Exploration and Development of Oral Herbal Compositions for Treating Rheumatoid Arthritis

THESIS

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By

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CERTIFICATE

This is to certify that the thesis titled **"Exploration and Development of Oral Herbal Compositions for Treating Rheumatoid Arthritis"** submitted by **Samrun Nessa** ID No**. 2017PHXF0404H** for award of Ph.D. of the Institute embodies original work done by her under my supervision.

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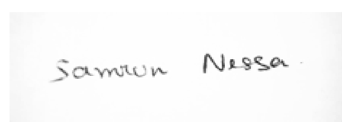
DECLARATION

I hereby declare that the work which is being presented in the thesis entitled "**Exploration and Development of Oral Herbal Compositions for Treating Rheumatoid Arthritis"** submitted by **Samrun Nessa** ID No **2017PHXF040H** for award of Ph.D. of the Institute embodies original work done. Any relevant material taken from open literature has been referred and cited as per established ethical norms and practices. The matter embodied in this thesis has not been submitted by me for the award of any other degree of any other University/Institute.



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Abstract

The aim of the study was to explore and develop oral herbal compositions for the treatment of Rheumatoid arthritis (RA). RA is a systemic chronic autoimmune disease characterized by inflammation of the synovium, synovial hyperplasia, angiogenesis and cartilage damage. Clinical treatment of RA primarily involves combinations of NSAIDs, glucocorticoids, and DMARDs. However, the long-term use of these drugs causes serious side-effects such as immunosuppression, gastrointestinal ulcers, osteoporosis, nausea, fatigue, cytopenia, rashes, liver damage, infections, psoriasis, etc., and therefore adds a limitation on their use for chronic conditions. Additionally, the high cost of DMARDs and the chronic nature of RA increases the economic burden on RA patients. Hence, there is a need to develop novel therapeutic strategies and cost-effective alternatives that are safe, efficacious and affordable. Herbal therapy is considered an alternative to conventional medicines due to their efficacy and lesser side effects and in some cases proved to be adjunct therapy.

For developing the oral herbal compositions for treating RA, two plants were selected. The major ingredient was *Halodule pinifolia,* a marine seagrass that grows in the backwaters of Bay of Bengal. This was selected for the present study based on the pro-inflammatory cytokine inhibition property exhibited by it in a pilot study conducted in our lab. After extensive literature study, another plant *Glycyrrhiza glabra* (liquorice), elaborated for its anti-inflammatory activity was selected in an attempt to understand if there would be an incremental effect in inhibiting the pro-inflammatory cytokines when the extracts of *H. pinifolia* and *G. glabra* were combined.

Further, considering the limitations encountered with the multi-herbal products during their standardization and formulation processes, two more HP-based compositions using anti-inflammatory natural compounds (glycyrrhizin and mono ammonium glycyrrhizinate) were designed and evaluated in the present study. Thus, three novel compositions were prepared using the medium polar extract of whole grass (HP) in combination with (i) polar extract of liquorice (LQ) (composition 1), (ii) glycyrrhizin (Gly) (composition 2) and (iii) mono ammonium glycyrrhizinate (MAG) (composition 3). Initially, the individual ingredients and the compositions were screened for their pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and NO inhibition

effect under in vitro (ELISA and Griess method) in LPS-stimulated RAW 264.7 cell lines. The results revealed significant pro-inflammatory cytokines and NO inhibition effect by all the tested samples with the compositions showing synergistic effect (CI score <1) and based on which 1:0.5 was fixed as the ratio of mixing of HP and LQ/Gly/MAG for the in-vivo experiments.

The samples HP and LQ used for the study were standardized by HPLC method using their anti-inflammatory constituents as marker compounds (rosemarinic acid, vanillic acid, ethyl protocatechuate and glycyrrhizin). Identification of these anti-inflammatory markers in HP and LQ signaled to further explore these extracts in in-vivo models of inflammation and arthritis. The heavy metal test on HP and LQ extracts using ICP-MS analysis showed the presence of As, Cd, Hg and Pb within the regulatory threshold values. Further, the acute toxicity study performed on Balb/c mice showed no adverse action or health-related acute toxicity due to the oral administration of HP (2000 mg/kg body weight). Thus, HP, a pro-inflammatory cytokine and NO inhibitory extract was proved to be safe for oral administration.

To evaluate the efficacy of compositions and their individual components under LPS-induced mouse endotoxemia model and carrageenan-induced mouse paw oedema model, oral suspension of 100 mg HP combined with 50 mg LQ/Gly/MAG (C1/C2/C3) was prepared (1:0.5 ratio optimized through in-vitro results). Results showed a significant decrease in LPS-induced plasma IL-1β, IL-6 and TNF-α levels by all. Further C1 and C2 reduced the paw oedema for 4 h and all compositions exhibited the attenuation of the mRNA expressions of cytokines, NO production, MPO activity and protein expression of NF-κB in paw tissue homogenate under carrageenan-induced oedema model. Thus, HP and the HP-based oral compositions were discovered as anti-inflammatory agents that could have potential anti-arthritic activity.

To prove this, HP, LQ and C1 were evaluated under Freund's complete adjuvant arthritis model. Results revealed that the oral treatment of arthritis-induced rats with HP, LQ and C1 for 14 days significantly ($P<0.001$) reduced the rat paw oedema, spleen index, controlled the hematological parameters, cytokine levels (IL-1β, IL-6, TNF-α), mRNA expression of cytokines and osteoclast markers (RANK, TRAP and Cathepsin K). HP showed better results among all. Histopathology and radiological scanning demonstrated lesser joint deterioration in sample-treated rats, as evident phenotypically. The downregulation of CD51 and MMP-3 corroborated the anti-arthritic effect of HP, LQ and C1.

Further, in order to improve the activity of C1, a lipid nano-emulsion of composition 1 containing HP and LQ in the same ratio of 1:0.5 (C1-N) was prepared. C1-N, a novel formulation was characterized for its particle size, PDI, zeta potential and stability and compared with that of C1.

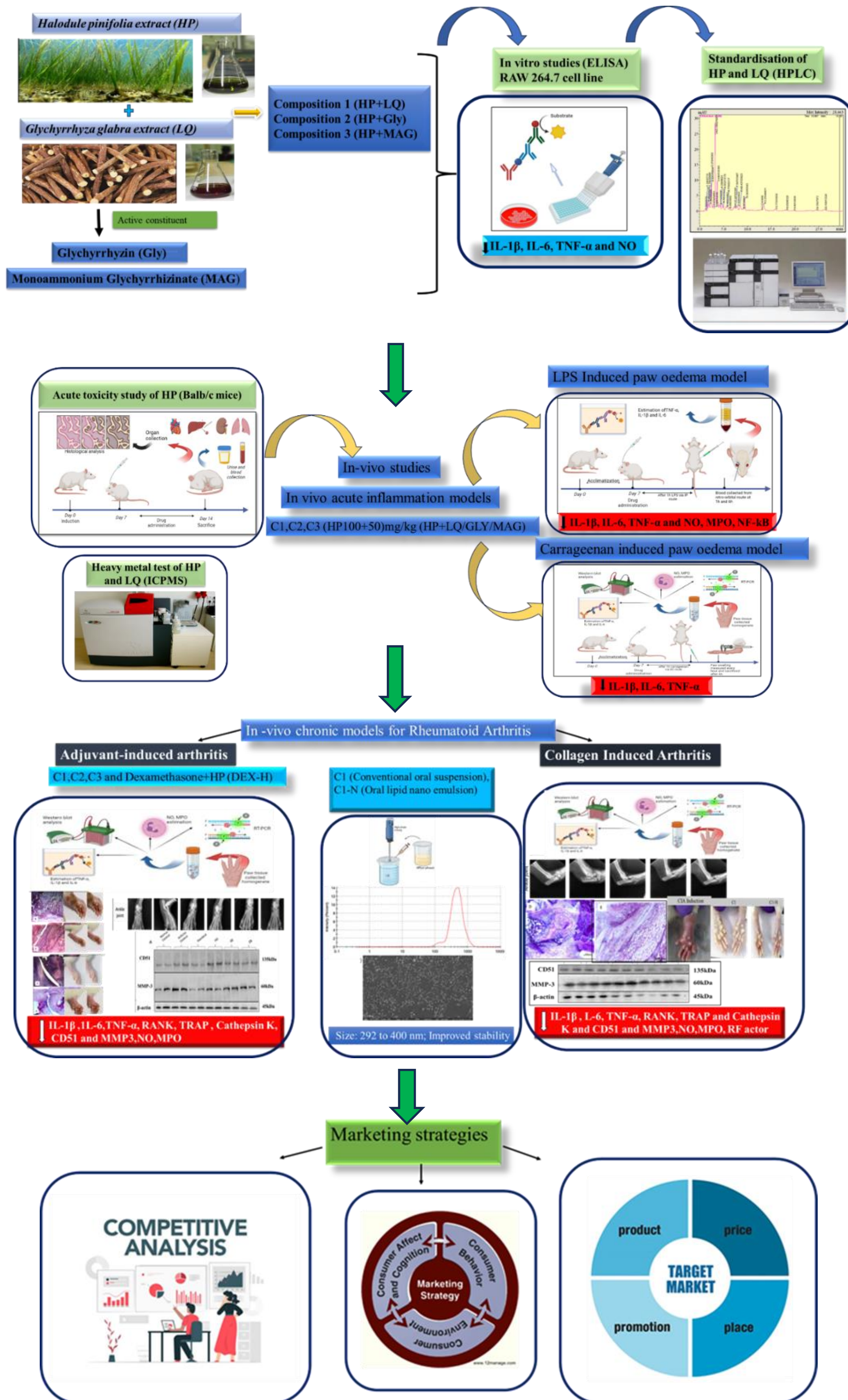
Both C1 and C1-N were found to be potentially active under collagen-induced arthritis model as evidenced by their effect on paw oedema, spleen index, hematological parameters, RF, cytokines, osteoclast markers, histology and X-rays. The results proved that nanonisation (292 to 400 nm) and formulation of C1 as lipid nano-emulsion (C1-N) improved the stability as well as the activity. Furthermore, evaluation of anti-arthritic activity (Freund's adjuvant model) of C2 and C3 was continued along with another novel combination DEX-H, containing 100 mg of HP + 0.05 mg of dexamethasone. DEX-H was designed to discover an alternative medicine having steroid-sparing effect that could reduce the usage of dexamethasone for chronic autoimmune diseases and thereby reduce the side effects in patients. It was interesting to observe treatment with DEX-H, C2 and C3 suppressed the arthritic progression and the effect was comparable to DEX alone treated group. DEX-H, C2 and C3 showed marked pro-inflammatory cytokines inhibition and downregulation of osteoclast markers. Paw swelling was significantly ($P<0.0001$) decreased by administering DEX-H and C2. The decrement in plasma concentration of IL-6 and TRAP by DEX-H, C2, and C3 (ELISA) was similar to that of DEX. The downregulation of mRNA expression of IL-1β by C2 (5.8-fold), Cathepsin K by DEX-H (6.3-fold) and C3 (4.2-fold) was higher than DEX alone treated group (IL-1β: 3.7-fold and Cathepsin K: 3.6-fold) which highlighted the steroid-sparing effect of the novel compositions. These results were supported by the radiographic and histological results of the ankle joint in arthritis. The expressions of CD51 and MMP-3 in western blot analysis were found to be downregulated by all the treatment groups with DEX-H and C2 showing a higher effect. The results reflected the potential of the new combinations as an alternative therapy for rheumatoid arthritis. Despite the reduced amount of dexamethasone present in DEX-H i.e. contained half that of DEX, a similar effect was observed between them, which proved the steroid-sparing effect of DEX-H.

Thus, the research explored the anti-arthritic effect of HP for the first time and yielded five novel oral compositions C1, C1-N, C2, C3 and DEX-H possessing significant anti-inflammatory and anti-rheumatoid arthritic activity.

Additionally, the research work was continued with an analysis of the challenges associated with the positioning and pre-launching of herbal products for rheumatoid arthritis to set forth marketing strategies. A thorough market analysis, competitor analysis, consumer analysis and marketing strategies were employed to understand the journey of our product from laboratory to market. The results obtained from thorough market analysis revealed that none of the marketed products contained any marine source which could add an edge in better positioning and pre-launching of our product. The buying behaviour and attitudes of consumers towards using herbal products in

treating RA were analysed by conducting a primary survey among RA patients. The results showed that patients with basic education (school/undergraduate) used complementary and alternative medicines (CAM) with a probability of 4.622 compared to an illiterate. It was found that patients experiencing moderate sensory pain had a probability of 31.176 to use CAM than the ones with no sensory pain. A significant relationship between depression and CAM usage was observed which revealed the CAM usage with a probability of 3.43 compared to patients with no depression. Comorbidities like hypertension, diabetes, thyroid, etc., discouraged CAM usage. Thus, it is concluded that CAM is preferred at the initial stage of pain among RA patients but as the pain reaches higher levels, patients opt for allopathy or allopathy along with CAM for faster relief. The in-depth analysis of marketing strategy and marketing mix, which include product, place, promotion and price helped to frame and provide a roadmap for creating and executing marketing activities that will deliver a return on the proposed product investment.

Graphical Abstract



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Abbreviations

APS	Ammonium Per Sulfate
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BSA	Bovine Serum Albumin
cDNA	Complimentary deoxyribonucleic acid
Cq	Cycle quantification
Cys	Cysteine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
EtOAc	Ethylacetate
EtOH	Ethanol
FBS	Fetal Bovine Serum
FESEM	Field Emission Scanning Electron Microscopy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gly	Glycyrrhizzin
His	Histidine
HPLC	High Performance Liquid Chromatography
IAEC	Institutional animal ethics committee
IC50	Half maximal inhibitory concentration
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
NFκB	nuclear factor kappa light chain enhancer of activated B cells
NO	Nitric oxide
π-GSH	Pi- Glutathione S transferase
PBS	Phosphate buffer saline
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay buffer
RTPCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate poly acrylamide gel electrophoresis
SEM	Standard Error of Mean
TBST 1X	Tris-Buffered Saline, 0.1% Tween
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	Tumour necrosis factor-alpha
TNFR	TNF receptor
TRAP	Tartarate-resistant acid Phosphatase

SYMBOLS

º A	Angstrom
°C	Degree Celsius
µg	Microgram
h	Hours
kDa	Kilodalton
nm	Nanometer
µL	Microlitre
mL	Millilitre
µM	Micromolar
mM	Millimolar

Chapter 1
Introduction

1.1 Arthritis

Arthritis is a broad term that describes more than 100 conditions that affect the joints, tissues around the joint, and other connective tissues (Krustev, Rioux, and McDougall, 2015). It can affect people of all ages, genders, and races, and it is the leading cause of disability in the United States. Arthritis affects more than 50 million adults and 300,000 children (CDC, 2023). The specific symptoms vary depending on the type of arthritis but usually include joint pain and stiffness. The most common types of arthritis include the following.

- Osteoarthritis - It is also known as "wear and tear arthritis" and it develops when joint cartilage breaks down from repeated stress.
- Ankylosing spondylitis - It is a type of arthritis that causes lower back pain and over time, vertebrae in the spinal column may fuse and become rigid (ankylosis).
- Juvenile arthritis - It is a disorder where the immune system attacks the tissue around joints. It typically affects children of age 16 or younger.
- Gout - It is a painful form of arthritis in which sharp crystals of extra uric acid in the body form in the joints (usually the big toe).
- Psoriatic arthritis - It is a form of inflammatory arthritis that develops in people with psoriasis. Both psoriasis and psoriatic arthritis are autoimmune diseases.
- Rheumatoid arthritis (RA) - It is a disease that causes the immune system to attack synovial membranes in joints.

Of these, the worldwide annual incidence and prevalence rate of RA is 3 cases per 10,000 population and 1%, respectively (Prasad et al., 2023). The prevalence of RA is reported to be three times higher in women than men (Wang et al., 2023). It is currently considered a chronic disease with no cure, but remission, has become an achievable goal with optimal treatment (Scott et al., 2020).

1.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by symmetrical joint involvement. It is an autoimmune condition in which the body's immune system attacks the lining of the joints, causing inflammation, pain and eventually erosion of cartilage and bone. RA can also affect other organs in the body, such as the lungs, heart and eyes. Symptoms of RA can vary from person to person, but common symptoms include joint pain, swelling and

warmth in the smaller joints of the hands and feet. Other symptoms can include fatigue, fever, weight loss and general malaise. The exact cause of RA is not yet fully understood, but it is thought to be a combination of genetic, environmental and lifestyle factors (Scherer, Häupl, and Burmester, 2020). Certain risk factors such as smoking, obesity and family history can increase the likelihood of developing RA. Diagnosis of RA typically involves a physical examination, blood tests for certain antibodies and imaging tests such as X-rays and MRI scans. Early diagnosis is the key to RA as early treatment can help to slow or prevent joint damage and improve the quality of life (Radu and Bungau, 2021).

1.3 Existing Treatment of Rheumatoid Arthritis

Pharmacologic treatment of RA involves combinations of nonsteroidal anti-inflammatory medications (NSAIDs), glucocorticoids (GCs) and disease-modifying antirheumatic drugs (DMARDs). NSAIDs like aspirin, diclofenac, ibuprofen alleviate acute joint inflammation, effectively reduce pain and swelling and improve joint function within the first weeks of the onset of RA (Brune and Patrignani, 2015). But they do not prevent additional joint damage. GCs like prednisone, hydrocortisone and dexamethasone have greater potency and efficacy than NSAIDs and are recommended from the beginning of the diagnosis of RA (Littlejohn, and Monrad, 2018.). They delay radiologic progression in early disease stages by general suppression of gene expression. Unlike NSAIDs or pain medication, DMARDs interfere with the signs and symptoms of RA, improve physical function, and inhibit the progression of structural joint damage. DMARDs prescribed for the management of RA are a class of immunosuppressive and immunomodulatory agents that can be divided into conventional synthetic DMARDs (csDMARDS), target synthetic DMARDs (tsDMARDs), and biological DMARDs (bDMARDs) (Singh et al., 2016). Figure 1.3 depicts the classification of currently prescribed DMARDS for treating RA.

