"Chemically synthesized derivatives of Boswellic Acid as modulators of cytokine mediated cell signaling implications in chronic inflammatory diseases"

Government of India, Ministry of Science & Technology
Department of Biotechnology Sponsored Project
(BT/PR11910/BRB/10/697/2009)

PROGRESS REPORT

(2011-12)

Dr. Latha Diwakar

Vittal Mallya Scientific Research Foundation
94/3 &94/5 ,23RD Cross, 29th Main, BTM II stage, Bengaluru – 560 076
Ph: 91-80-26687216, 26687223; Fax: 91-80-2668 7170
Email: vmsrf@vmsrf.org Website: www.vmsrf.org

November 2012

Ref. /File No. **BT/PR11910/BRB/10/697/2009**

PROFORMA FOR THE PROGRESS REPORT OF THE ON-GOING PROJECTS

Title of the Project. **"Chemically synthesized derivatives of Boswellic Acid as modulators of cytokine mediated cell signalling: implication in chronic inflammatory diseases"**

1. Principal Investigators and Co-Investigators.

a) Dr. Latha Diwakar
Scientist
Vittal Mallya Scientific Research Foundation
94/3 & 94/5, 23rd Cross, 29th Main,
BTM II Stage, Bengaluru – 560076
Ph:080-26687216 Fax: 080-26687170
E-mail: latha@vmsrf.org

b) Dr. Anil Kush
Research Director
Vittal Mallya Scientific Research Foundation
94/3 & 94/5, 23rd Cross, 29th Main,
BTM II Stage, Bengaluru – 560076
Ph:080-26687216 Fax: 080-26687170
E-mail: vmsrf@vmsrf.org

c) Dr G. Chadrashekar Reddy
Research coordinator
Vittal Mallya Scientific Research Foundation
94/3 & 94/5, 23rd Cross, 29th Main,
BTM II Stage, Bengaluru – 560076
Ph:080-26687216 Fax: 080-26687170
E-mail: gcreddy@vmsrf.org

d) Dr.Puja Ravikumar
Scientist
Vittal Mallya Scientific Research Foundation
94/3 & 94/5, 23rd Cross, 29th Main,
BTM II Stage, Bengaluru – 560076
Ph:080-26687216 Fax: 080-26687170
E-mail: puja@vmsrf.org

2. Implementing Institutions and other collaborating Institutions.

Vittal Mallya Scientific Research Foundation
94/3 & 94/5, 23rd Cross, 29th Main, BTM II Stage,
Bengaluru – 560076
Ph: 080-26687216 Fax: 080-26687170.

3. Date of commencement (Date of sanction order/receipt of grant).

No. BT/PR11910/BRB/10/697/2009 dated 29th October, 2010

4. Planned date of completion. **November 2013**

5. Proposed Objectives.

1. Exposure of synovial sarcoma cells to IL-1 and to investigate the role of signaling pathways like MAPK or ERK in the induction of NFkB which is key molecule in inflammation leading to cyclooxygenase expression.
2. Expression different cytokines like VEGF, ICAM, MMP-1 and MMP-2.
3. Extraction of boswellic acid and acetyl keto boswellic acid from gum serrata of *Boswellia Serrata* as well as to prepare chemically synthesized derivatives.
4. Development and evaluation of in vivo model for arthritis induced by complete freund's adjuvant (CFA) and carrageenan induced inflammatory in rat.
5. Effect of boswellic acid derivatives in synovial sarcoma cells in regulating signaling pathway in induction of NFkB and cyclooxygenase expression.
6. Effect of Boswellic acid derivatives in animal model of arthritis and inflammation.

6. Deviation made from original objectives if any, while implementing the project and reasons thereof.

a. Expression studies of different markers of MAPK/ERK signalling pathways which were proposed earlier could not be done because sanctioned money is less. The expression studies were done only for important inflammatory marker ‘NFkB’.
b. Instead of cytokine MMP-9 in study MMP-2 was selected as synovial sarcoma does not express MMP-9.
c. Arthritic study was done in Adjuvant induced model in Wistar rats as we could achieve complete arthritic induction.

