease progression**
- **Comparison for different grades of severity as disease progression**

The following figure shows the comparison of human disease like severity in developed models. Major severities were observed in groups sensitized with collagen and LPS as well as collagen, LPS and high fat induced groups. The human disease like symptoms were observed as-

Picture 1 shows **Boutonniere deformities** in left hind paw as were as symmetric paws which were seen in photographic and x-ray assessments.

Picture 2 shows **Swan neck deformities** in metaphalangeal joints which are major cause of severe and permanent deformities.

Picture 3 shows **High grade deformities** in the form of digestion of digits and sever edema and bone erosion at multiple metaphalangeal joints.

Picture 4 shows **Nodule formations** in different parts of body other than the site of disease induction which shows the auto immune expression of disease severity in the form of **uveitis in eye, blisters on mouth and upper limbs as well as severe nodule formation in tail of animals**.

Picture 5 shows the **severe bone erosion** where, in human this severity appears as cervical bone damages and in current study the spinal bones and cervical portion of rats showed the damages and **permanent deformity** as restriction of the movement of the animal.

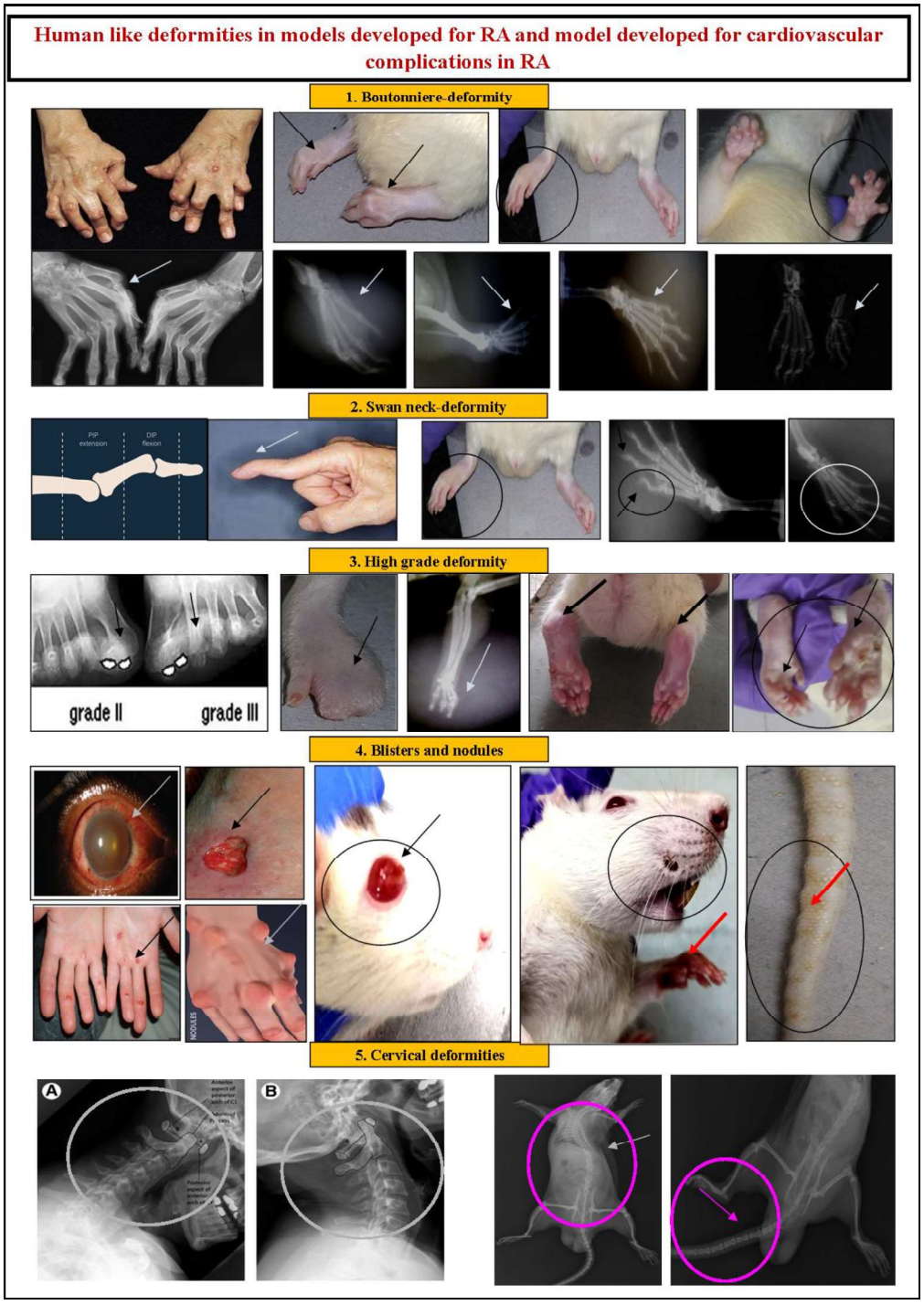


Fig. 5-36 Photographic assessment of human like deformities in developed models and test groups [(I-VII)]

5.4.4 Gross radiographic assessment of RA and disease severity

Radiography is confirmatory estimation to evaluate the damage occurred in bones and skeletal system due to induction of RA. Different chemical inducers used in this study were compared to check the maximum effect to select best inducer for RA. In the following figures different images shows the progression of disease on different stages.

