7 Summary

Rheumatoid arthritis (RA) is a chronic inflammatory lifelong disease which stimulates the auto immune drivers responsible for involvement of other organs; lung, renal, heart and skin which contribute in worsen the conditions of RA patients. Among these manifestations, development of cardiovascular complications (CVD) due to metabolic dysbiosis and pathophysiological insult is major area for prevalence in stable RA patients. Besides the markers generating due to compromised immune system the most commonly prescribed allopathic drugs like NSAIDs, Steroids, Methotrexate (MTX) and Biologics in the form of oral, injectable and topical preparation are also contributors in the progression of CVD associated with both morbidity and mortality.

The study design was aimed for distinct findings; Development of validated animal model for CVD in RA, individual effects of herbs in RA and or RA along with CVD and comparison of combination therapy with standard drug methotraxate. The results were analyzed statistically as well as with specific model validation tool. The entire study was divided in different sections to get better insight about each study design. All the methodologies, experimentations, observations, statistical values and comparisons indicate that-

In development of *in-vivo* rat models for Rheumatoid Arthritis-

The model developed with CFA alone was proved to give better inflammatory impacts on animals and the disease severity was slow in progression.

When, LPS was added with CFA, the model showed the prominent and early signs of inflammatory mediator release with an impact of immune responses.

Collagen induced model was used against CFA to check the immunological intervention, where collagen model proved to give early signs of high grade inflammation in animals as compared to CFA induction.

The incorporation of LPS in collagen again showed a boost in inflammatory and immune responses which was twofold higher and early in terms of onset and severity as compared to CFA and CFA with LPS.

On the basis of these results of *In-vivo* studies we can conclude that-

The model developed with CFA alone was proved to give better inflammatory impacts on animals and the disease severity was slow in progression. When LPS was added with CFA, the model showed the prominent and early signs of inflammatory mediator release with an impact of immune responses (moderately validity score 64%)

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CFA and collagen both are responsible for the RA induction but severity index was high in collagen induced groups showed the immunological intervention along with inflammatory markers (Model I and II).

Sensitization of LPS in 10 µg/ml dose along with CFA and collagen caused RA +Athero respectively and the disease severity was noticed in Collagen+HFD+LPS10µg/ml groups (95% validity) evident from face validity, constrict validity and predictive validity parameters.

In final conclusion developed model when validated the indicators for RA model I sensitized with CFA and LPS (64%), showed prominently inflammatory responses. On the other hand model II sensitized with collagen and LPS (82%) showed the systemic effects with the perceptible changes which proved combination of collagen and LPS in 1010µg/ml is suitable to develop model of RA with human resemblance.

In development and comparison of *in- vivo* rat models for cardiovascular complications in RA

When all four developed models -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) compared with different techniques where-

The basic pharmacological evaluation was done by traditional method of statistics comparison applied by using biostatics (one way ANOVA, two way ANOVA, and repeated measure ANOVA) and results were compared by graphical representation in form of histograms.

The statistical observations were counted as a part of pre validation and further utilized for scoring based external model validation measures on the basis of results of physical, biochemical and confirmatory tests among groups.

In second line of investigation, there was an exigency of model validation with optimization which was accomplished using higher validation method of same weight score system of framework to identify models for disease (FIMD).

On the basis of these results of model validation studies we can conclude that-

The initial pre validation results found to be useful only for initial stage error rectification for model development in terms of species, population size and basic validation by observational data.

The model created with collagen and LPS among the RA models was suited best in these predictive methods for all criteria of model validation.

The model developed with collagen, LPS and HFD were proved the best model among all four created models.

One important aspect in model validation is to maintain internal and external validity criteria to check the robustness of the method selected with same results on recreation of model for which, predictive validity, face validity and constrict validity were evaluated. Here when different animals were sensitized with CFA or collagen the time of onset of disease and disease severity was similar every time.

When LPS was incorporated the effects of higher dose of LPS (10µg/ml) was also given similar results with CFA and LPS but the severity and onset of disease was early with secondary lesions in collagen and LPS sensitized groups every time when model was recreated in different set of animals which resembles with clinical situation.

The incorporation of high fat diet with CFA and LPS induced group showed a moderate effect on disease severity but it was high in terms of severity and disease progression in collagen and LPS induction with high fat diet which was similar with the patients having metabolic syndrome and RA.

All the developed models able to recreate the similar results as per validation score of epidemiology, aetiological, and genetic validation criteria (kept similar for all the models) as per the radar values obtained by FIMD.

On the basis of validation parameters applied, the model developed for RA with LPS induction (collagen+ LPS) found validated on symptomatology, biochemical validation, pharmacological, histological and end point validation scores of radar plot which are more close to clinical evidences of RA in terms of secondary lesions and deformities and denoted as a highly validated model for RA.

The cardiovascular complications in RA were also resembled with the clinical evidences as per the confirmatory tests which also proved the validation of model on pharmacological, histological and endpoint validation by radar plot values.

With these results in pre validation also the model IV (CIA 0.1 ml+LPS 10µg/ml +HFD) scored the highest score (182) than other developed model. When these models were further optimized the model IV (CIA 0.1 ml+LPS 10µg/ml +HFD) secures highly validated

model score with 95% validity and only 5% uncertainty which shows that the method applied is suitable for comparison of models and the radar value showed that model IV is suitable to mimic human disease like conditions of CVD in RA.

In pharmacological evaluation of aqueous extracts of *Nigella sativa, Carica papaya* and *Momordica chirantia* seed in validated model of RA.

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

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

The herbs were further optimized by different combination doses where, selected combination dose (*Nigella sativa* 200 mg/kg +*Carica papaya* 50mg/kg +*Momordica Charantia* 400mg/kg) was proved to give better amelioration on RA and CVD as well as RA alone in preventive treatment.