7. Up-dated progress achieved vis-à-vis time schedule of objectives proposed.

Objectives proposed	Progress achieved
Extraction of boswellic acid and keto boswellic acid from gum serrata of *Boswellia Serrata* as well as to prepare chemically synthesized derivatives.	Boswellic acid and ketoboswellic acid extracted in pure form was coupled with few more NSAIDs like Indomethacin and Acelofenac
Development and evaluation of in vivo model for arthritis induced by complete freund's adjuvant (CFA) and carrageenan induced inflammatory in rat.	Wistar rats were injected with complete freund's adjuvant to induce complete arthritis and model was established by observing arthritic score, Cyclooxygenase activity and histopathology. Acute inflammation was achieved by injecting carrageenan in to paw of wistar rats and increased paw edema was measured by plethysmometer.

8. Experimental work giving full details of experimental set up, methods adopted, data collected, supported by necessary tables, charts, diagrams, photographs.

Background

Boswellic acid (BA) is an active component of *Boswellia serrata* and its derivatives has been studied extensively and has been used in the Ayurvedic system of medicine for the management of rheumatism, respiratory diseases, and liver disorders.

In present study we propose to synthesize hybrid molecules by hooking known anti inflammatory compounds to boswellic acid and keto boswellic acids to produce synergistic effect leading to enhanced activity. Project is focused on expression studies of specific targets for better understanding of inflammation in pathogenesis of disease.

Methodology and Results

a) Boswellic and Ketoboswellic hybrid molecules with NSAIDs

Total acid fraction is pharmacologically significant to give the therapeutic benefit. In our method we first purify 90% organic acids raw material in the chromatographic method from gum resins. These fractions were again subjected to repeated column chromatography over silica gel and eluted with organic solvent in different proportions furnishes individual component into pure forms. Crude extract of acids converted were into methyl esters of boswellic acids. Separated to pure individual compounds by subjecting them to silica column chromatography. The purified boswellic acid and ketoboswellic acid was combined with two non steroidal anti-inflammatory drugs Indomethacin and Aceclofenac. The hybrid molecules were further purified with thin layer chromatography. The purified samples were subjected to IR, NMR and LC-MS analysis to confirm the proper formation/ hooking of hybrid molecules (Fig 1,2,3).