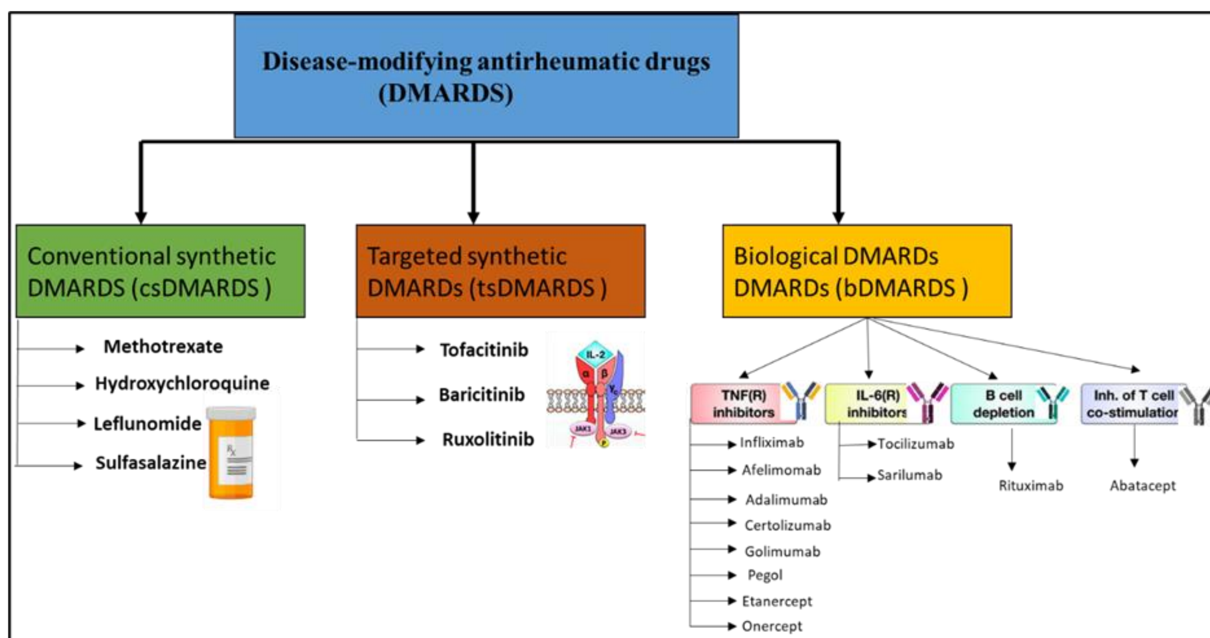


Figure 1.3. Classification of currently available DMARDs.

1.4 Limitations of current therapies of RA

Conventional RA management presents a challenge in terms of efficacy and safety. Long-term use of NSAIDs has a number of side effects like gastrointestinal ulcers and bleeding, altered renal functions and cardiovascular complications (Chiba et al., 2008). GCs can cause the development of a number of undesirable illnesses, including diabetes, weight gain, cushingoid symptoms, hypertension, peptic ulcers, and avascular necrosis of the bones (Paolino, Cutolo, and Pizzorni, 2017). DMARDs like methotrexate and leflunomide are teratogenic and women of childbearing age need effective birth control when taking these medications. Monotherapy with methotrexate requires regular monitoring for cytopenia and hepatotoxicity through periodic lab work. Biologic therapies are considered more potent immunosuppressive agents than csDMARDs and have serious side effects like increased risk of *Mycobacterium tuberculosis* infection/reactivation. There is evidence of an increased risk of serious infections and a dose-dependent increased risk of malignancies in patients with rheumatoid arthritis treated with anti-TNF antibody therapy (Lin, Anzaghe and Schülke, 2020). Despite advances in drug development, the use of these target-specific medicines has been limited due to negative side effects like vulnerability to severe infection and over activation of immune effect (Wang et al., 2021). In addition to this, the high cost of biological DMARDs presents a major challenge in RA treatment for the long term. Given these gaps, there is an urgent need for safer, more effective, and less expensive therapeutic agents to alleviate and treat RA.

1.5 Need for herbal drugs for RA management

Traditional medicines are used by 60-90% of arthritis patients and have earned a reputation as "the people's medicines" due to their ease of access, claimed safety, and low cost (Akram et al., 2021). To date, several extracts and bioactive compounds have exhibited anti-inflammatory, immunosuppressive and analgesic activities against rheumatoid arthritis. Although several herbal drugs are available in the market, there is still a need for newer oral drugs which are more potent with lesser side effects and at the same time available at low cost. Therefore, in view of the adverse effects presented by conventional RA drug regimen, exploration of new natural terrestrial or marine sources of herbal medicines have the potential to emerge as an alternative therapy.

1.6 Seagrass

Seagrasses are a group of angiosperms that are adapted to grow in estuaries and marine ecosystems. The group of seagrass includes 13 genera and approximately 72 species which belong to the families Zosteraceae, Potamogetonaceae, Posidoniaceae, Cymodoceaceae, Hydrocharitaceae and Ruppiaceae (Veettil et al., 2020). They are considered to be one of the most important and biodiverse coastal ecosystems (Jagtap, Komarpant, and Rodrigues, 2003). Reports on local knowledge of seagrasses dictate their usage in the treatment of various ailments including muscle pains, stings, wounds, stomach problems, fever, malaria and coughs (Newmaster et al., 2011; Rengasamy, Radjassegarin and Perumal, 2013). They have marked their medicinal importance traditionally in many communities like India, Africa, Canada, Mexico, etc. and have been used for various ailments like heart diseases, blood pressure, skin diseases, burns, etc., (Newmaster et al., 2011). Currently, 70 species of seagrasses have been explored for natural compounds and their bioactivity.

1.6.1 *Halodule pinifolia*

Halodule pinifolia (Miki) Hartog belongs to the family Cymodaceaceae and order Najadales. It mostly grows in tropical seas and in estuarine environments with temperatures ranging from 25 $^{\circ}$C to 30 $^{\circ}$C (Danaraj et al., 2021). It has been reported to have tremendous medicinal potential in the literature. It has been explored against human eye pathogens like *Escherichia coli*, *Enterococcus faecalis*, *Corynebacterium*, *Bacillussubtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and was found to have antimicrobial properties. It also showed significant inhibitory activity against methicillin-sensitive *Staphylococcus aureus*, *S.*

saprophyticus and *S. epidermidis* (Sangeetha and Asokan, 2015) and urinary tract infectious bacteria (Kannan, Arumugam and Anantharaman., 2012). However, the medicinal properties of *H. pinifolia* has been very little explored and existing literature suggests its potential to emerge as a hub of new compounds with inherent bioactivities.

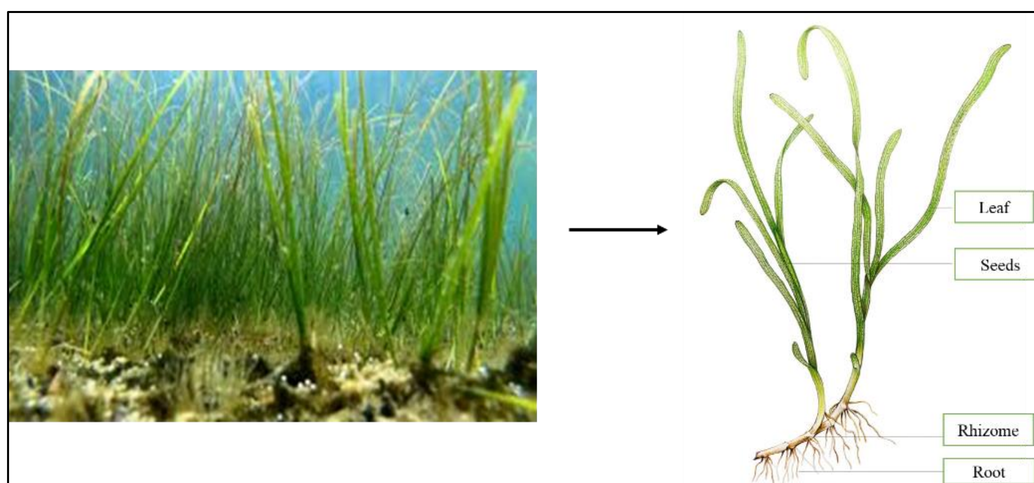


Figure 1.6.1. *Halodule pinifolia* plant (Picture courtesy: seagrassspotter.org/sighting/2342).

1.6.2 *Glycyrrhiza glabra*

Glycyrrhiza glabra commonly known as "liquorice" and "sweet wood" is known for its therapeutic properties since ancient times. The important phytoconstituents of liquorice includes flavonoids, triterpenoid saponins, coumarins, phenols and polysaccharides. Glycyrrhizin is recognized to be the most active ingredient in triterpene saponins (Jiang et al., 2020). Liquorice has been reported for anti-tussive & expectorant activity, antioxidant activity, skin lightening and skin tightening activity, anti-viral effects, anti-fungal activity, anti-bacterial activity, anti-malarial activity, anti-hyperglycaemic activity, immunostimulatory effects, memory enhancing activity, hepatoprotective activity, anticoagulant, hair growth stimulatory activity, etc. (Damle, 2014). Apart from these therapeutic properties liquorice is known for its underlining anti-inflammatory and anti-ulcer activity since ancient times. In Ayurvedic medicine, liquorice is suggested as a preventive medicine for gastric and duodenal ulcers (Ding et al., 2022). Liquorice has been extensively explored for anti-inflammatory and anti-ulcerative properties in modern studies (Yang et al., 2017, Fu et al., 2013).

Figure 1.6.2 Liquorice plant (Picture courtesy: allorganics.ng/product/fss-licorice-extract-os/).

1.7 Herbal Market

All traditional medicinal systems, including Ayurveda, Unani, and Chinese medicine, as well as homoeopathy and naturopathy, are primarily plant-based. The pharmacopoeia of modern drugs contains nearly 7,000 plant-based medicinal compounds. At least one active ingredient derived from plants is present in at least 25% of allopathic prescription drugs (Barrett et al., 1999). According to the 'Market Research Future,' the global market value of herbal medicine was US$ 62 billion in 2004, 80 billion in 2013, and is expected to surpass USD $ 129 billion by 2023 with a strong compound annual growth rate over the period 2018-2023. (Parveen et al., 2020). According to WHO, the herbal market will be worth $7 trillion by 2050 (Sen and Chakraborty, 2017). In India, approximately 70% of modern drugs are discovered from natural resources and a number of other synthetic analogues have been prepared from prototype compounds isolated from plants. The turnover of the Indian herbal industry is said to be more than Rs 80 billion per year. The high occurrence of rheumatic diseases supports a large market for treatment and medication. With the rising awareness, the herbal market has evolved as a potential market with the presence of companies such as Dabur, Himalaya, Zandu, Patanjali, Hamdard, Maharishi etc. The herbal-based small and big companies have marked their presence with the products such as Rumalaya, Forte-Himalaya, Rhumayog-Zandu, Majun surajan-Hamdard, Yograj Guggul- Patanjali, etc. Strong government initiatives like Ayush Mantralaya have positively influenced the herbal market of arthritis by-products like Artheal capsules.

1.8 Market and consumer analysis

The herbal medicine industry is one of the industries that still has a big opportunity with increasing competition. Herbal medicine marketing efforts are established by improving product quality, price, distribution, and promotion in order to persuade consumers to choose

products that fulfil their needs. Furthermore, cultural, social, personal, and psychological factors can all have an impact on consumer behaviour. Some of these characteristics are frequently overlooked by marketers, but they must be considered in order to determine how far these consumer behaviour factors can affect customer purchases (Khayru, and Issalillah, 2021). Knowledge of consumer behaviour especially in the marketing division, makes it easier to design effective marketing programs to increase product sales. Further, the implementation of marketing tools like “marketing mix” with variables like product, price, promotion and distribution often influences consumer behaviour, especially with regard to purchasing decisions (Sudari et al., 2019).

CHAPTER 2
Literature Review

2.1 Rheumatoid Arthritis

RA is a disease of unknown origin. It is one of the most prevalent chronic systemic autoimmune diseases that negatively affects bone health, cartilage health, and synovial membrane function leading to persistent joint inflammation and subsequent joint damage. One percent of the total global population has this disease which presents with persistent pain, stiffness, progressive joint destruction, functional disability, and progressive morbidity and mortality. It is currently considered a chronic disease with no cure, but remission, defined as the absence of active disease, has become an achievable goal with optimal treatment. (Scott et al., 2020). Furthermore, the disease's disability and enormous treatment cost serve as a massive burden that grows over time. Therefore, it is critical to detect RA early and begin treatment at the earliest to preserve function and quality of life.

2.2 Diagnosis of Rheumatoid Arthritis

RA is typically diagnosed based on a combination of the patient's symptoms, the results of the doctor's examination, the assessment of risk factors, the family history, joint ultrasound sonography, and the assessment of laboratory markers such as elevated levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in serum, as well as the detection of RA-specific autoantibodies. The challenge of treatment of early RA is not the lack of effective medicine but rather establishing an accurate diagnosis of RA. The 2010 classification of RA given by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) includes four domains namely: type of affected joints: serology: acute phase reactants and duration of symptoms (Table 2.2). The scores in each domain are added together for the evaluation and classification of RA. The maximum possible score is 10 and a score of 6 or more indicates the presence of RA (Aletaha et al., 2010).

Table 2.2. ACR/EULAR classification of RA

(A) Joint involvement	Score
1 large joint	0
2-10 large joint	1
1-3 small joint (with or without involvement of large joint)	2
4-10 small joint (with or without involvement of large joint)	3
> 10 joint (at least one small joint)	5
(B) Serology (at least one test result is needed for classification)	
Negative RF and negative ACPA	0
Low positive RF *or* low positive ACPA	2
High positive RF *or* high positive ACPA	3
(C) Acute phase reactants (at least one test result is needed for classification)	
Normal CRP *and* normal ESR	0
(D) Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1

ACPA-Anticitrullinated protein/peptide antibodies; CRP- C reactive protein; ESR- Erythrocyte sedimentation rate; RF- Rheumatoid factor

Source: Aletaha, D. et al., *Arthritis & Rheumatism*, 2010, *62*(9), pp.2569-2581.

2.3 Epidemiology and risk factors

The global prevalence of RA is estimated to be between 0.4% and 1.3% (Littlejohn and Monrad, 2018). Based on population-based studies between 1980 and 2019 the global population prevalence of RA was 460 per 100,000 population (Almutairi et al., 2021). Epidemiological studies revealed that North Africa, the Middle East and Asia have relatively lower prevalence at 0.16%, North America and Western Europe at 0.44% with Australasia having the highest at 0.46% (Yip and Navarro, 2021). All of the studies found that females had a 3-5-fold higher prevalence of RA than males (Radu and Bungau, 2021). Around 23.46 million individuals in India had osteoarthritis in 1990 which increased to 62.35 million in 2019 (Singh et al., 2019).

RA is most likely caused by a combination of genetic variation, epigenetic modification, and environmental factors triggered by a stochastic event (e.g., injury or infection) in genetically predisposed individuals and contributes to the multifactorial nature of RA (Lin, Anzaghe, and Schülke, 2020). Scientific studies estimate that a family history of RA increases an individual's likelihood of developing the disease by around 60% (Radu and Bungau, 2021; MacGregor et al., 2000). Major histocompatibility complex (MHC) molecules, which are found on T

lymphocytes, appear to play an important role in the majority of rheumatoid arthritis patients. The HLA DRB1 alleles, found on chromosome 6 of the human MHC, are the single most powerful genetic association for RA, accounting for at least 30% of the total genetic component of the disease (Dedmon, 2020). The disease-associated alleles share a five-amino acid sequence known as the 'shared epitope'. The shared epitope hypothesis proposes that certain alleles with this conserved sequence directly contribute to the pathogenesis of RA by allowing antigen-presenting cells to incorrectly present autoantigens to T cells, resulting in a T-cell mediated autoimmune response (Van Der et al., 2005; Du Mont et al., 2005). Other than HLA DR alleles tyrosine phosphatase non-receptor type 22 (PTPN22) risk alleles (Kokkonen et al., 2007; Källberg et al., 2007), tumour necrosis factor-receptor associated factor 1, complement component 5 (TRAF1/C5) and interferon regulatory factor 5 (IRF-5) also play a major role as genetic factors in RA patients (Gianfrancesco et al., 2020; Scherer, Häupl and Burmester, 2020; Zhang et al., 2013).

Smoking, air pollution, diet, obesity, and infections have all been suggested as environmental factors that cause RA among people who are genetically susceptible. The primary environmental factor that has been connected to a higher risk of developing RA is cigarette smoking (Gianfrancesco et al., 2020). It was found to be capable of inducing the production of rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), autoantibodies linked to joint destruction and systemic bone loss even in the early phases of RA (Ishikawa et al., 2019). The role of air pollutants and occupational exposure to organic (animal and textile) and inorganic dust (asbestos and silica) in RA development has been established in a study by Poole et al., (2019). Chronic exposure to silica can lead to rheumatoid pneumoconiosis, also known as Caplan's syndrome, a rare disease of RA patients who have developed silicosis (Alaya 2018). Obesity and dietary factors have also been evaluated over time. RA risk was higher for overweight and obese subjects compared to the normal-weight population (Feng et al., 2019). Evidences have shown that fasting periods and vegetarian diets slow the progression of RA (Pattison et al., 2004). Furthermore, avoiding red meat and increasing fruit and oily fish consumption has been linked to a lower risk of RA (Jin et al., 2021). Commensal microorganisms colonise the mucosal surfaces of the oral cavity, upper respiratory tract, and gut; this population is known as the microbiome. Changes in the microbiome may facilitate the development of innate and adaptive immunity, which predisposes to RA. Periodontal disease is caused by the pathogenic bacterium *Porphyromonas gingivalis*. An association between RA and periodontal disease has been reported due to its role in inducing citrullination and

promoting osteoclast genesis (Perricone et al., 2019). *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection may play a role in RA by inducing a dysregulation of macrophages and triggering citrullination (BOM 2019). Epigenetic modifications have been shown to play a role in the pathogenesis of RA. DNA methylation, histone protein modifications, and changes in gene expression by microRNA (miRNA) and other noncoding RNA are the major epigenetic mechanisms. Changes in histone acetylation and DNA methylation can influence synovial fibroblasts and leucocyte biology. MicroRNAs add another epigenetic layer by targeting mRNA for degradation and thus fine-tuning cellular responses. Many microRNAs have been identified as important regulators of lymphocytes, macrophages, and synovial fibrocytes (eg, miR146a or miR155) (Giannini et al., 2020).