All the photographs were taken from digital x-ray images and they were evaluated against normal control groups and treatment groups. The images were evaluated according to-

- **Edema and bone erosion occurred in injected paw of animals**
- **Edema and bone erosion seen in non injected paw of animals**
- **Bone disruption and erosion in upper limb as deformity and secondary lesion**
- **Deformities in the form of digestion of digits as severity of disease progression**
- **Nodule formation in tail of animals as secondary lesions and highest severity of disease**
- **Whole body x-ray to confirm the bone deformities in model control groups to check the human like deformity symptoms in animals**

Interpretations of Radiographic assessment

When X-ray was taken for confirmation of bone deformities in rats on 28 and 42 days of studies, various changes were observed in these animals.

(A) In this section picture 1 normal control x-ray when compared with picture 2 and 3 of model control group represents the severity in the form of bone deformities where, edema and inflammation with bone erosion with symmetric deformity is clearly visible in picture 2 where the spinal cord of rat is completely deformed and the animal was unable to walk properly which shows the human like severity and deformity in animal in collagen and LPS induced RA in rats.

(B) Here we can clearly seen that the x-ray of animals showed the bone deformity in left hind paw of animal in picture 1 and in picture 2 we can see the morphological as well as bone deformities of the paw of animals where upper and lower both limbs are affected which clearly indicates the symmetric patterns of diseases and in last images in picture 2 the deformed paw and its x-ray image is taken which confirms the human like deformity in the form of digestion of digits and bone deformity.