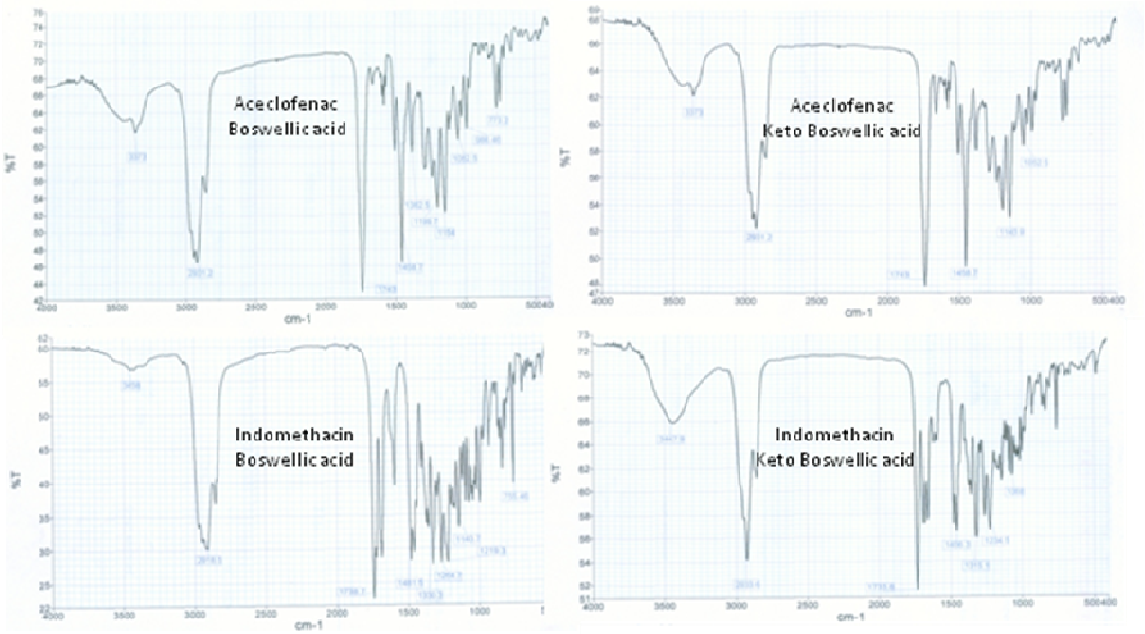

Fig. 1 IR-spectrum of boswellic and keto boswellic acid hybrid molecules with NSAIDs