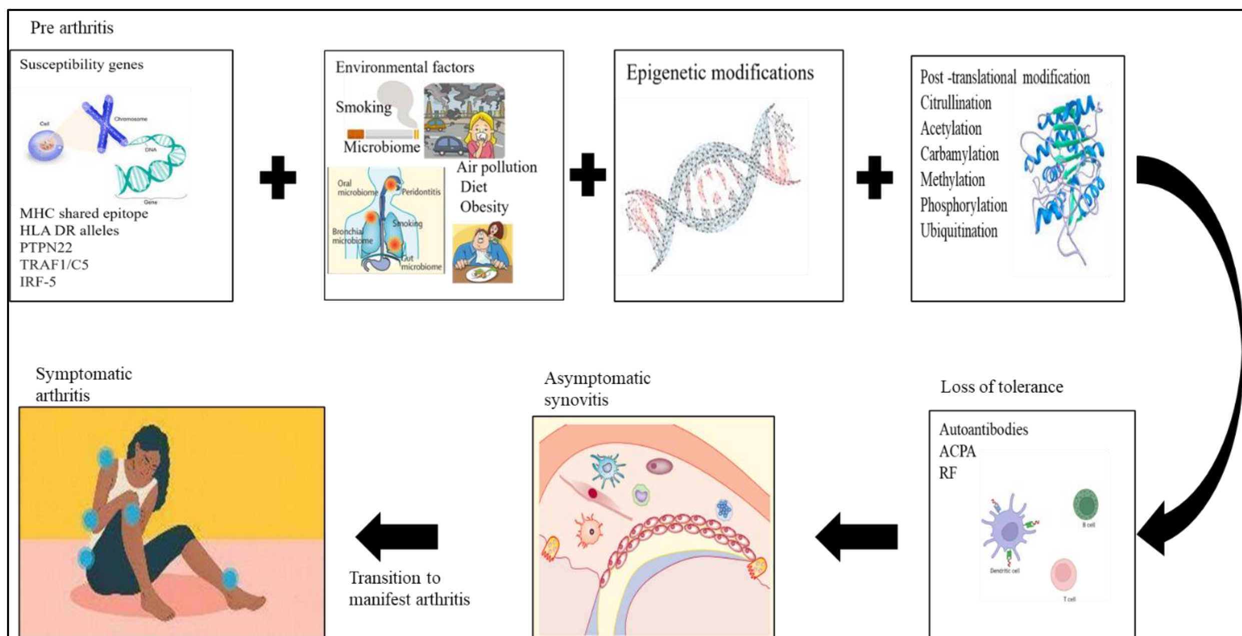


Figure 2.3. Disease prognosis in rheumatoid arthritis.

2.4 Pathogenesis of RA

RA is a systemic disease characterized by inflammation of the synovium, synovial hyperplasia, angiogenesis and cartilage damage, which in turn can lead to bone destruction. RA is an autoimmune disease in which the tolerance break occurs years before the inflammatory stage (Firestein, and McInnes, 2017). Synovial membrane hyperplasia and rheumatoid pannus formation occur as a result of autoimmune attack. The presence of ACPAs characterises the major subtype of RA. ACPAs are citrullinated protein-specific enzymes that catalyse the conversion of arginine (a positively charged amino acid) to citrulline (neutral). Citrullinated proteins are no longer regarded as self-structures leading to an abnormal antibody response.

Fibronectin, α-enolase, fibrin, Epstein-Barr Nuclear Antigen 1 (EBNA-1), type II collagen, and histones are among them (Guo et al., 2018).

Under normal conditions, the synovial membranous lining is almost acellular, consisting of a thin layer of cells composed primarily of macrophage and fibroblast called synoviocytes. These cells are very important in the pathogenesis of RA (Guo et al., 2018). In RA patients, it exhibits an inflammatory phenotype and becomes more hypertrophic (Alivernini et al., 2016). These include the activation and infiltration of various immune cells into the synovial tissue, primarily macrophages, T cells, and dendritic cells (DCs), as well as B cells, neutrophils, and mast cells, all of which are involved in the production of various chemical mediators and interleukins (ILs), causing the tissue to become inflammatory (Siouti, and Andreakos., 2019). CD4 T cells activate macrophages, resulting in the production of proinflammatory cytokines such as IL-1, IL-6, tumor necrosis factor (TNF), and other cytokines that promote osteoclastgenesis (Mrid et al., 2022). It is characterised by a disruption in the balance of osteoclasts (bone degrading) and osteoblast cells (bone forming). TNF-α, IL-6, and IL-1 are important mediators of cell migration and inflammation. IL-6, in particular, acts directly on neutrophils via membrane-bound IL-6R, contributing to inflammation and joint destruction by secreting proteolytic enzymes and reactive oxygen intermediates. The proteases essentially cause the cartilage to breakdown and cause cartilage degradation. The cartilage also produces proteases which is kind of feedback loop. All of these inflammatory cells along with resident synovial fibroblasts generate an exaggerated number of inflammatory cytokines, which along with locally produced autoantibodies, immune complexes, and complement, trigger the onset of chronic inflammation (Rosillo et al., 2016). Th17-derived IL-17A stimulates the production of pro-inflammatory cytokines IL-6, IL-8, and GM-CSF by epithelial, endothelial, and fibroblastic cells as well as neutrophil recruitment (Borregaard, 2010) resulting in local inflammation and disease progression. IL-17A contributes to bone erosion, cartilage destruction, and neoangiogenesis in RA patients through these actions (Robert and Miossec, 2019). Furthermore, the pro-inflammatory cytokines IL-1, IL-6, and TNF-α initiate and sustain the production of additional degradative enzymes (e.g., MMPs) and prostaglandins (Lin et al., 2020). RANK-L produced by cytokine-activated fibroblasts in combination with TNF-α and IL-6 produced by activated immune cells induces the differentiation of macrophages and preosteoclasts into osteoclasts specialised in bone material degradation (Redlich and Smolen, 2012; Smolen et al., 2016, Aletaha and Smolen, 2018).

Antibodies produced by abnormal activation of autoreactive B cells contribute significantly to the pathology of RA through immune complex formation and subsequent complement

activation (Holers and Banda, 2018). RF and ACPAs are the two most common types of autoantibodies found in RA. The presence of these two autoantibodies indicates that the patient has "seropositive" RA (Aletaha et al., 2010). While the pathology of RA can vary, the presence of RF and ACPA autoantibodies has been linked to worsened disease symptoms, joint damage, and an increased risk of death (Smolen et al., 2016).

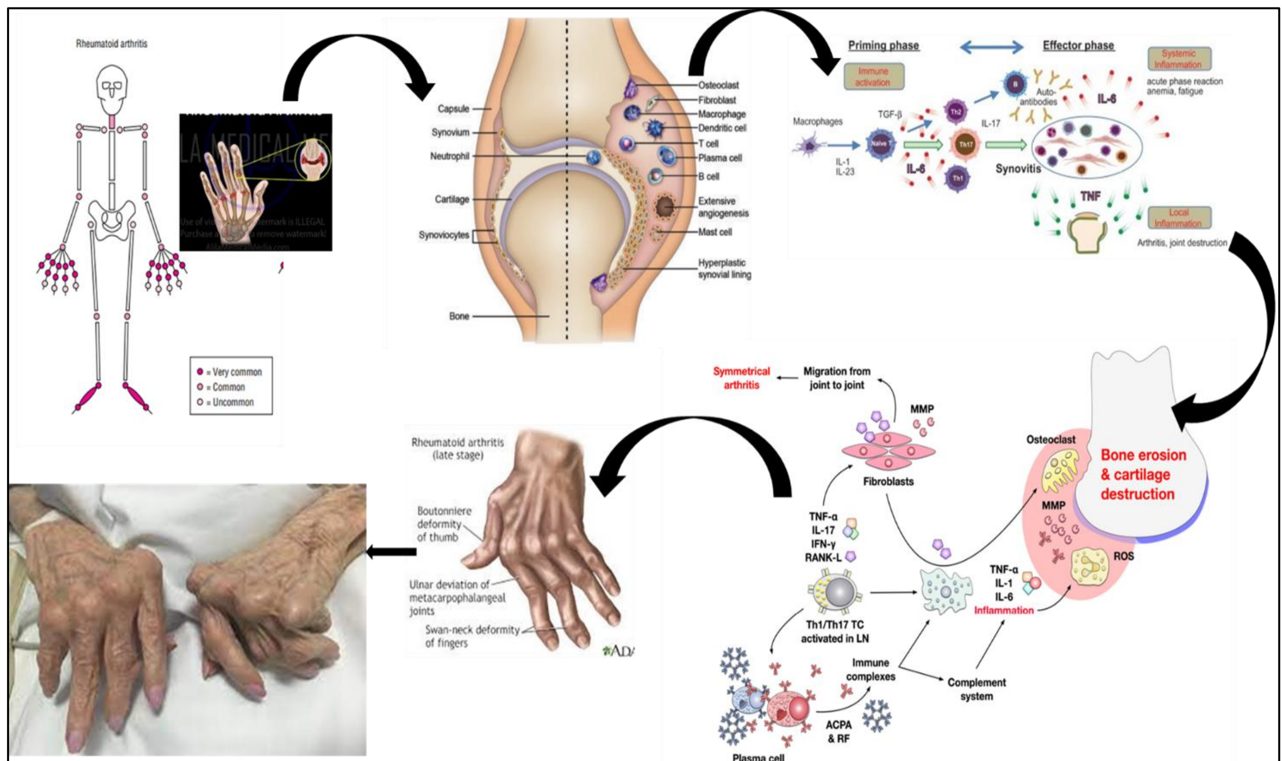


Figure 2.4. Pathomechanisms of rheumatoid arthritis.

2.5 Treatment of RA

In order to prevent joint degeneration, disability, and systemic indications of RA, the overall treatment goal is to achieve full remission or at least considerably reduce disease activity within a time frame of roughly 6 months (Burmester and Pope, 2017). To achieve the treatment goals, treatment should be initiated promptly and continuously, with frequent reassessment of both the disease's state and the effectiveness of the treatment strategy used. Many different medicines have been used to alleviate joint pain and inflammation in RA. These readily available medicines lessen joint inflammation, ease pain, delay joint deterioration, and lower disability. NSAIDs and corticosteroids, which act quickly to decrease pain and swelling brought on by RA, are used as first-line therapy to lower inflammation. The slow-acting treatments known as DMARDs, which include methotrexate, sulfasalazine, antimalarial medications, and gold compounds, can act on the immune system to reduce RA mortality rates

and radiographic progression. Figure 2.5 illustrates an overview of the available treatments of RA. Despite the fact that these therapeutic medications successfully reduced joint degeneration and improved physical function and quality of life, they have a variety of negative side effects that are detrimental to human health.

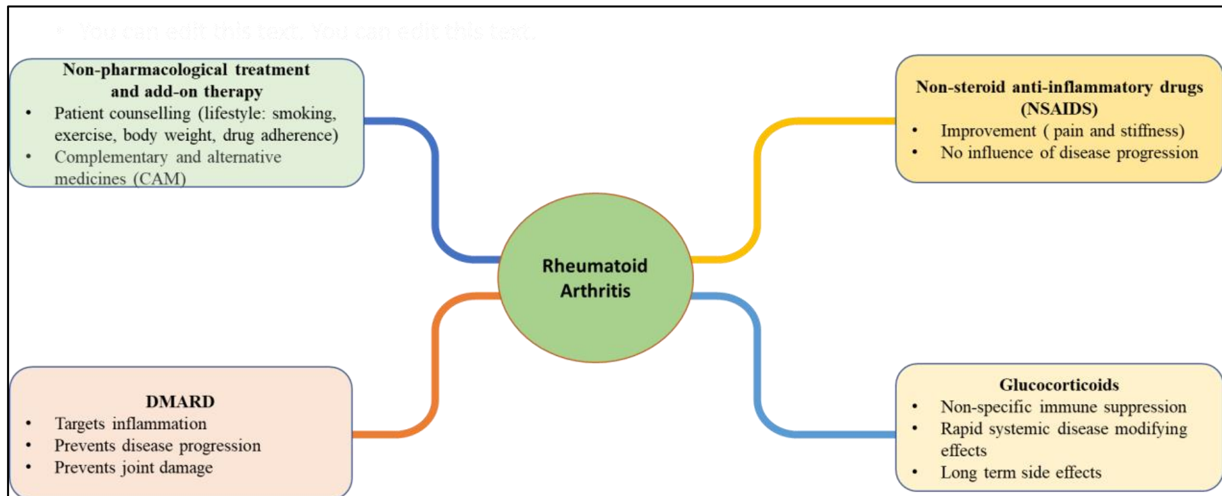


Figure 2.5. Overview of the available treatment strategies for RA patients.

2.5.1 NSAID

NSAIDs such as aspirin, diclofenac, or ibuprofen alleviate acute joint inflammation, effectively reduce pain and swelling and improve joint function within the first weeks of the onset of RA. But they do not prevent additional joint damage. Mechanistically, the anti-inflammatory properties of NSAIDs can be mainly attributed to the enzymatic activity of cyclooxygenase (COX), which is responsible for generating proinflammatory prostaglandin (PG) via arachidonic acid metabolism (Brune and Patrignani, 2015). NSAIDs are effective in the first week after RA onset; combination therapy with slow-acting DMARDs is effective in later stages of RA (Rosillo et al., 2016). Long-term use of NSAIDs, on the other hand, has a number of side effects, the most common of which are gastrointestinal disturbances such as ulcers and bleeding (Chiba et al., 2005). Renal functions are also harmed as a result of decreased PG production, which normally maintains blood flow and glomerular filtration rate in the kidneys. Prolonged NSAID use has also been linked to cardiovascular complications in RA patients.

2.5.2 Glucocorticoids

GCs like prednisone, hydrocortisone, prednisolone, dexamethasone have greater potency and efficacy than NSAIDs and are recommended by EULAR and ACR from the beginning of the diagnosis of RA (American College of Rheumatology 2002). They delay radiologic progression in early disease stages by general suppression of gene expression (Littlejohn and Monrad, 2018). GCs increase the expression of lipocortin-1, which inhibits the enzymatic activity of phospholipase A2 and the production of arachidonic acid. The transcriptional products of glucocorticoids inhibit the activities of NF-kB and activator protein 1 (AP-1) that are responsible for the upregulation of various proinflammatory cytokines such as tumour necrosis factor (TNF-α) and interleukin-1 (IL-1β) (Maity et al., 2021; Paolino et al., 2017). Long-term usage of these drugs can cause the development of a number of undesirable illnesses, including diabetes, weight gain, cushingoid symptoms, hypertension, peptic ulcers, and avascular necrosis of the bones. Glucocorticoids are primarily helpful as a short-term supplementary therapy. In combination with NSAID and DMARD therapy, they are utilised in chronic low doses in patients with severe disease-state RA (Ingawale and Mandlik, 2020; Sarzi et al., 2019).

2.5.3 Disease-modifying antirheumatic drugs

DMARDs unlike NSAIDs or pain medication, interfere with the signs and symptoms of RA, improve physical function, and inhibit the progression of structural joint damage. DMARDS prescribed for the management of RA are a class of immunosuppressive and immunomodulatory agents that can be divided into conventional synthetic DMARDs (csDMARDS), target synthetic DMARDs (tsDMARDs), and biological DMARDs (bDMARDs) (Sarzi et al., 2019).

2.5.3.1 Conventional synthetic DMARDs (csDMARD)

Methotrexate (MTX), a potential immune system suppressant is often used as first-line therapy for RA because of its proven efficacy, good safety profile, and low cost (Belani et al., 2022). The efficacy of bDMARDs or tsDMARDs is always higher when these medications are taken with MTX in combinatory regimens, suggesting the prospect of making MTX the cornerstone of any conventional treatment for RA. This fact further emphasises the significance of MTX. MTX, a folic acid antagonist, was initially created to treat leukemia by inhibiting the production of purines and pyrimidines, which causes the cell cycle to stop in the S phase and, ultimately, cell death (Cronstein and Aune, 2020). Through the inhibition of toxic polyamine

accumulation (which results in tissue damage in RA) the restoration of extracellular adenosine levels and the reduction of intracellular glutathione levels, it can promote a number of anti-inflammatory activities (Bedoui et al., 2019; Friedman and Cronstein, 2019). Unfortunately, this medication has a number of side effects, including cirrhosis of the liver, interstitial pneumonitis, and because of the medication's ability to block the absorption of folic acid, methotrexate can also cause stomatitis, mouth ulcers, tiredness, and gastrointestinal distress (Wang et al., 2018).

Hydroxychloroquine is an antimalarial drug introduced as a DMARD due to its immunomodulatory effects. It blocks the interaction between antigen-presenting macrophages and Th cells leading to a decrease in the overall inflammatory response and therefore can be an alternative option in the treatment of RA (Nirk et al., 2020). The toxicity of this drug includes retinopathy gastrointestinal and dermatological disorders (Kravvariti et al., 2020).

Leflunomide, an isoxazole derivative and its active metabolite, teriflunomide are potent inhibitor of de novo pyrimidine synthesis (Wostradowski et al., 2016; Wiese et al., 2021). It is also reported to inhibit tyrosine kinases, COX-2 activity and nuclear factor-kB (NF-κB) activation involved in the early signaling mechanisms of T-cell and B-cell (Wostradowski et al., 2016; Papadopoulou et al., 2006). Toxicities include headache, nausea, diarrhea, hypertension, liver failure, and loss of hair and body weight (Cui et al., 2020).

Sulfasalazine was introduced in RA therapy because of its antibiotic activity and is a classic DMARD that has been widely used to treat RA due to its anti-inflammatory and anti-microbial effects (Abbasi et al., 2018). Sulfasalazine has a relatively short lag time before onset of action and is thus frequently used as a more efficacious drug among traditional DMARDs. It suppresses the inflammatory microenvironment effectively by neutrophil inhibition, immunoglobulin level reduction, and NF-κB mediated T-cell function interference, halting RA progression (Niknahad et al., 2017; Volin et al., 2002; Augusto et al., 2009; DeMichele et al., 2012).

2.5.3.2 Target synthetic DMARDs (tsDMARD)

Synthetic DMARDs, also known as small molecule DMARDs, primarily participate in cytokine-mediated signalling pathways, specifically the JAK-STAT pathway (Boutet et al., 2021). When pro-inflammatory cytokines (e.g., IL-6, common-chain containing cytokines such as IL-2 or IL-15, type I and II interferons, or granulocyte-monocyte colony-stimulating factor (GM-CSF) bind to their respective receptors on the surface of immune cells, JAKs are recruited

and the intracytoplasmic parts of these receptors are phosphorylated. This, in turn, leads to the phosphorylation of various STATs. The phosphorylated STATs then homodimerize, causing them to translocate to the cell's nucleus, where they promote the expression of numerous pro-inflammatory genes that can initiate and sustain both joint inflammation (Kotyla, 2018). Tofacitinib is a powerful and selective JAK inhibitor which modulates inflammatory and immune responses by inhibiting JAK1/JAK3-mediated signalling of various cytokines such as IL-2, IL-4, IL-6, IL-7, IL-15, and IL-21, as well as IFNa and IFNc (Maeshima et al., 2012). Tofacitinib has also been linked to a decrease in the expression of various genes, including the chemokine (C-C motif) ligand 2 (CCL2), the C-X-C motif chemokine ligand 10 (CXCL10) and CXCL13, and MMP1 and MMP3, RANKL production all of which have been linked to the pathogenesis of RA (Boyle et al., 2015). The side effects of JAK inhibitors include increased frequency of infections (often with Herpes zoster), formation of blood clots, elevation of blood cholesterol levels, cytopenia and bowel perforation (Taylor, 2019; Winthrop, 2017).