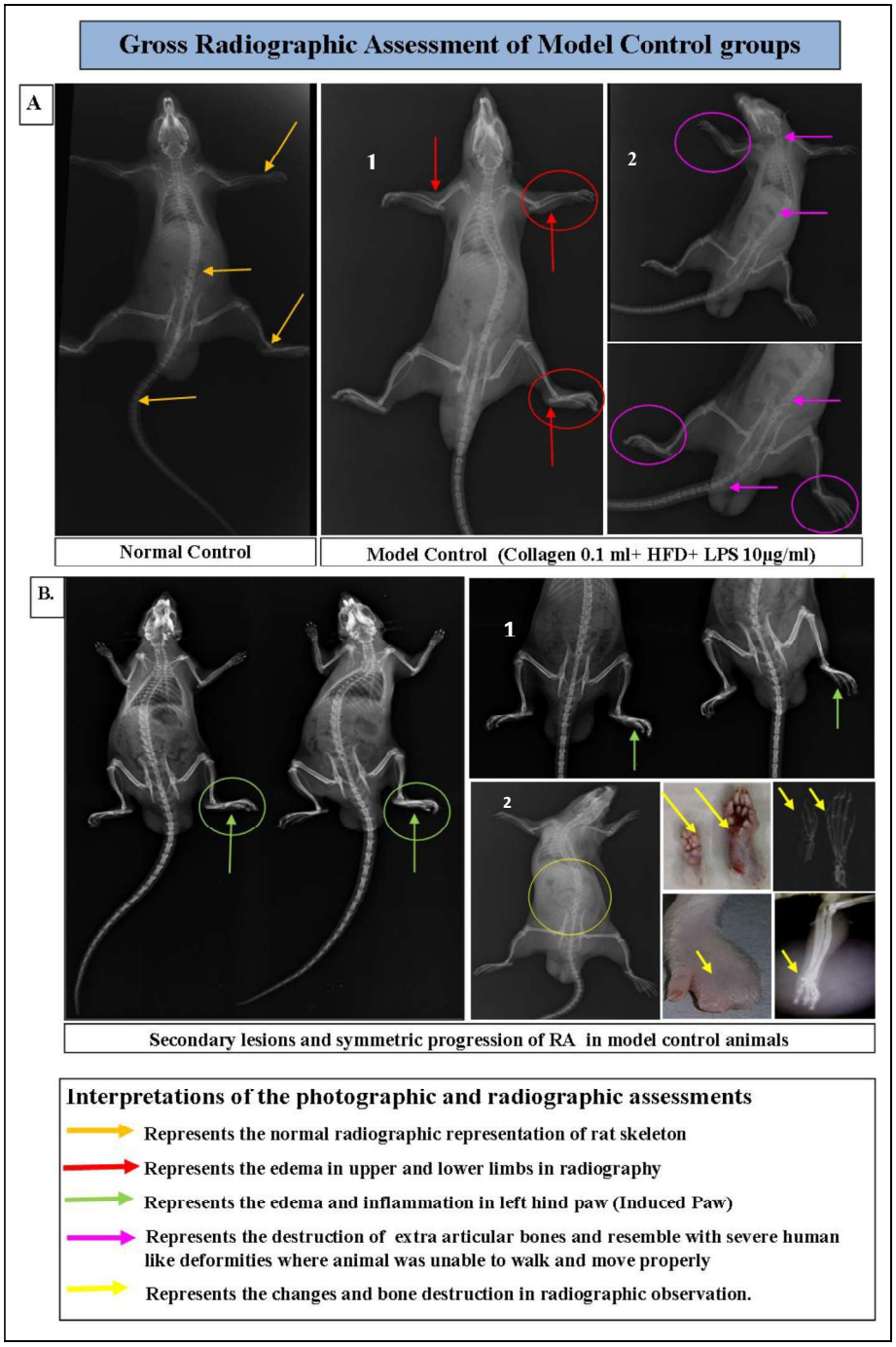


Fig. 5-37 Radiographic assessment of rats for assessment of RA

5.4.5 Histopathological assessment of combination treatment on disease progression

Histopathology is also one of the confirmatory tests which are required to compare the external and systemic pathogenesis of disease progression. In this study histopathology was used to assess-

- **Development of RA according to changes in cell cytology of ankle joint and connected tissues.**
- **In these sections pannus formation, infiltration of cells and changes in cell structures were seen in developed models for RA as well as in models for cardiovascular complications along with RA.**
- **In model developed for RA with cardiovascular complications, sections of heart were analyzed for modifications in cell cytology.**
- **The vastus medialis and biceps femoris muscles were also analyzed via histopathology to check the progression of atherosclerosis and fat depositions in muscles.**

Following figures are representation of histopathological changes in different groups in study design.

Interpretations of histopathological changes in model control groups

Image 1 shows the normal cytology of rat paw and synovium

Image 1(A) is representation of final models selected for comparison in validation.

a) Representation of model I (CFA 0.1ml+LPS 10µg/ml)

b) Representation of model II (CIA 0.1ml+LPS 10µg/ml)

c) Representation of model III (CFA 0.1 ml+LPS 10µg/ml +HFD)

d) Representation of model IV (CIA 0.1 ml+LPS 10µg/ml +HFD)

Among all these groups the cartilage damage and synovial damage is clearly visible. But the representative model of RA along with CVD model IV (CIA 0.1 ml+LPS 10µg/ml +HFD) and representative model of RA model II (CIA 0.1ml+LPS 10µg/ml) showed the Pannus formation which clearly shows the cell infiltration due to synovial damage and severe cartilage damage.

Image 2 shows the normal cytology for biceps femoris and vastus medialis muscles which were assessed for progression of atherosclerosis as initiation of cardiovascular complications.

In **figure 2(A) and 2 (B)** - a, b, c and d are representative of Model I, II, III and IV as mentioned above. And this clearly indicates that the muscular damages due to metabolic

dysbiosis, fat deposition and initiation of atherosclerosis the muscle fibers of model IV (CIA 0.1 ml+LPS 10µg/ml +HFD) were short in both the muscles (vastus medialis and biceps femoris). Moreover tearing of muscles is also observed due to HFD consumption which clearly confirms the induction of atherosclerosis in RA.

