7.1 Summary and Conclusion

This study describes the evaluation anti-arthritic activity of *Annona squamosa*, *Annona reticulata* and *Annona muricata*. Inflammation is typically a body’s self-protective mechanism that is triggered in response to noxious stimuli or infection to guard the body and to accelerate the recovery process. However the neglected acute inflammation leads to the chronic inflammation disorders. Arachidonic acid (AA) metabolism plays a crucial role in inflammatory process and associated diseases. Most of the anti-inflammatory medications inhibit the lipoxygenase or cyclooxygenase pathways and these two pathways are potential interventions against inflammation. Most of the anti-inflammatory drugs such as steroids and COX inhibitors are often associated with adverse effects like gastrointestinal irritation, ulcers, hypertension and cardiovascular problems.

Herbal medicines have been extended impetus use for arthritis in the recent years. The COX inhibitors have some concern over the use for therapeutic intervention, some of the COX-2 inhibitors either withdrawn from the market or warned by the US FDA. 5-LOX inhibitors of herbal origin on the other hand are reported to offer significant relief and devoid of adverse effects 5-LOX inhibitors are thus becoming first choice of treatment for chronic inflammatory disease such as arthritis. TNF-α is a pleotropic inflammatory cytokine produced by the immune system that suppresses tumor cell proliferation and is a key mediator of inflammation. IL1-α and IL1-β triggers the prostaglandin E2 and can stimulate T cell proliferation and is potent mediator in response to infection and injury.
In the present study, extracts of the selected medicinal plants were analyzed as a screening of chronic inflammatory disease like arthritis. The plants taken for the study include traditionally used three medicinal plants namely *Annona squamosa*, *Annona reticulata* and *Annona muricata*. The complete review elucidates literature of selected medicinal plants *Annona squamosa*, *Annona reticulata* and *Annona muricata*. In which the historical perspective of medicinal plants, general characteristics and scientific classification of three plants, also describes about phytochemical and pharmacological actions of selected three plants.

The phytochemical constituents present in all the extracts of the selected medicinal plants analyzed as a preliminary study. The plants taken for the study include traditionally used three medicinal plants namely *Annona squamosa*, *Annona reticulata* and *Annona muricata*. The leaves of three plants were collected and bioactive extracts was extracted with different solvents such as hexane, ethyl acetate, methanol, aqueous methanol and water by using soxhlet apparatus.

The qualitative phytochemical analysis showed the presence of different bioactive compounds phytosterols, triterpinoids, glycosides, tannins, phenols, alkaloids, saponins, flavonoids, carbohydrates, proteins and lipids whereas the common phytochemical constituents among the *A. squamosa*, *A. reticulata* and *A. muricata* are glycosides, tannins, alkaloids, saponins, flavonoids, proteins, lipids and carbohydrates. However, the methonolic extracts of all the plants showing presence of more phytochemical constituents when compared to other extracts. Quantitative estimation of all extracts and its fractions reveals that methanolic extracts has more phenolic and flavonoid content than its fractions hexane, ethyl acetate, aqueous methanol and aqueous extracts.
The in vitro analysis describes antioxidant properties of hexane, ethyl acetate, methanolic, aqueous methanol and aqueous extracts of all the three plants by using in vitro DPPH, Superoxide, ABTS, DMPD free radical scavenging and FRAP antioxidant power assay. Methanol, aqueous methanol and water extracts showed a significant scavenging activity all the antioxidant assays, however the hexane and ethyl acetate extracts failed to exhibit the potent free radical scavenging activity. The anti-inflammatory activity of the all the extracts of three plants was screened by using 5-Lipoxygenase enzyme inhibitory and TNF-α inhibitory action in LPS induced cytokine model in THP-1 cells.

The methanol, aqueous methanol and water extracts of three study plants showed potent 5-Lipoxygenase enzyme inhibitory activity when compared to the extracts of hexane and ethyl acetate. The methanol, aqueous methanol and ethyl acetate extracts of three study plants showed potent TNF-α pro-inflammatory cytokine inhibitory activity when compared to the extracts of hexane and aqueous extracts. The brine shrimp lethality test for screening the cytotoxicity effects of Annona squamosa, Annona reticulata and Annona muricata plant leaves extracts.