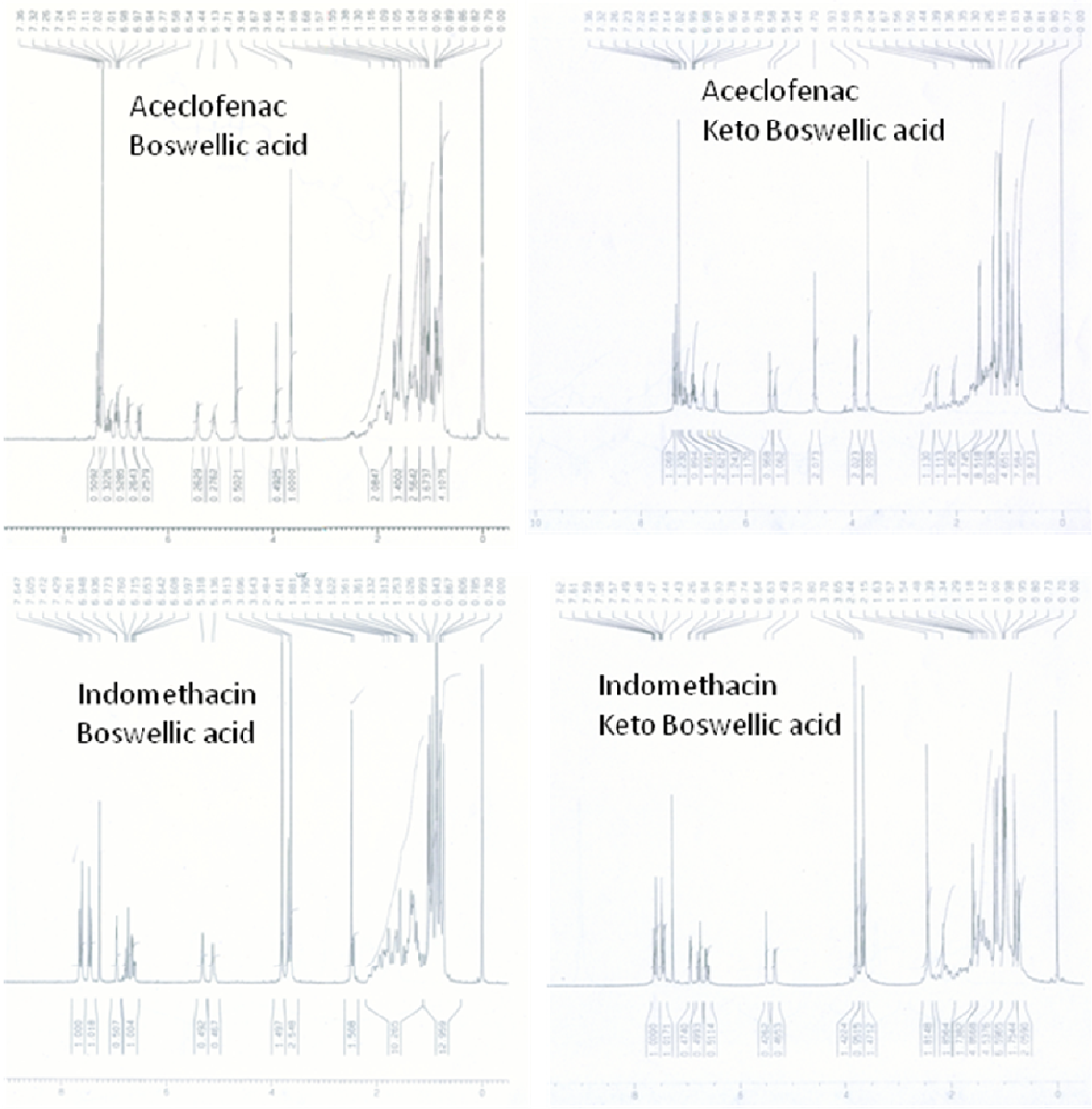

Fig. 2 NMR analysis of boswellic acid and keto boswellic acid hybrid molecules with NSAIDs.

The NMR analysis of hybrid molecules revealed proper identification of boswellic and keto boswellic acids with Aceclofenac and Indomethacin. IR-spectrum showed identification of carbonyl ester groups in both the form of hybrid molecules of boswellic acids.

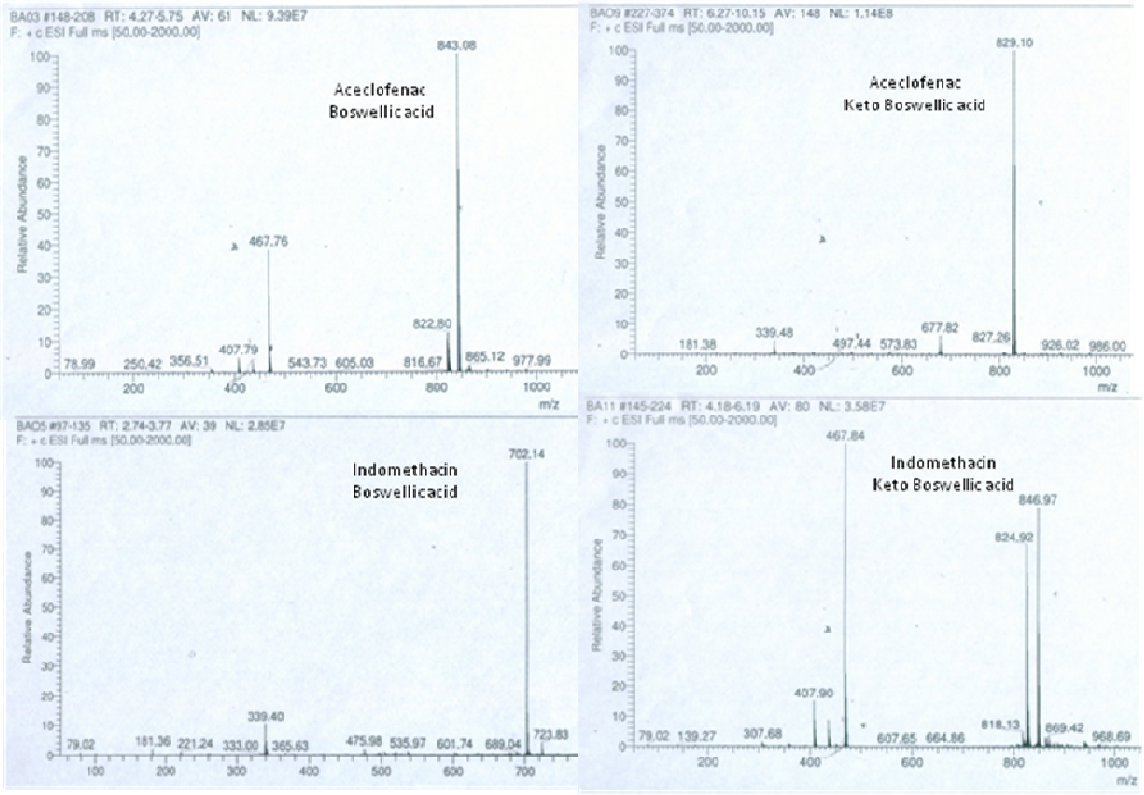

Fig. 3 LC-MS analysis of boswellic acid and keto boswellic acid hybrid molecules with NSAIDs.