On the basis of results obtained the combination therapy proved to give systemic effect on inflammatory as well as on immunological parameters which shows the sequential blockage of initiated pathways in generation of RA as well as CVD.

The animal doses of extract were converted in human doses for development of formulation and standardization.

The combination of (*Nigella sativa* 200 mg/kg +*Carica papaya* 50mg/kg +*Momordica Charantia* 400mg/kg) was proved compatible for formulation development in form of caplets using the effective markers.

Marker identification in developed formulation was also done which was similar to initial primary assessment with extracts which showed the potential of combination to serve as a therapeutic option for management of RA and CVD in RA.

On the basis of above points the treatment control group (NSAE 200mg/kg+CPAE50mg/kg+MCAE400mg/kg) showed the anti arthritic activity in

preventive treatment as there was 100% decrease in paw volume -75% decrease in arthritic score, 98% decrease in CRP levels,-69% decreases in ESR100% decrease in ACPA.

On the other hand the decrease in IL-6 levels (93%) TNF- α (50%) and other immunological markers showed that the combination is proved as immunomodulator which is may be blocking the markers sequentially to prevent disease progression.

Furthermore the lipid profile and atherogenic criteria was also seen prevented by the combination as there was a decrease in Hyc levels by 96%, 90% decrease in TG levels, 99% decrease in cholesterol levels and significant decrease in CRR by 80% proves the cardioprotective effects of this combination of NSAE200mg/kg+ CPAE 50mg/kg+MCAE 400mg/kg in prevention of CVD in RA.

In Formulation of combination of aqueous extracts of *Nigella sativa, Carica papaya and Momordica chirantia* seed was developed-

The preliminary marker identification at formulation development stage proved the presence of thymoquinone, phenolic acids and gallic acid in *Nigella sativa*, *Carica papaya* and *Momordica charantia* seed aqueous extract respectively.

The HPTLC was run for the quantification of markers present in extracts as well as for prepared formulation (for gallic acid, chlorogenic acid and thymoquinone).

Tablet standardization processes were followed for physicochemical, heavy metal and microbiological standardization of prepared formulation.

On the basis of these results of treatment studies we can conclude that-

The prepared formulation with *Nigella sativa*, *Carica papaya* and *Momordica charantia* seed aqueous extract showed the presence of markers identify at laboratory level, which shows the hypothesis correct.

The final formulation was prepared with extract sample evaluated in animals and they showed the compatibility during formulation development which indicates the physical, chemical as well as biological compatibility of selected herbs.

The HPTLC data showed the presence of markers identified for the pharmacological activity in RA as well as in CVD in RA, shows the right path of hypothesis which can further proved by proper pharmacological studies.

With the above standardization procedures for extracts and formulation there is a proven data to support the hypothesis where different biomarker combination is working in synergy to block the pathways responsible for disease generation and progression in developed models.

8 Applications

Rheumatoid Arthritis (RA) has complex pathogenesis with different factors and symptoms in individuals due to interplay of inflammation as well as auto immune responses which can be triggered by the microbial insult and the biomechanical disruption by any stimulant. As there is no set regimen for treatment of RA and the different agencies working in the field of RA also suggesting the drugs given for management of RA are itself contributors in extra organ manifestations and co morbid conditions specially the cardiovascular complications.

In this experimentation we tried to imply the methods which can give a clinical interpretation of both the situations as till date no single model is available which can represents the cardiovascular complications in existing RA. The prepared and validated model with this method can be used as a single model for RA with co morbid conditions which can be used for future pharmacological evaluation for drug discovery for RA with associated complications with maximum human resemblance.

Limitations of this study

FIMD is a validation method where researcher can get a quantitative data on the basis of same weight scoring system of statistics to compare the developed model/models or we can take this statistics for competence of existing models to select the suitable one for particular condition of our interest. This method is useful for the comparison and validation but it also has some limitations.

The questionnaire gives us the quantitative data based on the questions answered and the score calculated by researcher itself. Here sections secured (*yes partially* and *yes*) are dialectical situation because some markers were seen in all the models such as inflammation and they are irrespective of severity of actual situation of animal. In this type of situation this system cannot be completely reliable as the severity in terms of perceptive and biochemical changes in each and every animal of similar group will also vary and then this system will not generate the valid score.

This method is useful for comparison between developed models as well as existing models available for the diseases. It can give a better insight for pharmacological

standardization in terms of biomarkers, etiology and genetic basis to get advanced level results in validation tool. In some cases where models were not developed or evaluated by the same researcher and data is gathered by different sources or existing models there are chances of biasness in reporting of results which can mislead the other research and resources.

9 Future Scope

Model Development

On the basis of all the discussed points the present study also has scope for refinement of models on- i) Genetic validation which was done partially on the basis of framed questionnaire ii) In epidemiological domain also the animals were outbred which does not make a big difference as the animals were assured about not treated with any substances before and after birth till the accommodation in lab but the results may vary due to such condition also. iii) Again in epidemiology gender specific studies were partially performed but the similar situation for both the model is not performed due to some limitations. iv) Pharmacological validation was also not able to similitude the desired results as the doses selected were having therapeutic effects in both the situations which can be further assessed using other techniques.

Treatment approaches

In the current study preventive treatment of combination of aqueous extracts of *Nigella sativa, Carica papaya* and *Momordica charantia* seed were evaluated alone and in combination for RA as well as CVD in RA. In later part the selected combination was used for formulation development as a caplet (capsule shaped tablet) for clinical approaches. The study concluded at formulation development only but there are lot more future development were possible with this combination as well as with prepared formulation via pharmacological screening of formulation as well as screening of formulation with clinical evidences.