2.5.3.3 Biological DMARDs (bDMARD)

TNF-α Inhibitors: The first wave of biologic drugs to transform RA treatment was TNF inhibitors. TNF-neutralizing medications can be further divided into soluble TNF receptor constructions (etanercept, onercept), antibody fragments (certolizumab pegol), and neutralising monoclonal antibodies (afelimomab, infliximab, certolizumab, adalimumab, golimumab) (Smolen, 2016). These substances effectively suppress joint inflammation as well as cartilage and bone damage by neutralising TNF-α and the inflammatory processes induced by this cytokine. TNF-inhibitors can be used in conjunction with methotrexate or other DMARDs, and they are also commonly used as second-line treatments when patients do not respond to synthetic DMARDs alone (Law and Taylor, 2019). Adalimumab is a human monoclonal antibody that is subcutaneously administered every two weeks. Etanercept is a TNF-receptor fusion protein that is administered subcutaneously on a weekly basis. Infliximab is an intravenous chimeric monoclonal antibody that is administered every eight weeks. Golimumab is a human monoclonal antibody to TNF that can be given subcutaneously or intravenously every four weeks. Certolizumab pegol is a humanised monoclonal anti-TNF antigen-binding fragment that is injected subcutaneously every two to four weeks. The choice of DMARD therapy is determined by the patient's comorbidities and the presence of treatment contraindications. Infliximab was associated with the reactivation of tuberculosis and hepatitis B, as well as a higher incidence of serious infections (Guo et al., 2018). Adalimumab was also

linked to an increased risk of infections (Atzeni et al., 2012), and at high doses (100 mg) of golimumab, demyelinating events and lymphoma were reported (Singh et al., 2010).

IL-6 inhibition: The development of IL-6 blockers adds another treatment option for RA. Monoclonal antibodies used to inhibit IL-6 signalling in RA patients are classified as (1) antibodies that directly neutralise IL-6 (elsilimomab, siltuximab, sirukumab) and (2) antibodies that bind to the IL-6R and block the pro-inflammatory signalling induced by IL-6 binding (tocilizumab, satralizumab, sarilumab) (Lin et al., 2020). Tocilizumab was the first humanised IL-6 receptor (IL6R) antagonist developed for the treatment of moderate to severe RA patients (Dayer and Choy, 2010). Because of its safety profile and rapid and sustained effects, this monoclonal antibody has sparked a lot of interest (Biggioggero et al., 2018). Sarilumab is a fully humanised monoclonal antibody that binds to the IL-6R receptor (Rafique, A 2013). Preclinical trials showed that sarilumab treatment reduced cartilage matrix component loss, as well as inflammatory (synovitis and pannus formation) and erosive (bone erosion and tissue architecture loss) parameters when compared to control antibody treatment. The most common side effects in sarilumab-treated RA patients were infections, alanine aminotransferase elevations, and neutropenia (Genovese et al., 2015; Huizinga et al., 2013).

B-cell depleters: Rituximab is a chimeric monoclonal antibody that has been genetically modified to target CD20+ B cells (Law and Taylor, 2019). By lowering autoantibody synthesis and preventing antigen presentation to T cells, B cell depletion is hypothesised to be beneficial in the treatment of RA. A meta-analysis conducted between rituximab and placebo showed that rituximab is well tolerated even at higher doses. Rituximab is preferred as the bDMARD of choice when there are concurrent comorbidities present, such as multiple sclerosis or lymphoproliferative diseases.

Inhibitors of Co-Stimulation: Abatacept is the first member of a new class of biological agents that inhibit inflammation induction upstream of the pro-inflammatory signalling cascade. It is a fusion protein made up of the human IgG1 Fc region fused to the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4. (CTLA-4) (Bozec et al., 2018). Abatacept prevents CD80/86-mediated transmission of co-stimulatory signals from activated APCs to T cells and thus subsequent T cell activation by neutralising CTLA-4-part binding on the surface of activated APCs (Maxwell and Singh., 2010). Abatacept was approved for the treatment of RA patients who do not respond to other DMARDs or TNF-α inhibitors (Peichl et al., 2019).

The most frequent side-effects associated with Abatacept include upper respiratory tract infections, headaches, and nausea (Blair and Deekds, 2017).

2.5.3.4 Novel Strategies and future direction in the treatment of RA

Over the last few decades, RA management has evolved significantly, resulting in improved quality of life and outcomes for RA patients. The successful discovery of several pathways involved in the pathogenesis of RA has made this possible. However, the mechanisms underlying inflammatory processes as well as the pharmacological effects of therapeutic molecules remain unknown, leaving some gaps in RA management. These include gaining a better understanding of how different therapies have such comparable efficacies; determining why some patients become less responsive over time; detecting pre-RA and initiating an early and aggressive treatment regimen; and improving the efficacy and safety profiles of novel compounds, particularly JAK (Smolen et al., 2018). Several approaches to improving RA treatment are currently being tested in various experimental models. Numerous new therapeutic targets are being investigated, and potential therapeutic agents are being tested at various stages in order to achieve complete RA remission (Guo et al., 2018; Huang et al., 2021). Mesenchymal stem cells (MSCs) are another promising therapeutic approach because of their ability to differentiate into new tissues such as bone and cartilage, and they have been shown in vitro to have immunosuppressive properties by suppressing T cell activation. Furthermore, treatment with MSCs has been shown in both animal model studies and clinical trials in RA patients to reduce the proinflammatory response and improve RA symptoms by lowering blood levels of IL-1, IL-6, IL-8, and TNF- α (Lin et al., 2020; Zhang et al., 2019). Toll-like receptor 4 has been linked to the pathogenesis of RA by promoting joint inflammation. Therapeutic compounds that target this receptor or its ligands, such as heat shock protein crystalline or tenascin C, can thus be optimised (Lin et al., 2020). Therapeutic options for RA are becoming more diverse, and numerous ongoing studies have the potential to significantly improve the lives of RA patients by identifying new molecular targets, therapeutic agents, and methods to mitigate side effects. A personalised approach based on genetic studies, combined with evidence-based medicine, has the potential to transform the future of medicine and cure the incurable (Köhler et al., 2019).

2.6 Limitations of DMARD Therapy

In terms of efficacy and safety, conventional RA management presents a challenge. Patients frequently consider herbal medicine for the treatment of RA due to adverse reactions in long term to modern medications. RA treatment includes the long-term use of medications such as NSAIDs, corticosteroids, DMARDs, and biologics, which can cause gastric irritation, impaired wound healing, osteoporosis, and peptic ulcers. Despite advances in drug development, the use of these target-specific medicines has been limited due to negative side effects (vulnerability to severe infection and over activation of immune effect in vivo) (Lin et al., 2020) and the exorbitant prices of these orthodox medicines. Given these gaps, there is an urgent need for safer, more effective, and less expensive therapeutic agents to alleviate and treat RA. Table 2.6 illustrates the side effects associated with prescribed drug regimens for the treatment of RA.

Table 2.6. Overview of the different classes of drugs used in current approved RA treatments and the side effects associated with the drug regimen

Generic name of the drug	Side effects	Reference
NSAIDs		
Naproxen, Aspirin, Ibuprofen, Etodolac, Sulindac diclofenac Meloxicam Nabumetone Piroxicam, Indomethacin Mefenamic acid Meclofenamate, Tolmetin, Ketorolac, Rofecoxib, celecoxib lumiracoxib	Gastric irritation, Peptic ulcer, Gastrointestinal bleeding, Nausea, Abdominal discomfort, Kidney impairment, Cardiovascular effects, Rash	Crofford et al., 2013; Altman et al., 2015; Lie et al., 2017; Anjum et al., 2020
Corticosteroids		
Dexamethasone, Methylprednisolone, Prednisone, Triamcinolone	Diabetes, Osteoporosis, Metabolic syndrome, Cataract, Peptic ulcers	Buttgereit et al., 2019; Wang et al., 2020; Yoshii et al., 2020; Paglia et al., 2021
DMARDs		
MTX	Gastrointestinal disorders, Hepatic dysregulations, Pneumonitis, Hematologic disorders, Infections, Nephrotoxicity	Wang et al., 2018; Bedoui et al., 2019; Friedman et al., 2019
Hydroxychloroquine	Gastrointestinal disorders Dermatological disorders Retinal toxicity	Sames et al., 2016; Kim et al., 2017
Sulfasalazine	Renal injury, Leukopenia, Agranulocytosis, Central nervous system toxicity, Infertility	Niknahad et al., 2017; Augusto et al., 2009; Michele et al., 2012

Leflunomide	Headache, Nausea, Diarrhea, Hypertension, Liver failure	Antony et al.,2006; Cui et al., 2020
Tofacitinib	Infections, Hypercholesterolemia, Creatininemia, Elevation of serum transaminases	Meyer et al., 2010; Maeshima et al.,2012; Sonthalia et al., 2020
Baricitinib	Infections	Pandolfi et al., 2020
Ruxolitinib	Neutrophil decrease	Broglie et al., 2017
Iguratimod	Skin disorders (rash; edema; hair loss) Gastrointestinal disorders (Diarrhea; nausea, abdominal pain), Mucosal disorders	Xiao et al., 2018
Tocilizumab	Infections and infestations, High blood pressure, Skin rashes	Biggioggero et al., 2018
Sarilumab	Infections and infestations, Neutropenia, Increased low-density lipoprotein (LDL) cholesterol	Raimondo et al., 2017; Lee et al., 2018; McCarty et al., 2018
Etanercept	Infection, Nausea, Dyspnea, Hypertension	Radner et al., 2015; Puttini et al., 2019
Adalimumab	Chest discomfort	Bahardeeen et al., 2019

2.7 Need for herbal drugs for the treatment of RA

Herbal medicines have earned a reputation as "the people's medicines" due to their ease of access, claimed safety, and ease of preparation, and are now gradually scorning the mainstream systems of medical treatment in many countries, as advances in quality control of herbal drugs, as well as advances in clinical research, are now moulding the general opinion in their favour. In terms of efficacy and safety, conventional RA management presents a challenge. Patients frequently consider herbal medicine for the treatment of RA due to adverse reactions (mentioned in the above sections) to modern medications. The study found that people suffering from chronic pain, such as RA, and those who are resistant to conventional treatment are more likely to consider traditional medicine. Traditional medicines are used by 60-90% of arthritis patients (Akram et al., 2021). With the growing interest in traditional RA treatments, it is critical to investigate new drugs that are both safe and effective. The treatment of RA entails a multidisciplinary approach to reducing pain, reducing inflammation, and restoring joint activity. Inflammation is the focus of aggressive treatment. Traditional medicines have grown in popularity for the treatment of RA around the world. Traditional medicines linked to

inflammatory mediators are prescribed for the treatment of RA. Numerous studies on the efficacy of anti-inflammatory plant extracts and their bioactive compounds, including in vitro, in vivo, preclinical, and clinical studies, have been conducted in an effort to identify potential phytomedicine resources for the treatment of RA. Despite medicinal plant extracts' low toxicity profile, more research on dosage optimization, improved stability, and efficacy is needed before these phytomedicine resources can be used to treat diseases (Gandhi et al., 2022).

A thorough literature review was done on herbal drugs having anti arthritic activity. *Smilax chinensis* (Liliaceae) contains active components such as saponins, sterols, and alkaloids. It is used to treat rheumatoid arthritis, musculoskeletal scatters, and gouty arthritis (Xu et al., 2008). Sieboldogenin found in *S. chinensis* showed lipoxygenase inhibition in carrageenan-induced paw edema with doses of 10 or 50 mg/kg. It is advised for rheumatoid joint pain. *Piper nigrum* (Piperaceae) constitutes phytoconstituents like riboflavin, thiamine, iron, piperine, starch, oils, terpenes, piperolein B, piperolein A, piperettin, piperidine, alpha thujone, and carotene. The extract of *Piper nigrum* fruit and metabolite piperine are reported to possess anti-inflammatory and (Tasleem *et. al*., 2014) anti-arthritic effect against joint inflammation (Takooree et al., 2019). The ethanol extract of *Trigonella foenum-graecum* (Papilionaceae) seeds has been reported to ameliorate Freund's adjuvant-induced arthritis in albino rats (Suresh et al., 2012). *Linum usitatissimum* (Linaceae) contains secoisolariciresinol diglucoside, caffeic acid, and flavonoids and is reported to reduce vascular permeability and prostaglandin formation in RA along with anti-inflammatory activity (Wang et al., 2017; Dupasquier et al., 2007). *Tanacetum parthenium* (Asteraceae) leaves contain flavonoids, quercetagetin, pinenes, pinenes, santin, apigenin, flavonols 6-hydroxykaempferol, sesquiterpene lactones, parthenolide, jaceidin, luteolin 7-glucuronides, centaureidin. Pareek et al., (2011) reported the potential of this plant in 41 patients with symptomatic RA. *Zingiber officinale* (Zingiberaceae) rhizome contains zingerone, zingiberene, tryptophan, shogaols, sesquiterpene hydrocarbons, selenium, paradol, lecithin, limonene, gingerol, curcumin, caffeic acid, capsaicin, beta-sitosterol, beta carotene, and ascorbic acid (Funk et al., 2016). It is used for rheumatism and has been prescribed for the management of arthritis in traditional medicine. *Justicia gendarussa* leaves contain friedelin, beta-sitosterol, lupeol, amines, flavonoids, alkaloids, and saponins (Pal et al., 2015). Table 2.7 shows anti-RA effect of few more medicinal plants reported in literature.

Table 2.7. List of few more medicinal plants reported in literature having anti-arthritic activity

Classification	Components	Plants source	Mechanisms	Reference
Diterpenoids				
	Glaucocalyxin A	*Rabdosia japonica* var. Glaucocalyx	STAT3 pathway↓	Zhang et al. (2021)
	Andrographolide	*Andrographis paniculata* (Burm.f.) Nees	Anti-inflammation and anti-oxidation	Luo et al. (2020)
	Triptolide	*Tripterygium wilfordii* Hook. f.	apoptosis of OCP↑, the generation of OC and bone resorption↓	Wang et al. (2018a)
Triterpenoid				
	Taraxasterol	*Taraxacum mongolicum* Hand. - Mazz	NF-κB and NLRP3 pathways↓	Chen et al. (2019)
	Madecassoside	*Centella asiatica* (L.) Urb.	NF-κB/MMP-13 pathway↓	Yu et al. (2018)
	Celastrol	*Tripterygium wilfordii* Hook.f.	Oxidative stress↓	Gao et al. (2020)
	Betulinic acid	*E. ulmoides*	NF-κB signal pathway↓	Li et al. (2019b)
	Betulinic acid derivativesSH 479	*E. ulmoides*	JAK2/STAT3 pathways↓	Chen et al., (2017a)
Phenolic compound				
	Caffeic acid	*Solidago decurrens* Lour.; Crataegus pinnatifida Bunge; Valeriana officinalis L.	Inflammatory signals, chitinase-3-like protein-1 and angiogenesis↓	Fikry et al. (2019)
	Paeonol	*Paeonia lactiflora* Pal.	FOXO3 level↓ miR-155 expression↓	Liu et al. (2017b)
	Salvianolic acid B	*Salvia miltiorrhiza* Bunge	miR-142–3p expression↓ NF-κB and JNK pathways↓ Macrophage apoptosis↑	Meng et al. (2019)
	Curculigoside	*Curculigo orchioides* Gaertn	Nrf2↑ and NF-κB pathway↓	(Liu et al., 2021b)

	Curcumin	*Curcuma longa* L.	NF-κB signaling pathway↓ Macrophage apoptosis↑	Wang et al. (2019b)
	Resveratrol	*Polygonum cuspidatum*	BK-induced COX-2/PGE2 production↓	Yang et al. (2017)
Coumarin				
	Osthole	*Cnidium monnieri* (L.) Cusson	Inflammation and cellular stress↓	(Xia et al., 2018)
	Imperatorin	*Angelica dahurica* and *Angelica archangelica*	Mitochondrial/caspasemediated pathways	Zhai et al. (2018)
Alkaloid				
	Sinomenine	*Sinomenium acutum* (Thunb.) Rehd. Et Wils	Angiogenesis↓	Feng et al. (2019)
Flavonoid				
	Hesperidin	*Citrus aurantium* L.	Inflammation and oxidative stress↓	Adefegha et al. (2020)
	Liquiritin	*Glycyrrhiza uralensis* Fisch.	Inflammation↓ MAPK pathways↓ Angiogenesis↓	Zhai et al. (2019)
	Tangeretin	*Citrus reticulata* Blanco	Regulating the ROS-AKT/ mTOR signal axis	Yang et al. (2021)
	Genistein	Leguminous plants	JAK2/STAT3/VEGF pathway↓	Cheng et al. (2019)
	Apigenin	*Apium graveolens* L.	Maturation and migration of DCs↓	Li et al. (2016)
	Quercetin	*Tussilago farfara* L./*Taxillus sutchuenensis* (Lecomte) Danser	Neutrophil activities↓	Yuan et al. (2020)

2.8 Seagrass

Seagrasses are currently classified into four families: Cymodoceaceae, Hydrocharitaceae, Posidoniaceae, and Zosteraceae (Larkam, 2006) and two orders namely Alismatales and Potamogetonales. Alismatales includes seagrasses from the Hydrocharitaceae family and Potamogetonales which includes seagrasses from the Cymodoceaceae, Posidoniaceae, and Zosteraceae families (Les and Tippery, 2013). The importance of seagrasses has been underlined in literature as primary producers, shelter and food for fish, turtles, and invertebrates as well as spawning areas for these organisms and as habitats for epiphytes (Gillanders, 2006; Gobert et al., 2006; Ruiz et al., 2009). In folk medicine, seagrasses have been used to treat a variety of ailments, including fever and skin diseases, muscle pains, wounds, and stomach problems, stings from various types of rays, and as a tranquillizer for babies (de la Torre-Castro & Rönnbäck, 2004). Bioactivities from seagrass extracts and pure compounds derived from seagrass extracts have shown to have anti-cancer, anti-microbial, anti-viral, ant-feedant, antimicrofouling and anti-oxidant activity. Cymodienol from Cymodocea nodosa and crude extracts of T. ciliatum exhibited cytotoxic activity against selected human cancer cell lines, hepatitis A and herpes simplex viruses in vitro studies (Hamdy et al., 2012). Devi et al. (1997) tested extracts from sixteen marine species against 15 strains of marine fouling bacteria, including seagrasses *Halodule pinifolia*, *Halophila ovalis*, and *S. isoetifolium* and found *H. pinifolia* weakly inhibited an Anterobacteriaceae strain. Engel et al. (2006) surveyed extracts of three seagrass species: *Halodule beaudettei*, *S. filiforme*, and *T. testudinum* and found that *H. beaudettei* extracts were the most active, inhibiting the growth of *H. spinosa*, *S. aggregatum*, and *P. bacteriolytica*. The extracts from *S. filiforme* were active against *S. aggregatum*, and *P. bacteriolytica*, while the extracts from *T. testudinum* were only active against *H. spinosa*. extracts from *T. testudinum* were only active against *H. spinosa*. Ross et al. (2008) studied the antifungal activities of organic extracts from five seagrass namely *H. wrightii, Halophila decipiens, R. maritima, S. filiforme, and T. testudinum,* for their antifungal activities and were found to be effective by 35%. Zosteric acid from *Z. marina* showed moderate anti-Dengue-virus activity against all four types of Dengue virus s (Rees et al., 2008; Kadir et al., 2013). Iyapparaj et al. (2014) reported anti-macrofouling activities for extracts of *C. serrulata* and *S. isoetifolium*. Extracts of *H. ovalis* showed dose-dependent antioxidant activity and extracts of *H. ovalis* showed dose-dependent anti-inflammatory activity (Yuvaraj et al., 2012).