Image 3 shows the effects of treatment on test groups where pannus formation and edema was not seen and the cell cytology of bone is in normal condition which suggests the positive effects of preventive treatment of combination test drug.

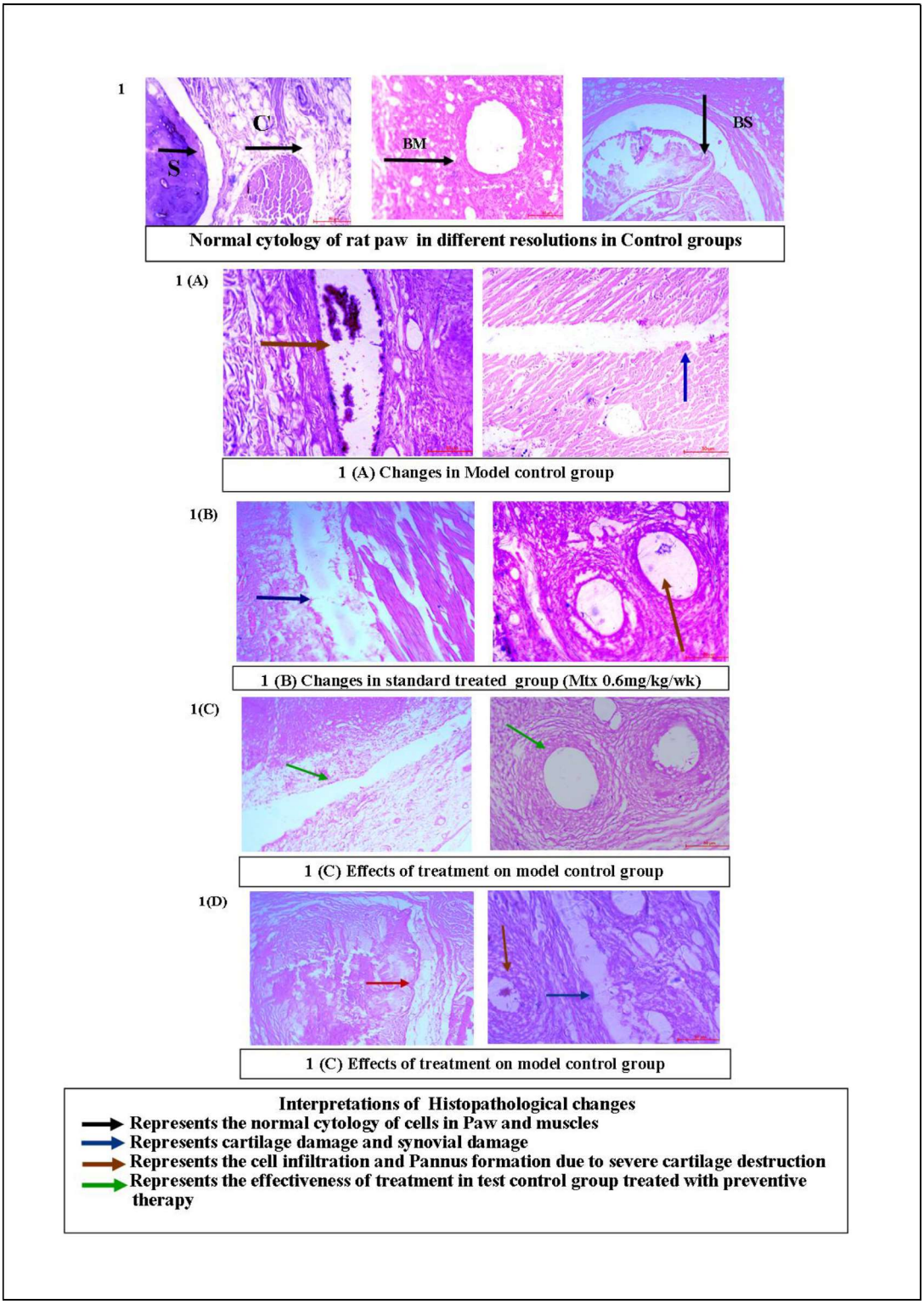


Fig. 5-38Histopathological assessment of Preventive treatment on RA and CVD

5.5 Results of Section V

5.5.1 Results of standardization of formulation

All the mentioned standardizations for prepared formulation are performed at research centre of Vasu Healthcare Pvt. Ltd. Vadodara, Gujarat.