b) Carrageenan induced inflammatory model in rats

Animal experiments were approved by the Institutional Animal Ethics Committee. 3-4 months male Wistar rats were obtained from animal house. To produce acute inflammation, carrageenan, 2 mg in 0.1 ml saline, was injected subcutaneous into the plantar region of the rat right hindpaw. The edema of the right hindpaw was measured using the plethysmometer before treatment and at 2, 4, 6 h and 24 hrs after treatment (Fig.4). The model of inflammation used, the carrageenan induced edema, is a commonly used model for studies of inflammation and for testing novel anti-inflammatory drugs.

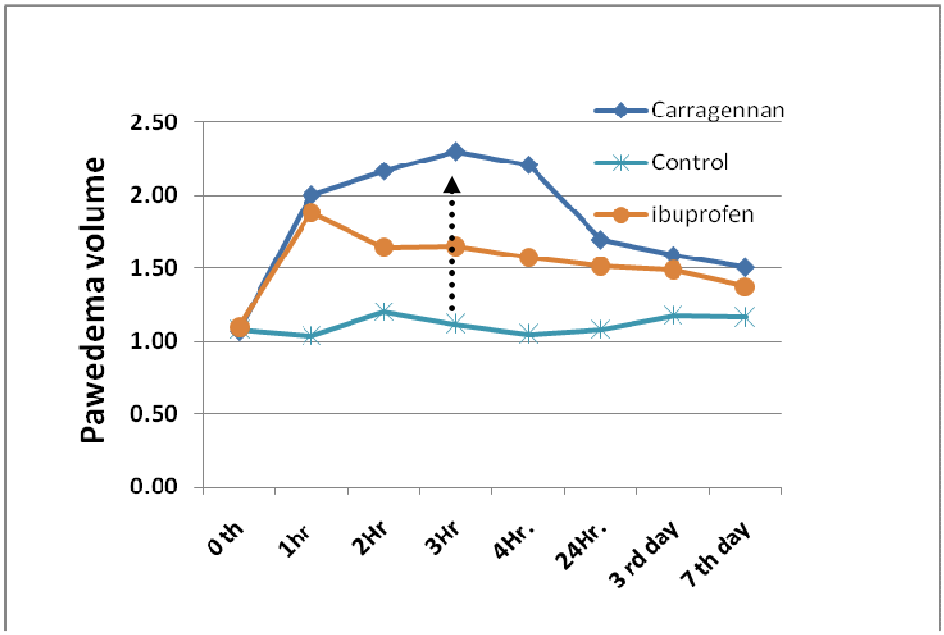

Fig. 4. Increase in paw volume after inducing with carrageenan for inflammatory model in rat.

c) Complete Freund's Adjuvant induced arthritis in rats

Animal experiments were approved by the Institutional Animal Ethical Committee. Wistar rats 3-4 months old were obtained from animal house. Complete Freund's adjuvant (CFA-200 ul), heat-killed mycobacterium in mineral oil at 3 mg/mL from sigma was injected intradermally to base of hind paw. Arthritic score was measured morphologically and graded from 0-4. Cyclooxygenase activity was measured from blood sample collected at different time points. General parameters like feed intake (Fig. 5) and water intake (Fig. 6) was measured for whole study period of three months. Rats were sacrificed after three months of induction and histopathology of joints was done by haematoxylin eosin staining to study the changes in synovial membrane and cartilage.