The detailed literature review on seagrasses revealed that though there is a wealth of information on the bioactivity of seagrasses scattered throughout the literature, many gaps

remain. The most obvious gaps concern the species of seagrasses that have not yet been studied. However, with the introduction of new analytical techniques such as HPLC-MS, comparative studies for the presence or absence of compounds already known from at least one species of seagrass would be relatively quick and simple to perform.

2.9 *Halodule pinifolia*

H. pinifolia is a commonly observed species of seagrass abundantly widespread in the pacific and Indian Ocean. According to the IUCN Red List of Threatened species the distribution of Halodule species in pacific stretches from southern Japan, Taiwan, the Philippines, Malaysia, Indonesia, throughout the Gulf of Thailand and along the coast of Vietnam and southern China. The abundance has also been observed in the Indian ocean from mid-Western Australia to the Timor Sea, the south coast of Indonesia, to the Andaman Sea and extending around the Bay of Bengal to the Coromandel Coast of India (Short et al., 2010). Halodule pinifolia belongs to the family Cymodaceaceae and order Najadales. It mostly grows in the tropical seas and in estuarine environment with temperature ranging from 25 °C to 30 °C It is ephemeral and can withstand high levels of disturbance due to its high seed set and quick turnover. This species spreads and grows quickly. Severe epiphytization is relatively common. *H. pinifolia* has male and female plants, as well as slender and branched rhizomes and creeping roots at each node. It thrives on sandy to muddy soils along coasts, mangrove creeks, coral platforms, and other similar habitats. The leaves are linear with an entire margin and three prominent midribs, 1-6 cm long and 1-5 mm wide, and lateral. It is common and widespread, but its population is likely to be declining due to a variety of localised threats across its range. This species spreads quickly and can recolonize areas where it has been removed; it forms colonies. (Skelton et al., 2006).

H. pinifolia has been reported to have tremendous medicinal potential in the literature. It has been explored against human eye pathogens like *Escherichia coli*, *Enterococcus faecalis*, *Corynebacterium*, *Bacillussubtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and was found to have antimicrobial properties. It also showed significant inhibitory activity against methicillin sensitive *Staphylococcus aureus*, *S. saprophyticus* and *S. epidermidis* (Sangeetha and Asokan, 2015) and urinary tract infectious bacteria (Kannan et al., 2012). A recent study by Kannan et al established the anticoagulant and antioxidant properties of *H. pinifolia*. Vijayakumar et al concluded that, the root extract of *H. pinifolia* can be used as potential larvicidal agent against Culex quinquefasciatus mosquito larvae. Apart from the medicinal properties Halodule pinifolia has also been reported to have high nutritional value with dietary fiber, mineral and vitamin content and high calcium and magnesium content (Jeevitha *et al.*,

2013). A high phenolic, tannins and vitamin E content of *H. pinifolia* have been reported which adds to its nutraceutical applications (Kannan *et al.*, 2013). Recently, the anti-inflammatory potential of *H.pinifolia* has been explored and reported to have pro-inflammatory cytokine inhibition effect (Begum et al., 2021). However, the medicinal properties of *H. pinifolia* has been very little explored and existing literature suggests its potential to emerge as a hub of new compounds with inherent bioactivities.

2.10 Liquorice

G. glabra commonly known as "licorice" or "liquorice" has been used since ancient times in traditional medical systems. *G. glabra* roots contain several active compounds including flavonoids, such as liquirtin, rhamnoliquirilin, liquiritigenin, prenyllicoflavone A, glucoliquiritin apioside, 1-metho-xyphaseolin, shinpterocarpin, shinflavanone, licopyranocoumarin, glisoflavone, licoarylcoumarin, coumarin-GU-12 and saponins namely glycyrrhizin. In addition, four isoprenoid-substituted phenolic constituents (isoangustone A, semilicoisoflavone B, licoriphenone, and 1-methoxyficifolinol), kanzonol R and several volatile components (pentanol, tetramethyl pyrazine, hexanol, terpinen-4-ol, linalool oxide A and B, geraniol, and α-terpineol) have also been reported (Bisht et al., 2022). The scientific investigations have proven its pharmacological effects, including those that are anti-demulcent, expectorant, antiulcer, anti-inflammatory, anticancer, and antidiabetic. (Pandey et al 2017; Sharma et al., 2018). Liquorice has been reported for anti-tussive & expectorant activity, antioxidant activity, skin lightening and skin tightening activity, anti-viral effects, anti-fungal, anti-bacterial, anti-malarial, anti-hyperglycaemic, immunostimulatory, memory enhancing, hepatoprotective, anticoagulant, hair growth stimulatory, etc, (Damle, 2014). Apart from these therapeutic properties liquorice is known for its underlining anti-inflammatory and anti-ulcer activity since ancient times. In Ayurvedic medicine, liquorice is suggested as a preventive medicine for gastric and duodenal ulcers (Ding et al., 2022). Liquorice has been extensively explored for anti-inflammatory and anti-ulcerative properties in modern studies (Yang et al., 2017, Fu et al., 2013). Liquorice extract was found to have similar anti-inflammatory properties like diclofenac and it could decrease plasma levels of TNF-α and IL-6, and increase production of IL-10 in LPS treated mice (Aly et al., 2005; Kim et al., 2006). It has been reported to inhibit intracellular signalling inflammatory proteins pathways including NF-κB p65, Jun proto-oncogene-encoded activator protein (AP)− 1 transcription factor, COX and LOX (Bodet et al., 2008; Trombetta et al., 2014). Glycyrrhetinic acid and glycyrrhizin has been reported to inhibit IL-3, IL-5, IL-6, IL-10, IL-12, IL-13, IL-1β, TNF- α expression (Jahromi et al., 2019; Richard,

2021). Glychyrrhizin treatment reduced serum levels of inflammatory factors: IL-1, IL-6, TNF-α, tartrate-resistant acid phosphatase positive (TRAP+) osteoclasts and cathepsins B and K in mice (Yamada et al., 2021; Huang et al., 2022). Monoammonium glycyrrhizinate (MAG) – salt of glychyrrhizin has been reported to have protective effect in LPS- induced acute lung injury in mice (Shi et al., 2010). A study by Kim et al (2010) revealed that liquorice have the potential to attenuate the pro-inflammatory cytokines and MMP-3 expression in joints of collagen-induced arthritic rats.

2.11 Global herbal market

All traditional medical systems, including Ayurveda, Unani, and Chinese medicine, as well as homoeopathy and naturopathy, are primarily plant-based. However, because these medical practices were regarded as primitive and unscientific, they were ignored and even declared illegal by authorities during the colonial era. When chemical analysis became available in the early nineteenth century, scientists began to extract and modify the active medicinal compounds found in plants in order to use them as ingredients in allopathic medicines. Chemists later began synthesising these compounds and creating their own versions of plant compounds. At least one active ingredient derived from plants is present in at least 25% of allopathic prescription drugs (Barrett et al., 1999).

The pharmacopoeia of modern drugs contains nearly 7,000 plant-based medicinal compounds. The market for herbal medicine is expanding at a rate of 7-30% per year. To name a few, annual revenue from herbal medicinal products in the United States reached US$ 17 billion (2002), 5 billion (2003-2004) in Western Europe, 14 billion (2005) in China, and 160 million (2007) in Brazil (Ghani, 2013). In Malaysia, approximately US$ 500 million is spent on herbal health care each year, compared to nearly US$ 300 million on allopathic medicine. According to the 'Market Research Future,' the global market value of herbal medicine was US$ 62 billion in 2004, 80 billion in 2013, and is expected to surpass USD$ 129 billion by 2023 with a strong CAGR (compound annual growth rate) over the period 2018-2023. (MRFR, 2019). According to WHO, the herbal market will be worth $7 trillion by 2050. The market for herbal products is also expected to grow in the near future, owing to the growing demand for plant-based veterinary medicine that does not harm animal health and can serve as a viable alternative to conventional synthetic pest-control products.

The industry comprises companies that manufacture biological, medicinal and pharmaceutical drug products in various forms including ampoules, tablets, capsules, vials, ointments,

powders, solutions, oils and suspensions that are used for the treatment of patients with RA in India. It also includes drug products developed as drug-device combination. It was estimated that around 23.5% of women and 18% of men in the United States had doctor-diagnosed arthritis for the period of 2013 to 2015.

The high occurrence of rheumatic diseases supports a large market for treatment and medication. In spring of 2017, it was estimated that just over 26 million people bought medications in the U.S. in the past year because of arthritis. Furthermore, the value of the rheumatoid arthritis market in 2013 was estimated at 6.4 billion U.S. dollars and is forecast to reach 9.3 billion dollars by the year 2020. In 2015, there were around 5,000 rheumatologists, doctors with specific training in diagnosing and treating rheumatic diseases, in the United States. However, over the next 15 years, this number is expected to decrease to fewer than 3,500 (Arthritis and rheumatic disease, 2022). With the rising awareness, herbal market has evolved as a potential market with the presence of companies such as Dabur, Himalaya, Zandu, Patanjali, Hamdard, Maharishi etc. Herbal products of small and big companies that have been increasingly penetrated in the market and have marked their presence with the products like: Rumalaya Forte- Himalaya, Rhumayog- Zandu, Majun surajan-Hamdard, Yograj Guggul-Patanjali. Strong government initiatives like Ayush Mantralaya have positively influenced the herbal market of arthritis by products like Artheal capsules.

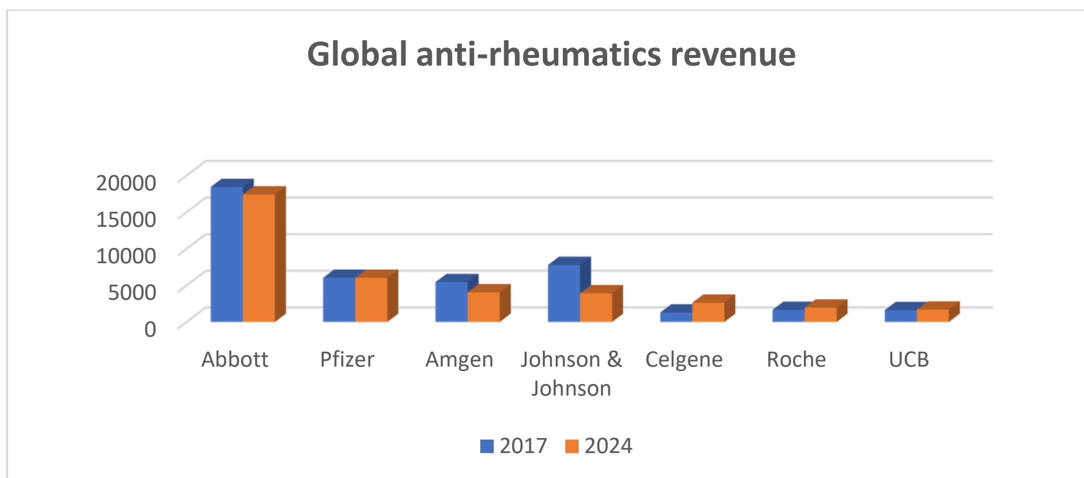


Figure 2.11. Statistics representing global revenue of anti-rheumatic drugs. (Source:*https://www.statista.com/statistics/309735/top-pharmaceutical-companies-by-anti-rheumatics-revenue-worldwide/*)

2.12 Complementary alternative medicine (CAM)

Herbal medicines are as effective as conventional medicines and are extremely safe, according to modern research. According to estimates, only 15% of the world's estimated 250-400 thousand plant species have been investigated phytochemically and 6% have been systematically screened for biological activity (Patwardhan et al., 2005). Over 60% of clinically used drugs contain natural compounds or their derivatives, and over 120 chemical products/moieties derived from herbal sources are used as lifesaving drugs (Yuan et al., 2016; PBW, 2018). Traditional herbal medicines are used by 80% of the population in some Asian and African countries for primary healthcare, whereas in many developed countries, 70-80% of the population uses CAM, which primarily consist of herbal products. 'Kampo' drugs are prescribed by 60-70 percent of allopathic doctors in Japan (largely composed of herbal products). Plant-based medicines are prescribed by approximately 70% of German physicians (Ghani et al., 2013).

According to the WHO Regional Office for the Americas, 71% and 40% of Chilean and Colombian populations, respectively, use herbal medicine. CAM is thought to be used by nearly 46%, 49%, and 70% of the populations in Australia, France, and Canada, respectively. Natural or herbal medicine is used by approximately 158 million Americans (Ghani et al., 2013).

CAM is defined as a collection of diverse health-care practises and systems that are not part of a country's tradition or allopathic/conventional medicine (WHO 2004). It has been reported that 60%-90% of the patient population from various countries use CAM to accelerate or modify pain relief caused by RA (Chandrashekara et al., 2011). Mind-body interventions (e.g., mindfulness, yoga, Qigong, Tai qi, hypnosis, cognitive therapy, patient support groups, and prayer), energy-based therapies (e.g., healing touch, therapeutic touch, Reiki), and biologically based therapies (e.g., herbal products, foods, dietary constituents) are all examples of CAM (Gellis et al., 2017; Phang et al., 2018).

Since ancient times, India has been a home for traditional medicine systems such as Ayurveda, Siddha, and Unani. Traditional, complementary, and alternative medicine has always been an integral part of India's health system, serving as a normative foundation for the maintenance of good health. In India, 65% of the population uses CAM to treat chronic illnesses and improve their quality of life (WHO 2004). CAM is now emerging as a promising add-on therapy for chronic illnesses such as RA, thanks to government support and initiative.

Chapter 3
Objectives and Plan of Work

3.1 Objectives

RA is a systemic chronic autoimmune disease characterised by inflammation of the synovium, synovial hyperplasia, angiogenesis and cartilage damage. The inflammation of the synovium and synovium pannus formation causes erosion and destruction of cartilage and subchondral bone in the affected joints, eventually leading to joint deformities and loss of joint function (Prasad et al., 2023). The pathogenesis of RA involves a complex interplay between genetic, environmental and immunological factors that lead to chronic inflammation and joint damage. Cytokines like TNF-α, IL-6 and IL-1β play a crucial role in the pathogenesis of RA (Kondo et al., 2021). These pro-inflammatory cytokines trigger signalling pathways associated with RA. The release of inflammatory mediators initiates the recruitment of innate and adaptive immune cells and the activation of synovial cells leading to synovial inflammation and aggravating disease progression (Peng et al., 2023).

Pharmacologic treatment of RA involves combinations of NSAIDs, GCs, and DMARDs. Though these drugs have been proven to be effective in delaying the progression of the disease, the clearly defined side effects of these drugs like immunosuppression, gastrointestinal ulcers, osteoporosis, nausea, fatigue, cytopenia, rashes, liver damage, infections, psoriasis, etc., limit their long-term use (Ding et al., 2023). In addition, the high cost of biological DMARDs presents a major challenge in RA treatment that needs to be addressed (Radu and Bungau, 2021). Therefore, there is a crucial need to develop cost-effective alternatives and novel therapeutics that have enhanced efficacy and reduced toxicity. Herbal therapy is considered an alternative to conventional medicines due to their better safety and efficacy (Akram et al., 2021). In view of the above facts, an investigation to explore and develop oral herbal compositions for the treatment of RA was planned.

The detailed objectives of the study included the following:

I. To investigate and identify herbal compositions possessing pro-inflammatory cytokine inhibitory activity based on in-vitro assays.

II. To perform standardisation of the herbal ingredients of the compositions and acute toxicity study

III. To evaluate the effectiveness of the compositions under acute and chronic in-vivo (inflammation and rheumatoid arthritis) models.

IV. To analyse the current challenges associated with the positioning and pre-launching of herbal products for rheumatoid arthritis to set forth marketing strategies.

3.2 Plan of work

The work plan was divided into the following phases:

Phase-1: Selection of herbal materials based on literature review

Phase-2: Extraction of herbal materials, preparation of compositions, and testing their pro-inflammatory cytokine and NO inhibitory activity through in-vitro assays.

Phase 3: Standardisation of the herbal ingredients of the compositions.

Phase-4: Evaluation of toxicity of the major herbal ingredient of the compositions.

Phase-5: Evaluation of the compositions under acute in-vivo inflammatory models

Phase 6: Evaluation of the compositions under RA animal models

Phase 7: Analysis of the challenges associated with the positioning and pre-launching of the herbal products for RA through a questionnaire-based survey.

Chapter 4
Materials and Methods

4.1 General

The experiments were carried out using analytical and molecular biology-grade reagents. For in-vitro studies, mouse RAW 264.7 cell lines obtained from National Centre for Cell Science in Pune, India were used. The test extracts and standard prednisolone were dissolved in DMSO and directly added to the culture media at a final concentration of less than 0.1%. To conduct the in-vivo study, animals BALB/c mice and Sprague-Dawley rats) were procured from Vab BioSciences in Hyderabad, India. Table 4.1.1. and 4.1.2. lists the chemicals and instruments used for the various experiments of the study.