5.5.1.1 Physicochemical analysis of formulation

The organoleptic evaluation was done for appearance of tablet with its general characteristics and physicochemical analysis was performed for validation of tablet on pharmacopoeia standards for herbal tablet.

Here physicochemical analysis for aqueous dry extract was performed individually and then organoleptic, physicochemical, heavy metal and micro biological results were compared together following table shows all the obtained results for this evaluation.

Table 5-20 combined results of analysis of organoleptic, physicochemical and microbial analysis for developed formulation

Sr. no.	Parameters	Result	Limit as per API
ORGANOLEPTIC ANALYSIS			
1	Description	Brown colored caplet	NA
2	Diameter	8.29 mm	NA
3	Thickness	5.04 mm	NA
PHYSICO-CHEMICAL ANALYSIS			
1	Hardness	2.8 kg/cm^2	NA
2	Friability	0.08 %	NA
3	Disintegration Time	32 min.	NA
4	Average Weight	746.22 mg	NA
5	Thymoquinone by UV	0.32 %	NA
6	Total Saponin by Gravimetry	0.55 %	NA
7	Total Bitter by Gravimetry	14.04 %	NA
HEAVY METAL ANALYSIS			
1	Lead	0.205 ppm	NMT 10 ppm
2	Cadmium	0.008 ppm	NMT 0.3 ppm
3	Mercury	0.005 ppm	NMT 1 ppm
4	Arsenic	0.119 ppm	NMT 3 ppm
MICROBIOLOGICAL ANALYSIS			
1	Total Microbial Plate Count	1265 cfu/g	10^5 cfu/g
2	Total Yeast & Mould Count	46 cfu/g	10^3 cfu/g
3	*Staphylococcus aureus*	Absent	Absent/g

4	*Salmonella sp.*	Absent	Absent/g
5	*Pseudomonas aeruginosa*	Absent	Absent/g
6	*Escherichia coli*	Absent	Absent/g
Notations**:** API **–** Ayurvedic Pharmacopoeia of India; % - Percentage w/w, ppm - Parts per millions, NA **–** Not applicable, cfu/g **–** Colony forming unit per gram.			

5.5.2 Quantification of markers in extracts for formulation development

The percentage values for active metabolites on the basis of preliminary data gathered in lab were further analyzed at Vasu Healthcare Pvt. Ltd. All three extracts for presence of effective metabolite viz; Thymoquinone in *Nigella sativa,* total saponin compound in *Carica papaya* and bitters in *Momordica charantia* evaluated. The results in percentage are summarized in following table-

Table 5-21 Quantification of *Nigella sativa, Carica papaya* and *Momordica charantia* dry extract

Sr. no	**Sample extract (Dry extract)**	**Targeted metabolite**	**Method for analysis**	**Results (% w/w)**
1	***Nigella sativa***	Thymoquinone	UV spectroscopy	1.72 %
2	***Carica papaya***	Total Saponin	Gravimetric method	11.14 %
3	***Momordica charantia***	Total Bitter	Gravimetric method	18.92 %

5.5.3 Results of instrumental analysis:

High Performance Thin Layer Chromatography (HPTLC) was performed as instrumental methods, where fingerprinting was done for formulation and dry extracts. The following figures represent the 3D chromatographic representations and different HPTLC runs for formulation against all three dry extracts at three different wavelengths as mentioned in methodology section.