There was no much change in feed and water intake when rats were induced arthritis with CFA. All the five animals showed induction of arthritis of grade-2 by 7 days, four animals showed complete induction of grade 4 by 21 days of induction and all the animals were having complete arthritis (Fig. 7).

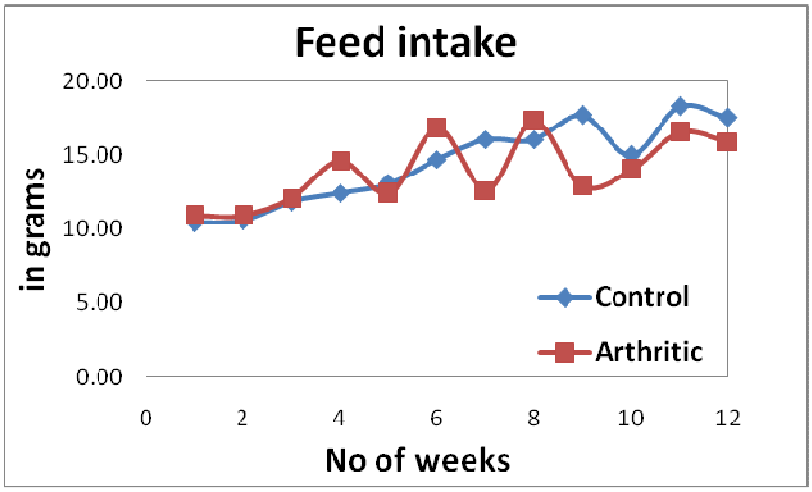

Fig. 5. Feed intake after complete freund's adjuvant induced arthritis in rats for 3 months.

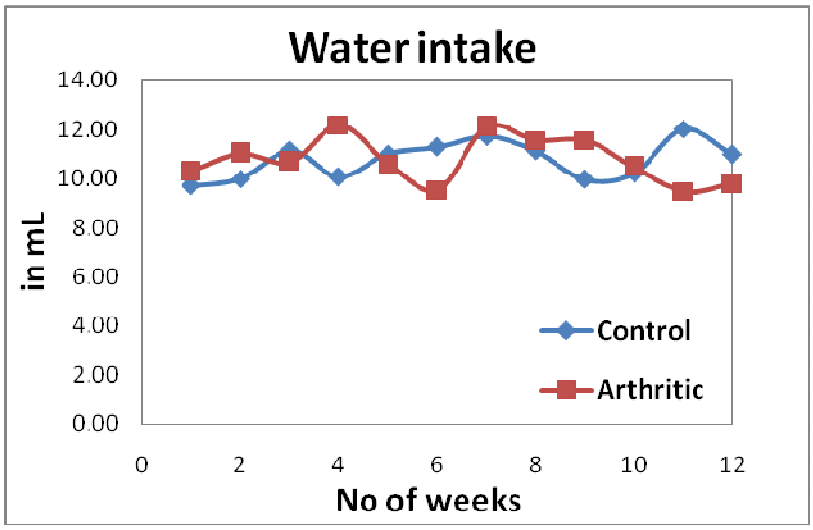

Fig. 6. Water intake after complete freund's adjuvant induced arthritis in rats for 3 months.

Histopathology of joints showed erosion in the structure of cartilage and fibrillation in cartilage matrix. In synovium, there was pannus formation with multiple epitheloid granulomata showing impression of granulomatus arthritis (Fig.7).