Table 4.1.1. Chemicals and reagents used in the study

Chemicals	Make
Dulbecco modified eagle media (DMEM), High Glucose, Diethyl pyrocarbonate (DEPC) water, Antibiotic solution, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT reagent), Dimethyl sulfoxide (DMSO), Sodium oxalate crystals, N,N,N′,N′- Tetramethylethylenediamine (TEMED).	Himedia Laboratories, India
Fetal bovine serum South American Origin (FBS)	Gibco™, USA
Lipo polysaccharide (LPS) (*E. coli*), λ-carrageenan, 2,4 Di-tertiary butyl phenol, 3,5-Di-tertiary-butyl-4-hydroxy phenyl propionic acid, Indole-3-carboxylic acid, 2-(4-Hydroxyphenyl) ethanol, Indomethacin, Prednisolone, Tween 20, RIPA lysis buffer, TMB substrate, BCA kit for protein estimation, Primers for TNF-α, IL-1β, IL-6, RANK, TRAP, Cathepsin and 18s.	Sigma-Aldrich, MO, USA
Organic solvents (Ethyl Acetate LR, Methanol LR, Ethyl Acetate AR, Methanol HPLC, Acetonitrile HPLC grade).	Finar, India
Glycine, Tris HCl, TRIS Base, Ammonium per sulphate, Sodium Dodecyl Sulphate and Acrylamide/Bisacrylamide, Phosphomolybdic acid, Hematoxylin and eosin stain.	Sisco Research Laboratories) Chemicals, India
Complete Freund’s adjuvant, Freund’s incomplete adjuvant.	MP Biomedicals, USA
Bovine type II collagen.	Chondrex Inc.USA
Calcium oxalate.	Alpha Aesar, USA
Duo Set ELISA kits for TNF-α, IL-1β and IL-6 estimations.	R & D Systems Biotechne, USA
Methyl cellulose 4000 CPS.	S. D. Fine-chem Ltd., India
SYBR Green PCR Master, cDNA synthesis kit, TB green advantage qPCR Premix.	Takara, USA
Trizol reagent	Thermo Scientific, USA
Prestained Precision Plus Protein™ (10-250 kDa), PVDF membrane , Clarity Western ECL Substrate.	Bio-Rad, Laboratories, USA
Primary antibody for MMP3, NFκB, Integrin α V, β-actin.	Cell Signalling Technology, USA

Table 4.1.2. Instruments used in the study

Instruments	Model/Make
Multiplate reader	Spectromax M4, USA
Digital Plethysmometer	Ugo Basil, Italy
Rota rod	LE8505 Pan Lab, Spain
Liquid Chromatography	LC-8A, Shimadzu, Japan equipped with an ODS column(Inertsil ODS-3, 25 cm×4.6 mm id GL Sciences, Japan)
Zeiss microscope	Axiovision software, Germany
Rotary evaporator	Buchi R-210, Switzerland
Centrifuge	Eppendorf, USA
Real time thermal cyclers	iCycleriQ apparatus with iCycler Optical System software (version 3.1), Bio-Rad Laboratories, USA
Enhanced Chemiluminescence detection system (FUSION FX)	Vilber Lourmat, France
Microscope	Leica microsystems, Germany
Nanodrop spectrophotometer	Eppendorf, Germany
ImageJ software 1.53a	NIH, USA
Graph pad prism 8	GraphPad Software, Inc, USA
Western blot apparatus	Bio-Rad Laboratories, USA
Isoflurane chamber	E-Z anaesthesia, USA
Zetasizer	Malvern Instruments, UK
Digital X-ray imaging system	i-sensorH1/H2, China
Blood cell counter	ADVIA-2120i, Siemens, Germany
FESEM	FEI-Apero Lo Vac/ Thermofisher USA
Inductively coupled plasma mass spectrometry	Perkin Elmer ICP-MS (Nex Ion 2000), USA

4.2 Plant material

H. pinifolia was collected from the Vellar Estuary, Tamil Nadu, India. The plant specimen was authenticated by Dr. Saravana Kumar, Centre for Advanced Studies in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, India. The other plant material, *Glycyrrhiza glabra* was supplied by an authenticated plant vendor Dr. V. Chelladurai, Research Officer Botanist (Retired), Central Council for Research in Ayurveda and Siddha, Govt. of India Tirunelveli, Tamilnadu, India. The plant sample of *H. pinifolia* was studied for macroscopic

characters and recorded. Thin sections of the plant sample were taken, stained with safranin solution and observed under light microscope for microscopic characterisation.

4.3 Preparation of extracts

The whole plant material of *H. pinifolia* (including roots and rhizome) was dried and pulverized to a coarse powder. The powder (417 g) was then extracted using ethyl acetate at room temperature for 48 h (thrice). The extract was filtered and the combined filtrate was evaporated under reduced pressure to make it a solvent-free residue (2.524 g). The residue (HP) obtained was then stored in an airtight container in a refrigerator for further studies. Similarly, the roots and rhizomes of *G. glabra* were dried and pulverized to a coarse powder (200 g) and extracted using water and ethanol (1:3) at 50 ℃ for 3 h (thrice). Then the extract was filtered and the combined filtrate was evaporated which yielded a residue (78 g) (LQ). The residue (LQ) was then stored in an airtight container in a refrigerator for further studies.

4.4 Preparation of herbal compositions

Composition 1 was prepared by mixing HP and LQ, respectively in different ratios 1:1, 1:0.5, 1:0.25 and 1:0.125. Composition 2 was prepared by mixing HP and glycyrrhizin (Gly), respectively in different ratios 1:0.5, 1:0.25, 1:0.125 and 1:0.0625. Composition 3 was prepared by mixing HP and mono ammonium glycyrrhizinate (MAG), respectively in different ratios 1:0.5, 1:0.25, 1:0.125 and 1:0.0625.

4.5 In-vitro studies

4.5.1 Cell Culture

Dulbecco’s modified eagle media supplemented with 10% FBS and 1% antibiotic was used for the culture of RAW 264.7 cells in a humidified atmosphere of 5% CO_2 at 37 °C. It was passaged to maintain logarithmic growth. After reaching 40% confluence, around 10,000 cells per well were seeded and kept overnight for adherence and growth.

4.5.2 Cytokine assay

The seeded cells were treated with various concentrations of samples, HP (500, 250, 100, 50, 25 and 12.5 µg/mL), LQ (500, 250, 100, 50, 25 and 12.5 µg/mL), composition 1 (HP:LQ 100:100, 100:50, 100:25 and 100:12.5 µg/mL), Gly (100, 30, 10 and 3 µM), composition 2 (HP µg/mL: Gly µM 100:50, 100:25, 100:12.5 and 100:6.25), MAG (100, 30, 10 and 3 µM) and composition 3 (HP µg/mL: MAG µM 100:50, 100:25, 100:12.5 and 100:6.25). Cells were pre-

incubated with different concentrations of samples for 1 h and then stimulated with 1 μg/mL of LPS for TNF-α and IL-6 estimations and with LPS+oxalate crystal for IL-1β estimation. After 6 h of incubation, the supernatant was collected, and cytokines concentrations were estimated (Hira and Sajeli 2021) according to the standard curve using ELISA kits as per the manufacturer's instruction.

4.5.3 Nitrite determination

Cells were seeded onto 96-well plates with 2 x 10^5 cells/well and allowed to adhere overnight. The seeded cells were supplemented with different concentrations of extracts, compounds, and their combinations. After 1 h of incubation, LPS stimulation was performed. LPS was added at a concentration of 1 μg/mL for 24 h. The cell supernatant was mixed with an equal volume of Griess reagent (equal volumes of 1 % (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamide–HCl), incubated at room temperature for 10 min, and then the absorbance was measured at 540 nm, using a microplate reader. The amount of nitrite present in the samples was calculated by means of a standard curve generated using serial dilutions of $NaNO_2$ (Moro et al. 2012, Begum et al. 2021).

4.6 Standardization of prepared extracts by HPLC method

4.6.1 Standardization of ethyl acetate extract of *H. pinifolia* (HP)

The ethyl acetate extract of *H. pinifolia* (HP) was analysed by HPLC using Phenomenex Kinetex 5μ C-18 100A (150 mm x 4.6 mm) column; Mobile Phase: 55A (Formic acid) and 45B (Methanol); Temperature - 28˚C; Flow rate – 1.2 mL/min. A serial dilution of standards resulting to 20, 40, 60, 80 and 100 ppm solutions were used for preparing the calibration curve (Concentration Vs Area Under Curve). The amount of vanillic acid (Rt 3.20 min), ethyl protocatechuate (Rt 7.097) and rosemarinic acid (Rt 8.507 min) present in HP was quantified from the standard graph.

4.6.2 Standardisation of aqueous alcoholic extract of *G. glabra* (LQ)

For the HPLC analysis of LQ, Phenomenex Kinetex 5μ C-18 100A (150 mm x 4.6 mm) column was used. A gradient analysis using 0.05% acetic acid and methanol as mobile phase at a flow rate of 1.2 mL/min was followed. A PDA detector set at 200-350 nm under room temperature was used. Glycyrrhizin, the principal bioactive constituent of liquorice was used as a marker compound for quantitative analysis. A serial dilution of glycyrrhizin sample was done to get

20, 40, 60, 80 and 100 ppm solutions, which were then used for preparing the calibration curve (Concentration Vs Area Under Curve; detection at 254 nm). The amount of glycyrrhizin (Rt 18.882 min) present in LQ was quantified from the standard graph.

4.7 Heavy metal analysis of ethyl acetate extract of *H. pinifolia* (HP)

The sample was prepared by dissolving HP (0.1 g) in a 1:1 mixture of HCl and HNO_3 and digested with a diluent for 45 min. An internal standard of 0.1 mL was added to the digested samples and the volume was made up to 10 mL using diluent. The prepared samples were then analysed for heavy metals using ICP-MS. The analytical conditions included Dual detector mode, Helium KED Gas mode and 3.5 mL/min Helium gas flow.

4.8 Acute toxicity study

The experiment was conducted in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines and was approved by the Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2019/08). Healthy male and female Balb/c mice (20–24 g) were used for acute toxicity studies. Animals were acclimatised under standard conditions (50% relative humidity, 27±2 °C temperature, and 12 h light/dark cycle) for around 1 week prior to the study. Animals were given standard pellet diet with free access to water. Animals were randomly divided into four groups with control (n=3) and treatment (n=5) of each sex. HP was suspended in tween 80 and sodium CMC to prepare an oral suspension before administration to the animals. The mice in the treated groups received doses of 500, 1000, and 2000 mg/kg via oral administration after one week of adaptation period and observed for 14 consecutive days following OECD guidelines. Mice in the control group received only the vehicle. During the experiment, body weight, food consumption, and water intake were recorded daily.

4.8.1 Morphological alteration

Mice were observed twice daily for signs of toxicity daily for 14 days. Morphological changes in skin, fur, eyes, mucous membrane, lacrimation, piloerection, pupil size, unusual respiration pattern, gait, posture, and response to handling; the presence of tonic-clonic movements, stereotypies (excessive grooming, repetitive circling), and bizarre behaviour (self-mutilation, walking backwards) were recorded before treatment once daily until 14 days (Variya et al., 2019).

4.8.2 Haematological and biochemical examination

At the end of 14 days, animals were anaesthetized under isoflurane anaesthesia and blood samples were collected from retro-orbital plexus into eppendorf tubes containing 20 μL ethylene-diamine tetra-acetic acid (EDTA) for various haematological examinations such as total red blood cell count and white blood cell count. In biochemical parameters random plasma glucose, blood urea, and serum creatinine were investigated. Urine was collected and analysed for appearance, volume, protein, glucose and presence of any blood cells.

4.8.3 Macroscopic gross necropsy

Animals were sacrificed after blood sampling, and various organs were collected for gross necropsy. The macroscopic morphology of vital organs such as the heart, kidneys, liver and lungs as well as organ weight was carried out.

4.8.4 Histopathological assessment of vital organs

For microscopic histological examination, the heart, spleen, liver, lungs, and kidney were fixed in 10% formalin for 48 h. After that, specimens were placed in increasing concentrations of isopropyl alcohol for dehydrating the tissue. Then, the tissued were infiltrated with paraffin followed by a xylene incubation step. After preparing the blocks using paraffin-embedding system, 5µm sections were taken on poly-L-lysine coated slides using a microtome. Slides were incubated for 2h at 60˚C in a hot air oven for fixing the tissue to the slides. Next the slides were rehydrated after dipping in decreasing concentrations of isopropyl alcohol. After processing the tissues Haematoxylin was added to the slide and incubated for 3min. Following washing, sections were processed in acid alcohol for 2min. Then, the sections were incubated with eosin Y solution for 30s. After giving the washings, the tissues were mounted with coverslips. Then examined for histological changes by light microscope (Zeiss microscope, Axiovision software, Germany).

4.9 In-vivo acute models of inflammation

4.9.1 LPS-induced mice endotoxaemia model

The study protocol was approved by Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2019/29). Male BALB/c mice of 24-30 g body weight were divided into seventeen

groups (n=5 in each group) which included control, LPS control, prednisolone (standard), and fourteen treatment groups to test the dose-dependent effect of HP, LQ, Gly, MAG and optimized compositions (C1, C2 and C3) on LPS-induced endotoxemia condition. Table 4.9.1 details the groups, treatments and doses used for the study. The treatment doses were given as a suspension prepared in a vehicle containing 0.5% methylcellulose and 0.025% tween 20. Samples were administered one hour prior to LPS administration *via* oral route using gavage. Blood was withdrawn through the retro-orbital route at different time points (1 h and 6 h after LPS administration) for cytokines estimations using the ELISA kit as per the manufacturer's instruction

Table 4.9.1. Details of the groups, treatments and doses (mg/kg body weight) administered in LPS-induced endotoxemia model

Group	Treatment & Dose (mg/kg)	Group	Treatment & Dose (mg/kg)	Group	Treatment & Dose (mg/kg)
Group 1	HP (100)	Group 6	MAG (50)	Group 11	Gly (50) *2
Group 2	LQ (50)	Group 7	C3 – HP+MAG	Group 12	C2 – (HP+Gly) *2
Group 3	C1 - HP+LQ	Group 8	HP (100) *2	Group 13	MAG (50) *2
Group 4	Gly (50)	Group 9	LQ (50) *2	Group 14	C3 – (HP+MAG) *2
Group 5	C2 - HP+Gly	Group 10	C1 - (HP+LQ) *2		

4.9.2 Carrageenan-induced paw oedema model

The study protocol was approved by Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2019/29). Male BALB/c mice weighing between 24-30 g were randomly divided into five groups (n=5): Group 1 - normal control, Group 2 - carrageenan control group, Group 3 - C1 (HP+LQ), Group 4 - C2 (HP+Gly) and Group 5 - C3 (HP+MAG). The treatment groups received 100+50 mg/kg body weight doses containing HP+LQ/Gly/MAG, respectively. Carrageenan (1% w/v solution in 0.9% sterile saline) of 25 μL was injected into the paw of the mouse to test the efficacy of the compositions in local inflammation. Treatment was given 1 h prior to carrageenan injection using oral gavage. Then carrageenan was injected in the sub-plantar region of the left hind paw subcutaneously. Paw volume was measured on an hourly basis using a digital Vernier Caliper for 4 h after carrageenan administration (Morris 2003 and Ou et al. 2019). Animals were sacrificed after the fourth paw volume reading. Paws were then collected and snap-frozen for further molecular studies.

4.9.2.1 Estimation of TNF-α by ELISA

The paw tissues were homogenized using liquid nitrogen in RIPA buffer supplemented with a protease inhibitor cocktail (Sigma, USA) and 1 mM phenylmethylsulphonyl fluoride. The prepared homogenate was then centrifuged at 15000 g for 15 min at 4 ºC and the supernatant was collected and tested to determine the concentration of TNF-α using standard ELISA kits as per the manufacturer's instruction.

4.9.2.2 Determination of NO levels

The supernatant obtained from the paw tissue homogenates of various groups was mixed with an equal quantity of Griess reagent (50 µL each) and incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm, using a microplate reader (Moro et al., 2012, Begum et al., 2021).

4.9.2.3 Determination of myeloperoxidase activity in paw tissues

Myeloperoxidase (MPO) activity is considered an index of neutrophil infiltration and was measured in snap-frozen paw tissue samples as described in the literature (Pulli et al., 2013, George et al., 2016; Alavala et al., 2020,). The tissues were minced in liquid nitrogen using a mortar and pestle. The minced tissues were then homogenized in CTAB (Cetyl Trimethyl Ammonium Bromide) buffer containing 50 mM phosphate buffer (pH 6.0). The tissue homogenates were centrifuged at 15000 g for 20 min at 4˚ C. For the determination of MPO activity, 10 µL supernatant was combined with 80 µL of 0.75 mM H_2O_2 and 110 µL of TMB solution (sigma) in a microwell plate. The plate was then incubated at 37˚ C for 5 min. The reaction was then stopped by adding 50 µL of 2M H_2SO_4 solution and the absorbance was measured at 450 nm to estimate the MPO activity.

4.9.2.4 Estimation of gene expression of TNF-α, IL-6 and IL-1β levels by RT-PCR

The expression of IL-1β, IL-6 and TNF-α in paw tissue was also examined using RT-PCR method. Briefly, total RNA was isolated using Trizol reagent (Takara) according to manufacturer's protocol. Absorbance ratios of 260/280 and 260/230 were measured to determine the purity and concentration of RNA. The 260/280 ratio of 2.0 and the 260/230 ratio of 2.0-2.2 were considered pure. To prepare the first strand of complementary DNA (cDNA), 2 µL of RNA from the total extracted RNA was reverse transcribed using the cDNA Synthesis Kit (Takara). The resulting cDNA was used to quantify the mRNA levels of IL-1β, IL-6 and

TNF-α. The housekeeping gene 18s rRNA was used to normalize all Cq values. Furthermore, RT-PCR study was carried out for determining inflammatory markers using primers 18 S (Forward CACGGACAGGATTGACAGATT; Reverse GCCAGAGTCTCGTTCGTTATC), TNF-α (Forward CTACCTTGTTGCCTCCTCTTT; Reverse GAGCAGAGGTTCAGTGATGTAG) IL-6 (Forward GCACTACAGGCTCCGAGATGAA; Reverse GCCTCCGACTTGTGAAGTGGTA) and IL-1β (Forward GCACTACAGGCTCCGAGATGAA; Reverse GTCGTTGCTTGGTTCTCCTTGT). Finally, the amplified PCR products were observed by measuring the increase in fluorescence due to the increased binding of SYBR Green Dye to dsDNA produced and calculated by the $2^{-\Delta\Delta Ct}$ method as described by (Schmittgen et al., 2000).

4.9.2.5 Western Blot Analysis

Western blot analysis was performed on the paw tissue homogenates to study the mechanism of action of compositions C1, C2 and C3. Briefly, paw tissues were homogenized in RIPA lysis buffer containing protease inhibitor cocktail and 1 mM phenylmethylsulphonyl fluoride to obtain the whole tissue extracts. The protein concentration in each tissue extract was determined using a BCA protein assay kit with BSA as a standard. Equal amounts of protein (30 μg protein/lane) were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% BSA for 1 h and then incubated at 4 ℃ overnight with the primary antibodies. Antibody against NF-κB (1:1000) was employed to analyse the expression of the respective proteins. Horseradish-peroxidase (HRP)-conjugated anti-rabbit antibody (1:4000) was used as the secondary antibody. The membrane was washed and bands were visualized by enhanced chemiluminescence using Chemidoc and densitometrically analyzed using Image J software (NIH, USA). Blots were stripped and re-probed for β-actin to ensure equal protein loading. The compositions of resolving and stacking gel are described in detail in Table 4.9.2.5

Table 4.9.2.5. Composition of resolving gel and stacking gel for protein separation

12% Resolving gel (10 mL)		Stacking gel (10 mL)	
Components	**Volume**	**Components**	**Volume**
Water	3.35 mL	Water	3.35 mL
Acrylamide/Bis-acrylamide(30%/0.8% w/v)	4 mL	Acrylamide/Bis-acrylamide (30%/0.8% w/v)	4 mL
1.5 M Tris (pH of 8.8)	2.5 mL	0.5 M Tris-HCl, pH 6.8	2.5 mL
10% (w/v) SDS	100 µL	10% (w/v) SDS	100 µL
10% (w/v) ammonium persulfate	50 µL	10% (w/v) ammonium persulfate	50 µL
TEMED	15 µL	TEMED	15 µL

4.10 In-vivo evaluation in chronic models of arthritis

Adjuvant-induced arthritis and collagen-induced arthritis are two established animal models that share pathological similarities with RA and therefore the anti-arthritic potential of extracts and compositions was evaluated in these two models.