The Hexane fraction of three study plants showed potent cytotoxic effect against the brine shrimp nauplii. Whereas other fractions of three study plants also showed cytotoxic effect but when compared to standard phodophyllotoxin its effect is less. The anti-diabetic and anti-obesity properties of three plant leaves extracts was accessed by α-Amylase and α-Glucosidase enzyme inhibitory assay. The methanol, aqueous methanol and water extracts of three study plants showed potent α-Amylase and α-Glucosidase enzyme inhibitory activity when compared to the extracts of hexane and ethyl acetate. Nonetheless, the methanol extracts of three study plants
(ASME, ARME and AMME) was revealed potent activity. However, basing on this screening of selected three plant extracts against anti-oxidant screening, LPS activated THP-1 monocyte cell TNF-α inhibitory activity, 5- LOX inhibitory activity and cytotoxicity prioritize the methanolic extracts for their in depth in vivo anti-inflammatory and pyretic studies.

The toxic and mutagenic effects of the prioritized methanolic extracts of Annona squamosa, Annona reticulata and Annona muricata plant leaves based in vitro screening. The in vivo studies first started with acute oral toxicity study according the OECD guideline 423. Acute oral toxicity study was carried out at a dose of 2000 mg/kg body weight was found to be nontoxic. The mutagenic effects of the selected methanolic extracts of three plants screened by using two different methods namely micronucleus test (OECD 474) and chromosomal aberration test (OECD 475). The two methods was carried with 2000 mg/kg body weight dose orally did not showed any mutagenic effects. The above results suggest that, the selected plant methanolic extracts are safer for therapeutic use without any toxic or mutagenic effects.

The therapeutic properties explain the selected plant methanolic extracts mainly deals with anti-inflammatory and anti-pyretic activity. Effect of methanolic leaf extract of A. squamosa, A. reticulata and A. muricata at 100 and 200 mg/kg screened for anti-pyretic activity on brewer’s yeast induced rectal temperature in rats. The three methanolic fractions maximum effect disclosed at dose of 200 mg/kg. The antipyretic effect started as early as 1st hour and the effect was maintained for 4th hour, after its administration. The standard drug paracetamol 100 mg/kg and tested drugs ASME, ARME and AMME fractions were significantly reduced the yeast-
elevated rectal temperature, at 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} hour compared to control group. However, the AMME fraction showed better rectal temperature reduction when compared to other two plants.

The acute anti-inflammatory activity of the selected ASME, ARME and AMME extracts were performed in an inflammation induced animal models using inflammatory agents like carrageenan, egg albumin and xylene with doses at 100 and 200 mg/kg. The results were compared with the inflammation control and with the activity of standard anti-inflammatory drugs (Indomethacin in carrageenan and egg albumin induced paw oedema; Dexamethasone in xylene induced ear oedema). The results revealed that the activity of AMME was found to be high in reducing the inflammation then the other two extracts ASME and ARME.

The chronic anti-inflammatory activity of the selected ASME, ARME and AMME extracts were carried out by inducing FCA induced rat model at the doses of 100 and 200 mg/kg. The chronic inflammation was measured accessing the paw oedema, pro-inflammatory cytokines, inflammatory mediators, biochemistry, heumatology, histopathology and radiography. The adverse effect compounds ASME, ARME and AMME on liver and kidney was mark off by screening by biochemistry and the antioxidant properties was measured by estimating the levels of MDA, catalase, SOD and reduced glutathione in liver. All the compounds exhibited potent dose dependent significant activity ion reducing the paw oedema, which is comparable to positive control, However the \textit{Annona muricata} extract showed the highest reduction property against the arthritis when compared to other selected plant extracts.
The biochemical parameters measurement provides an excellent and simple tool to assess the anti-arthritic activity. The levels of AST, ALT, ALP, Total Bilurubin, Total Protein, Albumin, Cholestrol, Triglycerides, Creatinine, Urea and CRP levels of serum also significantly reduced in all the treated groups when compared to control. Which boosters the anti-arthritic constraining nature of ASME, ARME and AMME. Erythrocyte sedimentation rate (ESR) is an indirect measure of acute phase response to determine activity of rheumatoid arthritis, the increased levels of ESR was significantly restored by ASME, ARME, AMME (100 and 200 mg/Kg). The increase in RBC, haemoglobin and packed cell volume (PCV) and the restored levels of WBC and platelet count was clearly observed in treated groups dose dependently when compared to the adjuvant induced arthritic control.