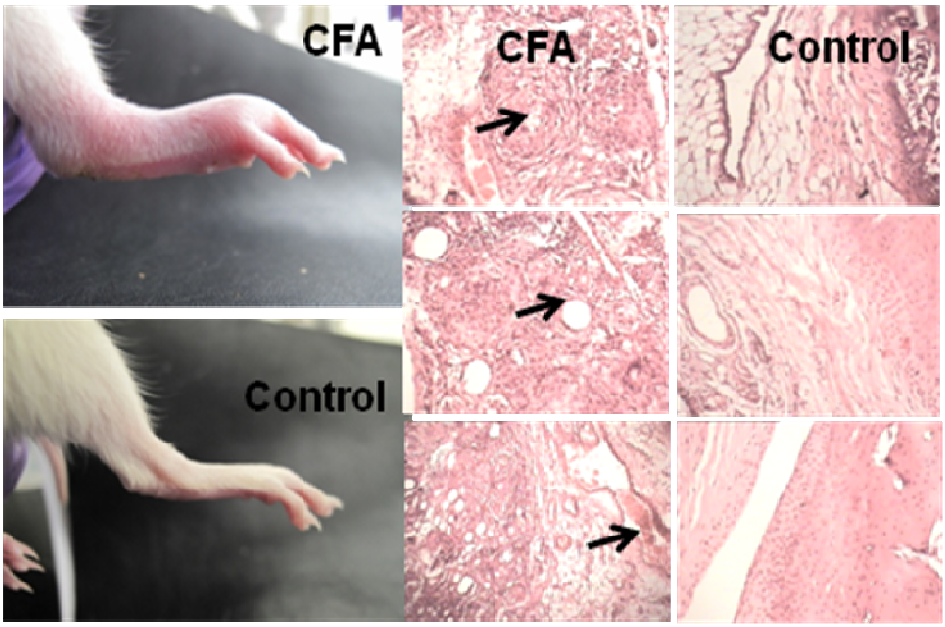

Fig.7. Haematoxylin eosin staining after complete arthritic induction showing bone and cartilage erosion, pannus formation in Wistar rat joints.

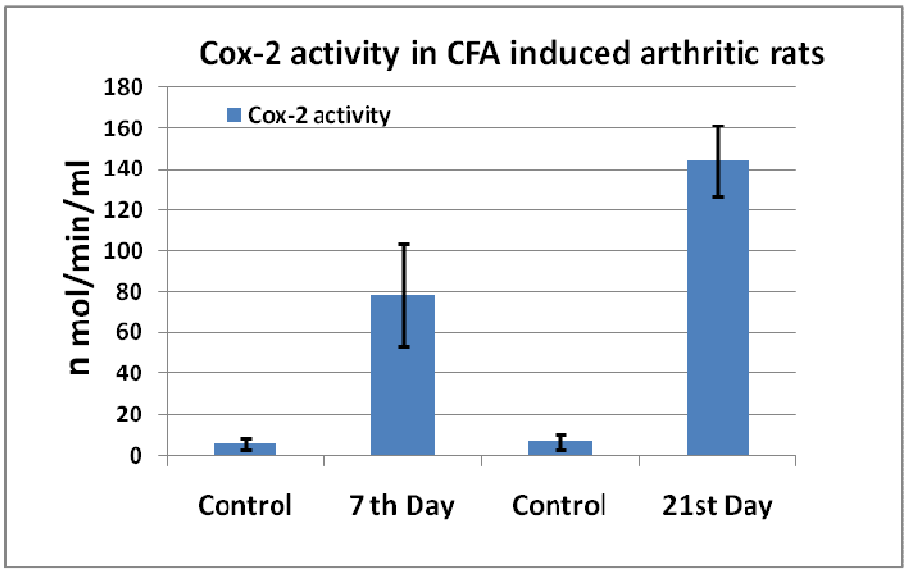

Fig. 8. Cyclooxygenase activity in Complete Freund's adjuvant induced arthritis rats at different time points.