4.10.1 Freund's complete adjuvant-induced arthritis model in rats

The experimental protocol was approved by the Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2019/29). Female Sprague Dawley (180-200 g) rats were used in the experiment after a one-week adaptation period. The animals were housed under a light/dark cycle of 12 h at 22 °C, and food and water were available *ad libitum*. The rats were weighed after 6 days of adaptive feeding. The volume of the right hind paw was measured. According to the measurement results, the animals were randomly divided into 6 groups with 8 rats in each group: G1 – normal control group, G2 - Freund's complete adjuvant arthritis group (arthritic control), G3 - dexamethasone group (standard; 0.1 mg/kg), G4 – HP treated group (100 mg/kg body weight), G5 – LQ treated group (50 mg/kg body weight) and G6 - C1 treated group (mixture of HP 100 mg/kg and LQ 50 mg/kg body weight). All the treatment samples were prepared as a suspension using tween 80 and sodium CMC in a clean motor and pestle. Animals of all groups except G1 were injected subcutaneously with 0.1 mL of Freund's complete adjuvant (FCA) on the right posterior sole. Rats of G1 group were injected with normal saline in the same way and modelled according to the traditional rats feeding conditions. While, on the 14th day after injection, rats of G3 – G6 groups were administered (using oral gavage) with the corresponding drugs, the control group and arthritic control group were administered with normal saline. The administration was once a day and continued for 14 consecutive days. At the end of the study, i.e., on the 28th day, blood samples were collected from the retro-orbital

plexus of experimental animals followed by euthanasia *via* cervical dislocation. The spleen and right paw tissues from each experimental animal were excised and weighed. Ankle joints were stored in 10% formalin for histopathological analysis and excised paws were stored at − 80 °C for further biochemical, immunoblot and ELISA analysis.

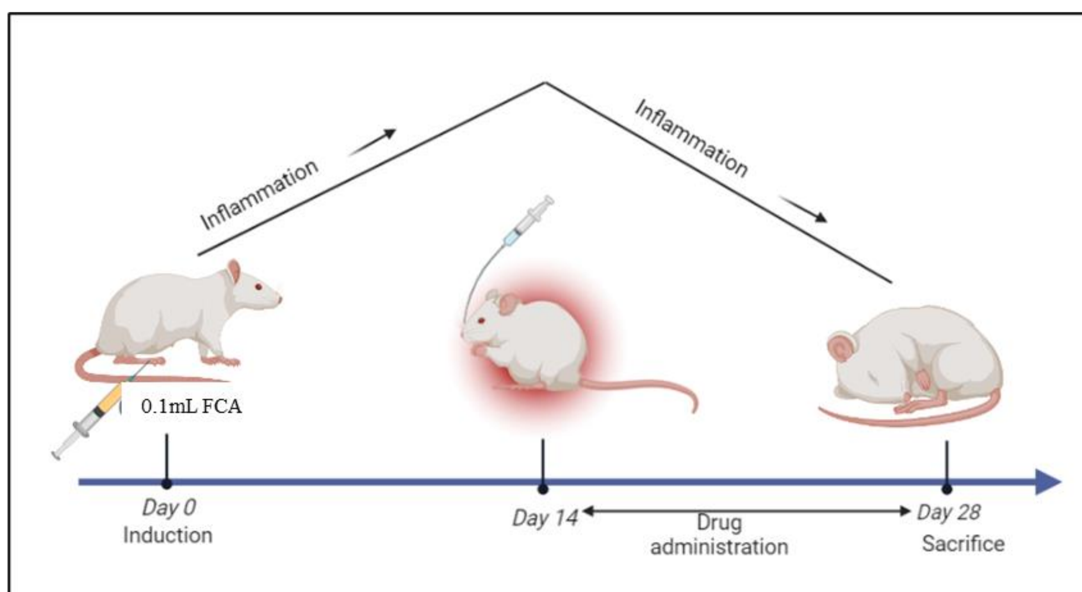


Figure 4.10.1. Schematic representation of disease induction with FCA and drug administration.

4.10.1.1 Assessment of arthritis in rats

The volume of the rat's right hind paws was measured using a digital Plethysmometer before the induction of arthritis. Later, the paw volume was measured weekly twice at the same position of the right hind paw of the rat until the 28th day, and the values were recorded.

4.10.1.2 Measurement of spleen index

Animals were sacrificed on the 28th day and the spleen was removed, washed with phosphate-buffered saline (PBS) and weighed. The index of spleen was expressed as the ratio (mg/g) of spleen wet weight to body weight.

4.10.1.3 Estimation of haematological parameters

On 28th day, blood samples were collected through the retro-orbital plexus in tubes containing anti-coagulant (disodium EDTA) from experimental animals of all groups. The haematological parameters platelet cells, white blood cells, and total leukocyte count were measured using a blood cell counter. Also, the erythrocyte sedimentation rate (ESR) was measured for all experimental animals.

4.10.1.4 Estimation of TNF-α, TRAP and IL-6 by ELISA

The paw tissues were homogenized using liquid nitrogen in RIPA buffer supplemented with protease inhibitor cocktail and 1 mM phenyl methyl sulfonyl fluoride. The tissue homogenate was centrifuged at 15000 g for 15 min at 4 ºC and the supernatant was collected and subjected to the estimation of TNF-α, TRAP and IL-6 using standard ELISA kits. The assay was carried out as per the standard protocol given by the manufacturer.

4.10.1.5 Estimation of gene expression of TNF-α, IL-1β, IL-6, TRAP, cathepsin and RANK by RTPCR

The expression of TNF-α, IL-1β, TRAP, Cathepsin and RANK were examined in paw tissue using RT-PCR. Briefly, total RNA was isolated using Trizol reagent (Takara) according to manufacturer's protocol. Absorbance ratios of 260/280 and 260/230 were measured to determine the purity and concentration of RNA. The 260/280 ratio of 2.0 and the 260/230 ratio of 2.0-2.2 were considered pure. To prepare the first strand complementary DNA (cDNA), 2 μL of RNA from the total extracted RNA was reverse transcribed using the cDNA Synthesis Kit. The resulting cDNA was used to quantify the mRNA levels of TNF-α, IL-1β, TRAP, Cathepsin and RANK. The housekeeping gene GAPDH rRNA was used to normalize all Cq values. Furthermore, RT-PCR study was carried out for determining inflammatory markers using primers TNF-α, IL-1β, TRAP, Cathepsin and RANK mentioned in Table 4.10.1.6. Finally, amplified PCR products were observed by measuring the increase in fluorescence due to the increased binding of SYBR Green Dye to dsDNA produced and calculated by the $2^{-\Delta\Delta Ct}$ method (Schmittgen et al., 2000).

Table 4.10.1.5. Primer sequence of TNF-α, IL-1β, IL-6, RANK, TRAP, Cathepsin K and GAPDH for RT-PCR study of Adjuvant Induced Arthritis model

Gene	Sense primer Antisense primer (5'-3')
TNF-α	TACTGAACTTCGGGGTGATCG
	CCTTGTCCCTTGAAGAGAACC
IL-1β	CAACAAAAATGCCTCGTGC
	TGCTGATGTACCAGTTGGG
IL-6	AAATCTGCTCTGGTCTTCTGG
	TTAGATACCCATCGACAGG
RANK	TTAAGCCAGTGCTTCACGGG
	ACGTAGACCACGATGATGTCGC
TRAP	CGCCAGAACCGTGCAGA
	TCAGGCTGCTGGCTGAC
Cathepsin K	CCCAGACTCCATCGACTATCG
	CTGTACCCTCTGCACTTAGCTGCC
GAPDH	ACCACAGTCCATGCCATCAC
	TCCACCACCCTGTTGCTGTA

4.10.1.6 Histopathological assessment of ankle joint

For microscopic histological examination, ankle joint samples were fixed in 10% formalin. After 48 h in fixative, specimens were placed in 5 % formic acid for decalcification. After 2 weeks, the ankle joints were evaluated for decalcification by adding 0.5 mL of formic acid aliquot of decalcified solution to a 10 mL of citrate-phosphate buffer (pH 3.2-3.6) mixed with 2.5 mL of 5% ammonium oxalate solution. The failure to form a cloudy white precipitate over the course of 20 min on two successive days of testing indicated complete decalcification. After evaluating decalcified tissue, the specimens were placed in increasing concentrations of isopropyl alcohol for dehydrating the tissue. Then, the tissues were infiltrated with paraffin followed by a xylene incubation step. After preparing the blocks using a paraffin-embedding system, 5 μm sections were taken on Poly-L-lysine coated slides using a microtome. Slides were incubated for 2 h at 60 °C in a hot air oven to fix the tissue to the slides. The detailed staining procedure is presented in Table 4.10.1.7. The tissue sections were then examined for histological changes by light microscopy (Schmitz, N., 2010). Histopathological characteristics

were assessed and scored under blinded conditions according to the following system: 0) no detectable change; 1) mild; 2) moderate and 3) severe.

Table 4.10.1.6. Staining protocol followed for the histological assessment of ankle joint

S. No	Solution	Time (min)
1	Xylene	4
2	Xylene	4
3	Xylene	4
4	95% Alcohol	1
5	95% Alcohol	1
6	70% Alcohol	1
7	Tap water rinse	1
8	Mayer hematoxylin solution	8
9	Running tap water	10
10	95% Alcohol	10 dips
11	Eosin Y solution	1
12	100% Alcohol	5
13	100% Alcohol	5
14	100% Alcohol	5
15	Xylene	5
Mount with xylene-based media		

4.10.1.7 Radiological assessment of ankle joint

To analyse the bone changes, X-ray radiographs of the adjuvant-injected hind paws were taken on 28th day. Radiographic measurement of the hind paws was performed at 65 kV peak, 7.5 mAs, with an exposure time of 2I sec. To evaluate the degree of soft tissue swelling/joint destruction and periarticular erosive changes, radiographs were also scored (Gowayed et al., 2015) as follows: a) For soft tissue swelling, radiographs were scored on a scale of 0–4 (0 = normal with no soft tissue swelling, 1 = minimal soft tissue swelling, 2 = mild to moderate soft tissue swelling, 3 = moderate soft tissue swelling, 4 = marked to severe soft tissue swelling); b) For the degree of joint destruction and periarticular erosive changes, radiographs were also scored from 0–4 (0 = no joint destruction, 1 = minimal joint destruction, 2 = mild to moderate joint destruction, 3 = moderate joint destruction, 4 = marked to severe joint destruction with evident periarticular erosive changes). All scores were assessed by an observer blinded to the experimental groups.

4.10.1.8 Western Blot analysis

Western blot analysis was performed to study the effect of HP, LQ and C1 on CD51 and MMP3 in the paw tissue samples of FCA model. Briefly, paw tissues were homogenized in RIPA lysis buffer containing protease inhibitor cocktail and 1 mM phenyl methyl sulfonyl fluoride to obtain whole tissue extracts. The protein concentration in each tissue extract was determined using BCA protein assay kit with BSA as a standard. Equal amounts of protein (40 μg protein/lane) were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% BSA for 1 h and then incubated at 4 °C overnight with the primary antibodies. Antibody against CD51 (1:1000) and MMP3 (1:1000) was employed to analyse the expression of the respective proteins. Horseradish-peroxidase (HRP)-conjugated anti-rabbit antibody (1:4000) was used as a secondary antibody. The membrane was washed and bands were visualized by enhanced chemiluminescence using Chemidoc (Bio-Rad) and densitometrically analysed using ImageJ software. Blots were stripped and reprobed for β-actin to ensure equal protein loading (Alavala et al., 2019).

After establishing the anti-arthritic activity of C1 in FCA model the study was taken forward to test its effect in collagen induced arthritis (CIA) model. Also, an attempt was made to prepare a nano-emulsion of C1 to investigate if the efficacy could be enhanced by reducing the particle size and developing it into a stable lipid nano-emulsion. Thus, both C1 and its lipid nano-emulsion (C1-N) were tested in CIA model.

4.10.2 Collagen-induced arthritis model

4.10.2.1 Preparation of C1-N

A lipid nano-emulsion of C1 (C1-N) was prepared by dissolving HP (2000 mg) in 20 mL ethanol at 50-55 ºC. To this solution, 10 g of egg phosphatidylcholine and 20 mL of soyabean oil were mixed and dissolved. The resultant solution (oil phase) was then gradually added (1 mL/min) with a syringe into 140 mL of water containing 1000 mg of LQ under high shear mixing (22000 rpm) at 50-55 ºC. After the complete addition of the oil phase, the high shear homogenization was continued for 15 min and the volume was made up to 200 mL.

4.10.2.2 Determination of particle size, polydispersity index and zeta potential

The average diameter and polydispersity index (PDI) of C1-N were determined using a Zetasizer Nano ZS that utilizes the Non-Invasive Back-Scattering technique. The samples were diluted with filtered (0.2 µm) milli Q water in 1:1000 dilution and the reading was carried at a 173° angle with respect to the incident beam. The zeta potential values were measured using a Zetasizer Nano ZS Malvern Instrument. The samples diluted in 1:1000 dilution with milli Q were analysed in triplicate. Each reported value was the average of three measurements.

4.10.2.3 Morphological studies with field emission scanning electron microscopy (FESEM)

Particle size and morphological features of C1-N were studied by using SEM at varied magnification. The micrographs of all prepared samples were recorded using FESEM at an acceleration voltage of 10kV. All the samples were sputtered with a gold coating of 10 nm thickness to prevent surface charge accumulation.

4.10.2.4 Estimation of total phenolic content

The total phenolic content of C1-N was estimated using Folin–Ciocalteu reagent following standard procedures. In brief, 1 mL of the formulation was combined with 2.5 mL of 10% (w/v) Folin-Ciocalteu reagent. After 5 min, 2.5 mL of $NaHCO_3$ (7.5%) was added to the mixture, which was then incubated at 50 °C for 10 min with intermittent agitation. After cooling, the sample was measured for absorbance at 765 nm using a UV Spectrophotometer against a blank without extract. The results were expressed as milligrams per gram of gallic acid equivalents (mg GAE/g) of dry extract of C1 (Singleton et al. 1999).

4.10.2.5 Stability studies

The stability test of the prepared lipid nano-emulsion of C1 was conducted at 5˚ C, 25˚ C /60% RH, and 40˚ C/75% RH for 3 months. Their particle size, PDI, zeta potential, shape and surface morphology, total phenolic content and physical appearance were observed

4.10.2.6 Induction of arthritis

The experiment was conducted in accordance with the OECD guidelines and was approved by the Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2019/29). Female Sprague Dawley rats (180-200g) were used in the experiment after a one-week adaptation period. The animals were housed under a light/dark cycle of 12 h at 22˚ C, and food and water were available *ad libitum*. The rats were weighed after 6 days of adaptive feeding. Arthritis in rats was induced by immunization with bovine collagen type II (CII), emulsified with incomplete

Freund's adjuvant. Briefly, Collagen was dissolved in 0.01N glacial acetic acid (2 mg/mL) by gently stirring overnight at 4 °C and emulsified with an equal volume of incomplete Freund's adjuvant (IFA). The prepared emulsion contained 250 µg of collagen in 125 µL of 0.01N acetic acid which was emulsified with 125 µL of IFA. Therefore, a total emulsion volume of 250 µL was administered subcutaneously into the base of the tail of each animal. A booster injection of 125 µL of the same emulsion was administered after 7 days. The volume of the right hind paw was measured before the induction of arthritis and recorded as 0th day reading. According to the measurement results, the animals were randomly divided into 5 groups with 5 rats in each group: G1- normal control group, G2 - collagen-induced arthritis group (arthritic control), G3 - dexamethasone treated group (standard), G-4 - C1 treated group and G5 - C1-N treated group. Sample C1 was prepared by suspending HP 100 mg/kg and LQ 50 mg/kg in tween 80 and sodium CMC. On the 3rd week after injection, the animals of standard group (0.1 mg/ kg), C1 group (100+50 mg/kg) and C1-N group (100+50 mg/kg) received the corresponding drugs orally, while the control group and arthritic control group were given the same amount of normal saline orally once a day for 28 consecutive days. At the end of the study i.e. on the 48th day, blood samples were collected from the retro-orbital plexus of experimental animals followed by euthanasia via cervical dislocation. The spleen, liver, and right paw tissues from each experimental animal were excised and weighed. Ankle joints were stored in 10% formalin for histopathological analysis and excised paws were stored at − 80 °C for further biochemical, immunoblot and ELISA analysis.

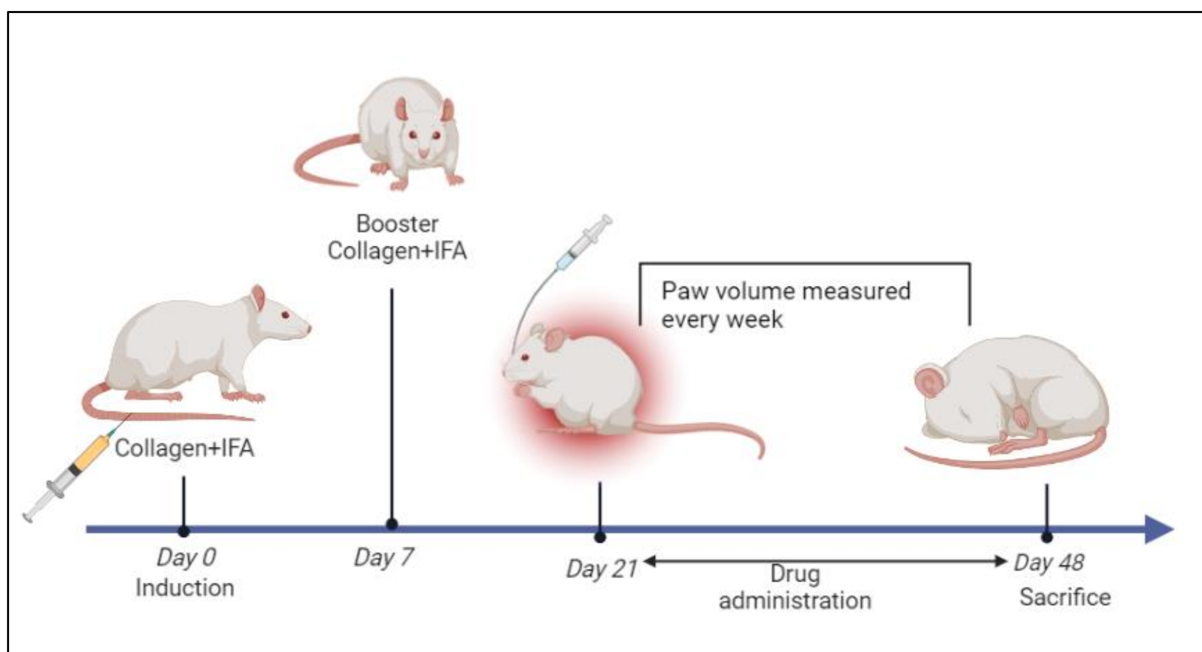


Figure 4.10.2.6. Schematic representation of disease induction with CIA and drug administration.