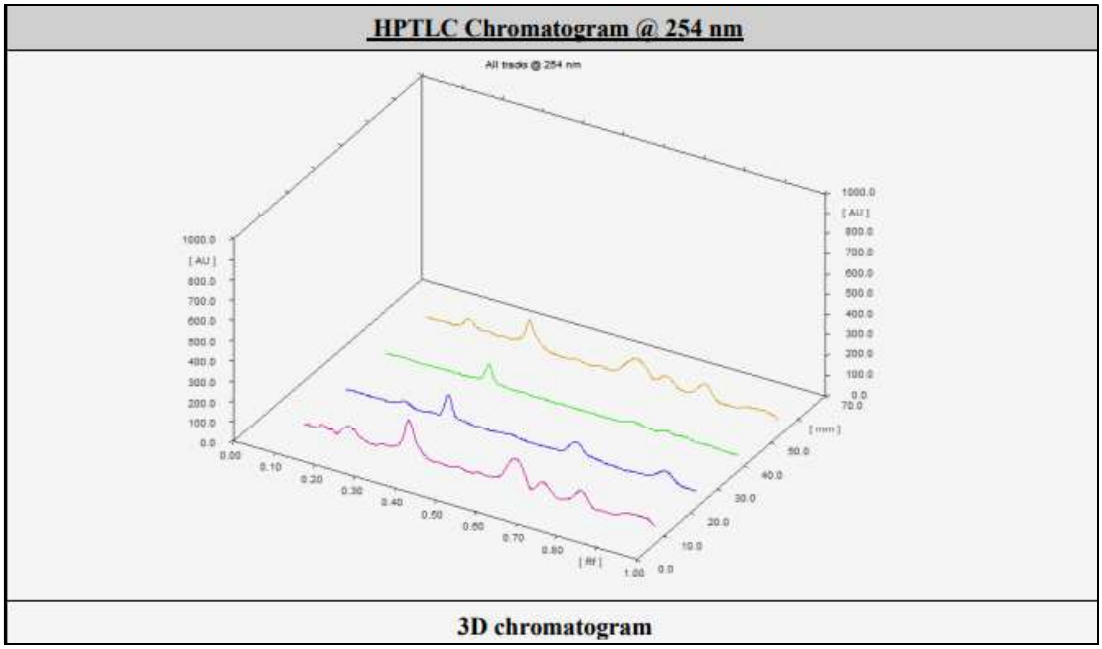


Fig. 5-39 HPTLC 3D Chromatogram of combination formulations of test drug at 254nm

R_f value

Spot No.	Track T1	Track T2	Track 3	Track 4
1	0.10	---	---	---
2	0.14	---	---	---
3	0.16	---	---	---
4	0.21	0.21	---	0.21
5	0.29	0.29	---	---
6	0.35	0.35	0.35	0.35
7	0.48	0.48	---	---
8	0.53	---	---	0.53
9	0.62	0.62	---	0.62
10	0.69	---	0.69	0.69
11	0.78	---	0.78	0.78
12	0.92	0.92	0.92	---
13	0.94	---	---	0.94

Track T1 : *Momordica Charantia* DE

Track T2 : *Nigella sativa* DE

Track T3 : *Carica papaya* DE

Track T4 : Herbal Tablet

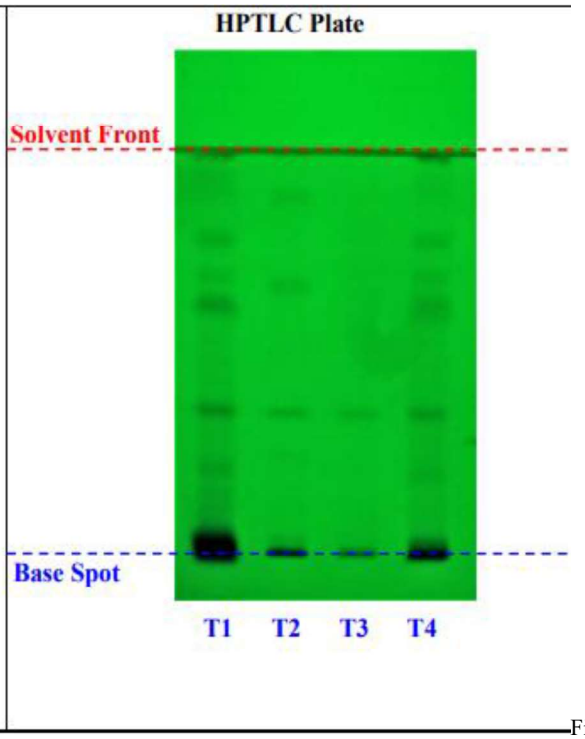


Fig. 5-40 Representation of HPTLC fingerprinting for Extracts vs. Herbal Tablet developed using select herb at 254 nm