9. Detailed analysis of Results indicating contributions made towards increasing the state and knowledge in the subject.

In present study we have synthesized hybrid molecules by hooking known anti inflammatory compounds to Boswellic acid and keto boswellic acids to produce synergistic effect to get enhanced activity. Further studies are focused on expression studies of specific targets in cell signaling pathway for better understanding of inflammation in pathogenesis of disease. The enrolment of the synthesized hybrid molecule may facilitate in better understanding of modulations in inflammatory response. Consequently, present work adds value in developing novel therapeutic strategies for treating chronic inflammatory diseases. We are reporting for first time involvement of hybrid molecules in establishing NFkB induction through IL-1 signalling in human synovial sarcoma cell lines. We are able to purify more than 90% pure boswellic and keto boswellic acid on improvising the standardized method available in literature.

10. Conclusions summarizing the achievements (Not more than 150 words).

We were able prepare pure form of methyl esters of boswellic acids as validated by NMR. We further hooked known NSAIDs aceclofenac and indomethacin purified them on thin layer chromatography and validated by NMR, IR and LC-MS analysis. All the analytical technique supported the hooking and validated structure of hybrid molecules. These molecules will be evaluated for synergistic effect in synovial cells and *in vivo* animal models next year. Carragennan induced acute inflammatory model was standardized and increased paw volume was measured by plethysmometer. Adjuvant induced arthritis model was established after inducing with complete freund's adjuvant showed complete arthritis. Chronic inflammatory condition in arthritic rats showed increased cyclooxygenase activity. Morphological grading of arthritic scoring from 0-4 showed that at the end of 21 days of induction complete arthritis was observed. Histopathological evaluation of knee joints of arthritic rats showed impression of arthritis.

11. Scope for future work.

Many derivatives of boswellic and keto boswellic acids have been hooked till now and evaluated for anti inflammatory activity have been reported in literature. However in present study the linkage used is to facilitate easy separation of acid moiety and NSAIDs moiety to have synergistic effect. The hybrid molecules synthesised will be evaluated for 'NFkB' activation after IL-1 induction in synovial sarcoma cell lines. Since animal model for inflammation and arthritis have been established, hybrid molecules will be evaluated for their anti arthritic activity. Such detailed evaluation from cell system to animal model may facilitate in better understanding of modulations in inflammatory response. Hence present work adds value in developing novel therapeutic strategies for treating chronic inflammatory diseases.

12. Likelyhood of product/process development, with time frame.

Hybrid molecules of pure boswellic and keto boswellic acid with four different NSAIDs (Ibuprofen, Indomethacin, Acelofenac and Naproxen) were synthesised. The molecules were subjected to different analytical parameters like NMR, IR and LC-MS established the credibility of structure. Acute inflammatory induced by carrageenan was established in Wistar rats. Adjuvant induced complete arthritis was evaluated by histopathology and other arthritic parameters established *in vivo* animal model for arthritis. The progress of the project is according to the time frame against committed objectives.

13. **Brief summary (not more than one Para) of significant outcome/achievement during the period**

The hybrid molecules for two NSAIDs Indomethacin and Aceclofenac were prepared for both the forms of boswellic acid and keto boswellic acids, validated through NMR, LC-MS and IR analytical analysis. All the above techniques proved the structure and formation of hybrid molecules. As per the

objective all the four NSAIDs were hooked and validated by analytical techniques. Acute inflammatory model with carragennan induction was standardized and increased paw volume was measured by plethysmometer. Complete arthritis was achieved by Adjuvant induced arthritis model showed swollen joints and morphologically graded at different time points from 0-4 over a period of 21 days. There was increased edema and cyclooxygenase activity indicating chronic inflammatory response. Haematoxylin eosin staining showed cartilage disruption, bone erosion and pannus formation indicating impression of grannulomatous arthritis. The progress of project is according to committed objective for two years of project period. Further these hybrid molecules will be evaluated next year for anti-inflammatory activity *in vitro* in synovial sarcoma cells and *in vivo* in animal model.

14. **List of Publication/patents granted or applied**

Manuscript under preparation

Name and Signature and Date

_____

(Dr. Latha Diwakar -Principal Investigator)

_____

(Dr.G.C.Reddy- Co-Investigator)

_____

(Dr.Puja Ravikumar- Co-Investigator)