The inflammatory cytokines TNF-α and IL-1β, inflammatory mediators PGE₂ and LTB₄ were analyzed in serum of all the experimental rats on day 35. In the present study ASME, ARME and AMME 100 and 200 mg/Kg treated animal serum samples showed dose dependent and significant reduction in TNF-α, IL-1β, PGE₂ and LTB₄ levels in serum and the results can be comparable with positive control. Hence, the three plant extracts exhibited potent significant activity in suppressing the pro-inflammatory cytokines and inflammatory mediators in adjuvant induced arthritis. Reduction of PGE₂ represents the inhibition of COX pathway and reduction of LTB₄ represents the inhibition of LOX pathway. As the rheumatoid arthritis is an autoimmune disease the inhibition of PGE₂ of COX pathway and LTB₄ of LOX pathways could help in ameliorating the effect of disease by controlling both pain and neutrophil activity. Further analysis of liver samples was performed to determine the
whether the ASME, ARME and AMME can reduce the oxidative stress condition which is generally observed in rheumatoid arthritis.

This study involves the assay of levels of LPO, Catalase, SOD and GSH. The compounds clearly and significantly decreased the dangerous levels of LPO and also significantly increased the levels of anti-oxidant enzymes catalase, SOD and GSH dose dependently. The results implies the compounds are potent anti-oxidants which might be the active principle behind the preventive role exerted by the ASME, ARME and AMME on rheumatoid arthritis development as observed in our study. In histopathological study the neutrophil infiltration, synovial hyperplasia, ankyloses, pannus formation, rough cartilage surface, small cracks and clumps of cartilage tissue and bone erosion were restored to normal stage in test item treated groups at 100 and 200 mg/Kg. The radiological analysis of the tibio tarsal joint in control and extracts treated animals further supports and confirms the potent anti-arthritic effect of Annona squamosa, Annona reticulata and Annona muricata in a dose dependent manner and it suppress the pathological changes such as pannus formation, bone destruction, degree of bone resorption and diminished joint space.

In conclusion from all these observations the methanolic leaves extracts of Annona squamosa, Annona reticulata and Annona muricata possess significant anti-oxidant, anti-pyretic, anti-inflammatory and anti-arthritic activity. This anti-inflammatory and anti-arthritic activity is might be due to the 5-Lipooxygenase enzyme inhibitory action along with the Cycloxygenase pathway (PGE₂). In our present investigation we have find out that there are many bioactive compounds in leaf methanolic extract mainly flavonoids, phenols, saponins, terpenes and glycosides etc. for pharmacological evaluations. It is concluded that the leaf extracts of Annona
squamosa, Annona reticulata and Annona muricata exhibited significant anti-arthritic activity without any adverse effects and these can be used for the treatment of rheumatoid arthritis.

The present study results reveals that good inhibitory activity in both acute and chronic inflammatory process similar to COX inhibitors and hence the methanolic extracts of selected plants were selected basing on 5 LOX inhibition and antioxidant property can be used in amelioration of arthritis without any side effects like mutagenic, hepatotoxic and nephrotoxic effects as generally we come across with the marketed COX inhibitors and the extracts also having anti pyretic activity. This research work opens new path for the treatment of chronic inflammatory diseases like rheumatoid arthritis by inhibiting the 5 LOX pathway. The antioxidant activity exerted by these extracts may be a part of their mechanism in preventing inflammation and arthritis. Even though animal models appeared to show effective and promising results in anti-inflammatory and anti-arthritic it is necessary for a detailed molecular study for the better understanding of the mechanism of action as well as specific compound involved.