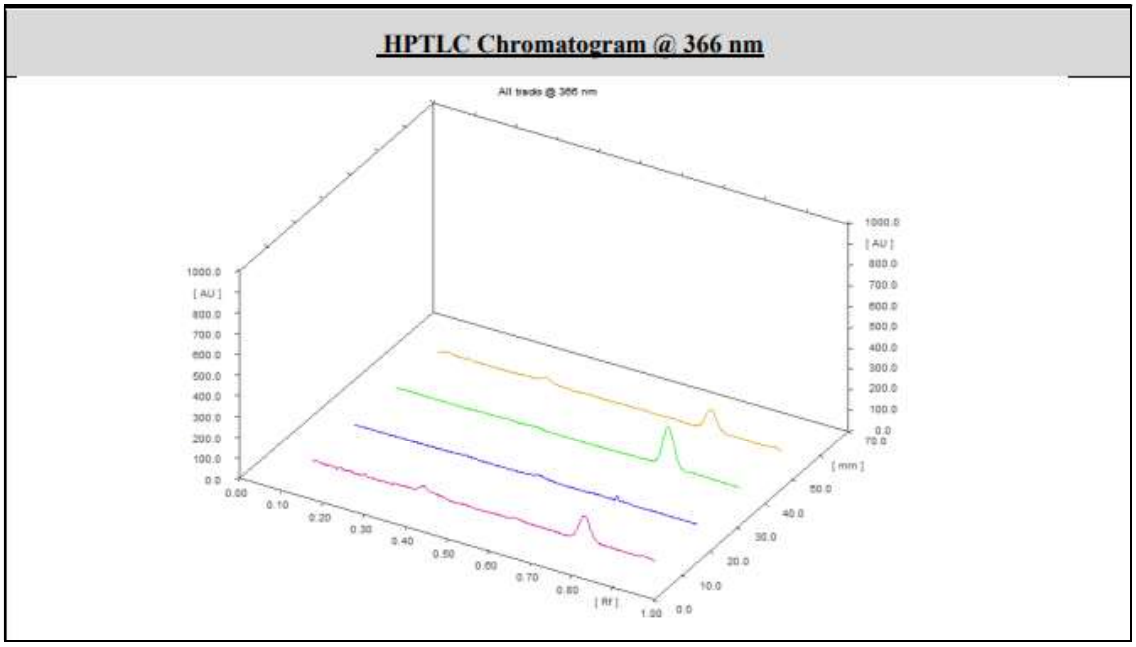


Fig. 5-41 HPTLC Chromatogram of prepared herbal formulation at 366nm

R_f value

Spot No.	Track T1	Track T2	Track 3	Track 4
1	0.10	---	---	---
2	0.21	---	---	---
3	0.35	---	---	0.35
4	---	0.54	---	---
5	0.75	0.75	0.75	0.75

Track T1 : *Momordica Charantia* DE

Track T2 : *Nigella sativa* DE

Track T3 : *Carica papaya* DE

Track T4 : Herbal Tablet

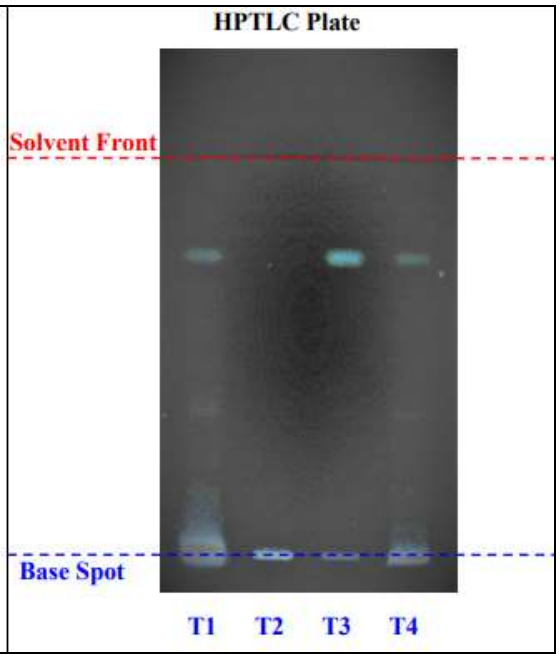


Fig. 5-42 Representation of HPTLC fingerprinting for Extracts vs. Herbal Tablet developed using select herb at 366nm

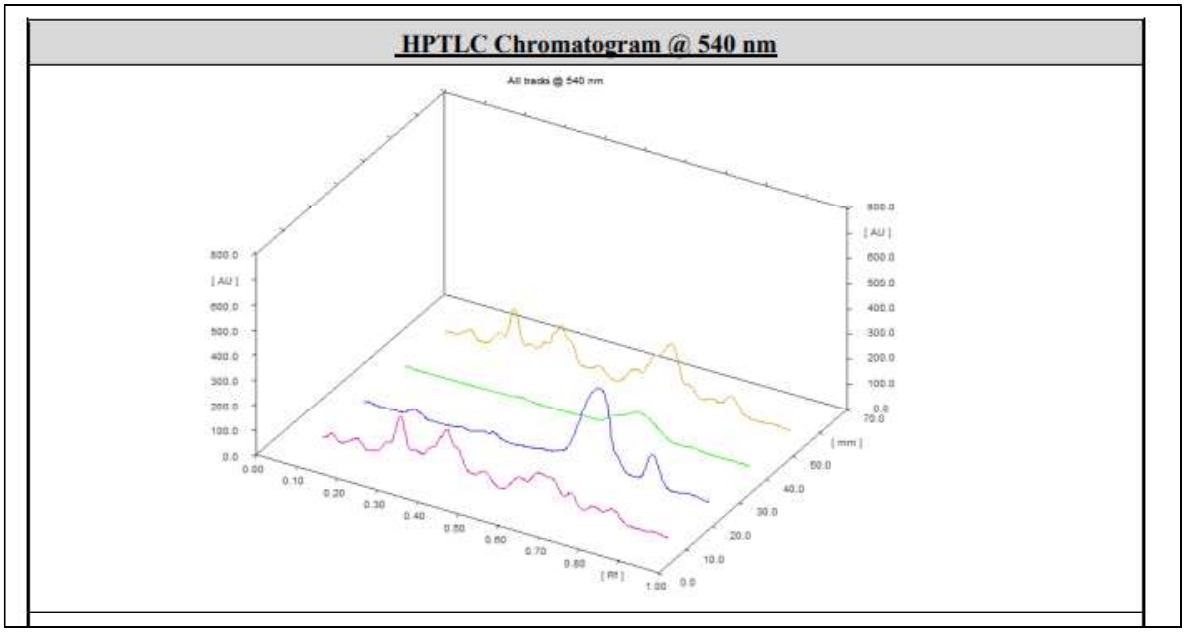


Fig. 5-43 HPTLC 3D Chromatogram at 540 nm

R_f value

Spot No.	Track T1	Track T2	Track 3	Track 4
1	0.10			
2	0.16			0.16
3	---	0.22	---	0.22
4	---	---	---	0.25
5	0.29	---	---	0.29
6	---	0.33	---	---
7	0.39	0.39	---	0.39
8	---	0.41	---	---
9	0.48	0.48	---	0.48
10	0.57	---	---	0.57
11	0.62	---	---	---
12	0.65	0.65	0.65	0.65
13	0.69	---	---	0.69
14	0.78	---	---	0.78
15	0.80	0.80	---	0.80

Track T1 : *Momordica Charantia* DE

Track T2 : *Nigella sativa* DE

Track T3 : *Carica papaya* DE

Track T4 : Herbal Tablet

HPTLC Plate

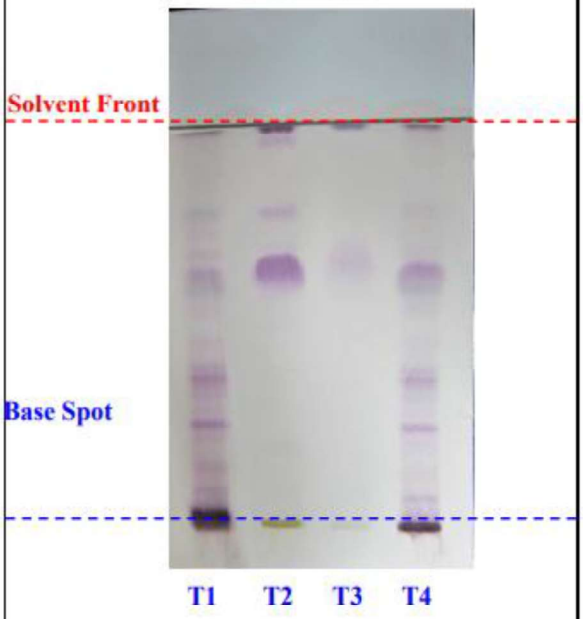


Fig. 5-44 Representation of HPTLC fingerprinting for Extracts vs. Herbal Tablet developed using select herb at 540 nm