4.10.2.7 Measurement of paw volume in rats

The volume of the rat's right hind paws was measured using a digital Plethysmometer before the induction of arthritis. Later, the paw volume was measured weekly twice at the same position of the right hind paw of the rat until the 48th day, and the values were recorded.

4.10.2.8 Measurement of spleen index

Animals were sacrificed on 48th day and the spleen was removed, washed with PBS and weighed. The indices of spleen, liver and thymus were expressed as the ratio (mg/g) of spleen, liver and thymus wet weight to body weight, respectively.

4.10.2.9 Estimation of hematological parameters

On 48th day, blood samples were collected through the retro-orbital plexus in tubes containing anti-coagulant (disodium EDTA) from experimental animals of all groups. The haematological parameters (red blood cells, white blood cells, haemoglobin, platelet cells and total leukocyte count) were measured using a blood cell counter. Erythrocyte sedimentation rate (ESR) was also measured for all experimental animals.

4.10.2.10 Estimation of TRAP and IL-6 by ELISA

The paw tissues were homogenized using liquid nitrogen in RIPA buffer supplemented with

protease inhibitor cocktail and 1 mM PMSF. The tissue homogenate was centrifuged at 15000 g for 15 min at 4 ºC and the supernatant was collected for the estimation of TRAP and IL-6 using standard ELISA kits as per the protocol of the manufacturer.

4.10.2.11 Estimation of gene expression of TNF-α, IL-1β, cathepsin and RANK by RTPCR

The expression of TNF-α, IL-1β, TRAP, cathepsin and RANK were examined in paw tissue using RT-PCR as per the procedure mentioned in section 4.10.1.5. The housekeeping gene GAPDH rRNA was used to normalise all Cq values. Furthermore, RT-PCR study was carried out for determining inflammatory markers using primers. Finally, amplified PCR products were observed by measuring the increase in fluorescence due to the increased binding of SYBR Green Dye to dsDNA produced and calculated by the $2^{-\Delta\Delta Ct}$ method.

4.10.2.12 Histopathological assessment of ankle joint

Histopathological assessment of the ankle joint was carried out as per the procedure mentioned in section 4.10.1.6. Briefly, for microscopic histological examination, paw tissue samples were fixed in 10% formalin. After 48 h in fixative, specimens were placed in 5% formic acid for decalcification. After evaluation of decalcified tissue, the specimens were processed, embedded in paraffin, sectioned at 5 µm thicknesses and stained with haematoxylin and eosin (H&E). The tissue sections were then examined for histological changes by light microscopy.

4.10.2.13 Radiological assessment of ankle joint

X-ray radiographs of both hind paws were taken digitally on the 48th day. Radiographic measurement of the hind paws was performed at 60 kv peak, 7 mA. with an exposure time of 2s. Hind paws were placed over a radiographic cassette containing standard X-ray film at a distance of 90 cm from X-ray source. To evaluate the degree of soft tissue swelling/joint destruction and periarticular erosive changes, radiographs were also scored (Gowayed et al., 2015) as follows: a) For soft tissue swelling, radiographs were scored on a scale of 0–4 (0 = normal with no soft tissue swelling, 1 = minimal soft tissue swelling, 2 = mild to moderate soft tissue swelling, 3 = moderate soft tissue swelling, 4 = marked to severe soft tissue swelling); b). For the degree of joint destruction and periarticular erosive changes, radiographs were also scored from 0–4 (0 = no joint destruction, 1 = minimal joint destruction, 2 = mild to moderate joint destruction, 3 = moderate joint destruction, 4 = marked to severe joint destruction with

evident periarticular erosive changes). All scores were assessed by an observer blinded to the experimental groups.

4.10.2.14 Determination of NO levels

The paw tissues were homogenized using liquid nitrogen in RIPA buffer supplemented with protease inhibitor cocktail and 1 mM PMSF. The tissue homogenate was centrifuged at 15000 g for 15 min at 4 ºC and the supernatant was obtained from paw tissue homogenates and mixed with an equal quantity of Griess reagent (50 µL each) and incubated at room temperature for 10 min. The absorbance was measured at 540 nm, using a microplate reader.

4.10.2.15 Determination of myeloperoxidase activity (MPO) in paw tissues

The tissues were minced in liquid nitrogen using a mortar and pestle. The minced tissues were then homogenized in cetyl trimethyl ammonium bromide buffer containing 50 mM phosphate buffer (pH 6.0). The tissue homogenates were centrifuged at 15000 g for 20 min at 4˚ C. Briefly, 10 µL supernatant was combined with 80 µL 0.75 mM H_2O_2 and 110 µL TMB solution and the plate was incubated at 37˚ C for 5 min. The reaction was then stopped by adding 50 µL of 2M H_2SO_4 solution and the absorbance was measured at 450 nm to estimate MPO activity.

4.10.2.16 Western Blot analysis

The paw tissues were homogenized in RIPA lysis buffer containing protease inhibitor cocktail and 1 mM PMSF to obtain whole tissue extracts. The protein concentration in each tissue extract was determined using BCA protein assay kit with BSA as a standard. Equal amounts of protein (40 µg protein/lane) were resolved by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. Membranes were blocked in 5% BSA for 1 h and then incubated at 4 °C overnight with the primary antibodies. Antibody against CD51 (1:1000) and MMP3 (1:1000) was employed to analyse the expression of the respective proteins. HRP-conjugated anti-rabbit antibody (1:4000) was used as a secondary antibody. The membrane was washed and bands were visualized by enhanced chemiluminescence using Chemidoc (Bio-Rad) and densitometrically analysed using Image J software. Blots were stripped and reprobed for β-actin to ensure equal protein loading.

4.10.3 Freund's complete adjuvant-induced arthritis model for anti-arthritic evaluation of DEX, DEX-H, C2 and C3

Dexamethasone (DEX) is a well-established glucocorticoid prescribed for the treatment of RA, particularly to delay the progression of the disease. A new combination of DEX with HP (DEX-H) was prepared and examined on the disease progression of adjuvant-induced arthritis in rats. Also, two new compositions made by mixing (i) glycyrrhizin with HP (C2) and (ii) mono ammonium glycyrrhizinate salt with HP (C3) were also investigated

4.10.3.1 Preparation of test samples

A total of four samples were prepared for the evaluation. DEX at a dose of 0.1 mg/kg was prepared as suspension using 0.5% methylcellulose and 0.025% Tween 80. Sample DEX-H suspension was prepared by combining 0.05 mg/kg of DEX with 100 mg/kg of HP. Similarly, C2 suspension contained 50 mg/kg dose of Gly and 100 mg/kg of HP extract. C3 suspension was prepared using 50 mg/kg dose of MAG and 100 mg/kg of HP extract. The suspensions were administered to rats via oral gavage from 14th to 28th day of arthritis induction.

4.10.3.2 Induction of Arthritis

The experimental protocol was approved by the Institutional Animal Ethics Committee (BITS-Hyd/IAEC/2019/29). Female Sprague Dawley (180-200 g) rats were used for the experiment after a one-week adaptation period. The animals were housed under a light/dark cycle of 12 h at 22 °C, and food and water were available *ad libitum*. After 6 days of adaptive feeding, the rats were weighed and the volume of the right hind paws was measured. According to the measurement results, the animals were randomly divided into 6 groups with 8 rats in each group: G1 – normal control group, G2 - Freund's complete adjuvant arthritis group (arthritic control), G3 - DEX group, G4 – DEX-H group, G5 – C2 and G6 – C3 group. Animals of all groups except G1 were injected subcutaneously with 0.1 mL of Freund's complete adjuvant on the right posterior sole. Rats of G1 group were injected with normal saline in the same way and modelled according to the traditional rats feeding conditions. On the 14th day after injection, rats of the G3 – G6 groups were administered (using oral gavage) with the corresponding drugs, and animals of G1 and G2 were administered with normal saline. The administration was once a day and continued for 14 consecutive days. On the 28th day, blood samples were collected from the retro-orbital plexus of rats followed by euthanasia *via* cervical dislocation. The spleen and right paw tissues from each experimental animal were excised and weighed. The ankle

joints were collected and stored in 10% formalin for histopathological analysis. The excised paws were stored at − 80 °C for further molecular analysis.

4.10.3.3 Evaluation of arthritis in rats

The volume of the right hind paws of the rats was measured using a digital Plethysmometer as mentioned in section 4.10.1.1.

4.10.3.4 Examination of haematological parameters and spleen index

On the 28th day, blood samples were collected from experimental animals of all groups through the retro-orbital plexus in tubes containing disodium EDTA. The haematological parameters such as platelets count, white blood cells, and total leukocyte count were measured using a blood cell counter. Also, the erythrocyte sedimentation rate (ESR) was measured for all the samples. Additionally, on the 28th day, after blood collection, the animals were sacrificed and the spleen was collected, washed with PBS and weighed. The index of the spleen was calculated as the ratio (mg/g) of spleen wet weight to the body weight of the rat.

4.10.3.5 Estimation of TRAP and IL-6 by ELISA

The estimation of TNF-α, TRAP and IL-6 in the paw tissue homogenate was done using standard ELISA kits as mentioned in section 4.10.1.4

4.10.3.6 Estimation of gene expression of TNF-α, IL-1β, cathepsin and RANK by RT-PCR

The mRNA expression of TNF-α, IL-1β, RANK and cathepsin were analysed in paw tissue using RT-PCR as mentioned in section 4.10.1.5

4.10.3.7 Histopathological analysis of ankle joint

The ankle joint samples were fixed in 10% formalin for microscopic histological analysis as mentioned in section 4.10.1.6

4.10.3.8 X-ray analysis of ankle joint

On the 28th day of the experiment, before sacrificing the animals, X-ray radiographs of the adjuvant-injected hind paws were taken to analyse the bone changes. The radiographic measurement of the hind paws was performed at 65 kV peak, 7.5 mAs, with an exposure time of 2 s. To evaluate the degree of soft tissue swelling/joint destruction and periarticular erosive changes, radiographs were also scored as follows: (i) For soft tissue swelling, radiographs were scored on a scale of 0–4 (0 = normal with no soft tissue swelling, 1 = minimal soft tissue

swelling, 2 = mild to moderate soft tissue swelling, 3 = moderate soft tissue swelling, 4 = marked to severe soft tissue swelling); (ii) For the degree of joint destruction and periarticular erosive changes, radiographs were also scored from 0–4 (0 = no joint destruction, 1 = minimal joint destruction, 2 = mild to moderate joint destruction, 3 = moderate joint destruction, 4 = marked to severe joint destruction with evident periarticular erosive changes) (Gowayed et al., 2015). All scores were assessed by an observer blinded to the experimental groups.

4.10.3.9 Rota-rod test

The rota-rod test was used to assess motor coordination (in terms of time). Animals with joint pain fall off quickly from rotating wheel and therefore this forced motor activity has subsequently been used to determine the functional recovery in rats. On the 28th day of FCA induction, rats were subjected to a rota-rod test. The test was performed by placing the rat on a rotating rod that rotated under continuous acceleration from 4 to 40 rpm over 300 s, and the latency (the time until the mouse falls off the rod) was measured. This experiment consisted of three trials separated by 15 min intervals.

4.10.3.10 Determination of NO and MPO levels

The NO and MPO levels were determined by following the method mentioned in sections 4.10.2.14 and section 4.10.2.15.

4.10.3.11 Western Blot analysis

To study the effect of administered samples on CD51 and MMP3 in the paw tissue samples, western blot analysis was performed as per the method described in 4.10.1.8

4.11 Statistical analysis

All values in the results were presented as mean ± SEM. The groups were compared using one-way ANOVA, followed by Dunnett's multiple comparisons using Graph Pad Prism 8.0 statistical software, and $P< 0.05$ was considered significant. The level of activity was calculated using the Bliss Independence model, where the calculation index (CI) of <1, >1 and =1 was considered as synergy, antagonism and additivity, respectively.

4.12 Marketing Strategies for proposed herbal product used by RA patients

4.12.1 Consumer analysis

The type and extent of complementary alternative medicine (CAM) usage among RA patients visiting rheumatology clinics was assessed taking into account the socio-demographic profile and perception of the RA patients towards CAM usage. A descriptive cross-sectional questionnaire-based study was conducted in the top Rheumatology clinics of Hyderabad, Telangana with 200 RA female patients and the obtained data were analysed using logistic regression.

4.12.2 Study Design

To understand the profiles of CAM users (a patient was termed a CAM user if he/she had ever tried CAM for RA till the time of the study), the role of psychological factors, as well as types of RA pain in the usage of CAM, following hypothesis were developed.

H_1: There will be differences between CAM and non-CAM users concerning; a) education, b) tenure of pain, c) family income, d) marital status, e) co-morbidities.

H_2: Patients experiencing more depression will show high usage of CAM.

H_3: Patients experiencing sensory and physiological pain will show high usage of CAM.

H_4: Patients experiencing severe inability in day-to-day physical activities will show high usage of CAM

Thus, a cross-sectional, questionnaire-based study was conducted to test the conceptual framework (Figure 4.12.2). The study protocol was approved by the Institutional Human Ethical committee (BITS-Hyd/IHEC/2020/07). The consent from the doctor and patients were taken before the survey.

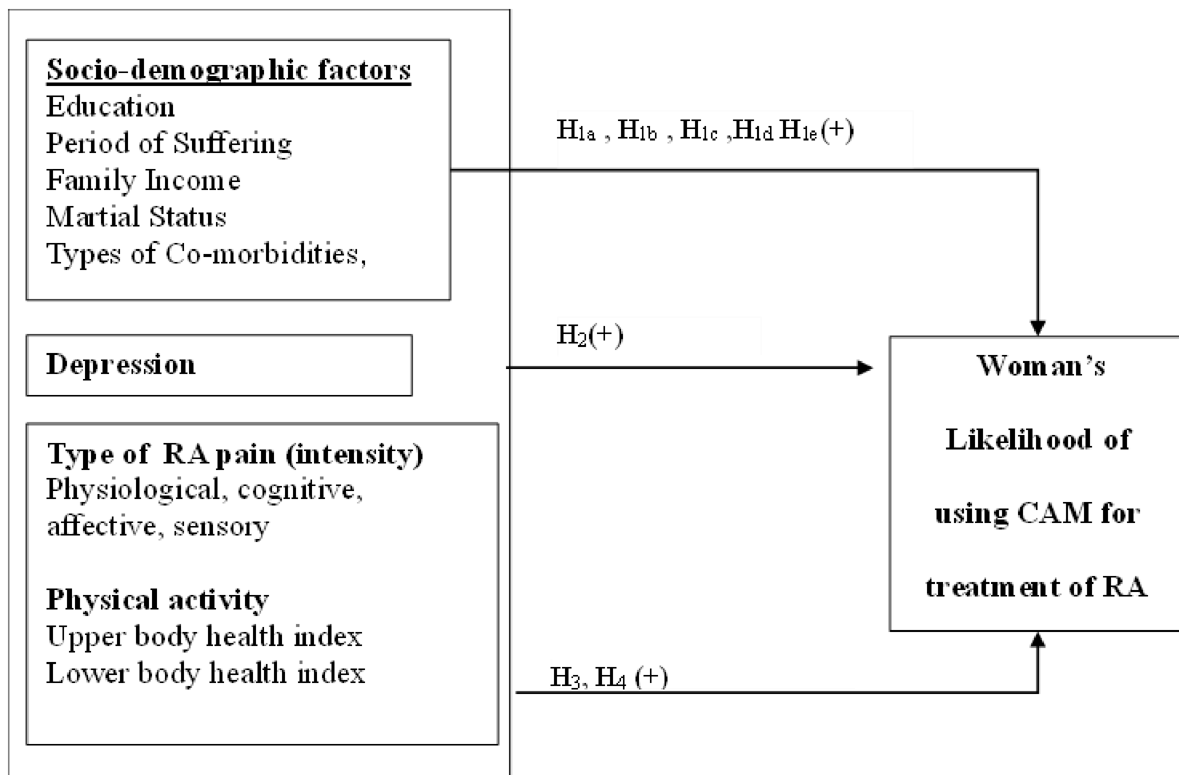


Figure 4.12.2. Theoretical Model: Factors Influencing CAM usage among RA women patients.

4.12.3 Sample size

A descriptive cross-sectional questionnaire-based study was conducted in the top Rheumatology Clinics of Hyderabad, Telangana. As per earlier studies, RA prevalence in India varies from 0.28 to 0.70 with Pune at 0.51 %, Jammu at 0.7%, and Hyderabad representing the national average of 0.3% (acrabstracts.org, 2020). At the same time, female to male ratio of RA prevalence in India is reported to be 3:1 (Van Vollenhoven 2009). Therefore, male patients with RA were excluded from the study. To understand the usage of CAM among menopausal women, we studied the behavioral pattern of women aging above 60 and used this information as a proxy to menopause. After establishing the fact that Hyderabad represents the national average, the study was very relevant to conducted in this location. According to the 2017 census, the population of Hyderabad is approximately 10.8 million. Taking the 0.3% average of RA prevalence, with 75% of them being women, the population of Hyderabad that may be suffering from RA would be 24,300 females (0.75* 0.003* 1, 08, 00,000). Using Cochran's formula at a 5 % margin of error and a 90% confidence interval, a sample size of approximately 200 was achieved. Hence, a target for 200 RA female patients was set, however, the response rate was only 75% with 150 out of 200 responses giving complete data.

The city was divided into 4 zones, geographically and the top 10 Rheumatology clinics from each zone were identified. Few examples include Advanced Rheumatology Centre, AB Rheumatology Centre, Sai Rheumatology Centre, Sri Deepti Rheumatology Centre, Hyderabad Rheumatology Centre, and Niveditha Orthopaedic Centre. Permission was taken from the doctors of each clinic to interact with their patients during the clinic hours for a week. Patients present during clinic hours were provided with an informed consent form (attached as annexure I) and only after receiving their consent, they were requested to fill the questionnaire.

4.12.4 Questionnaire design

The questionnaire (attached as Annexure II) was designed after a thorough literature search and segregated into various sections. The first section dealt with basic socio-demographic details like the financial and social status of the patient, drug regimen, and comorbidities if any. The second section focused on CAM usage - formulation, mode of awareness, communication to the physician, and monthly expenditure (Almuhareb et al., 2019). A dedicated section to assess depression states was provided on a Likert 4-point scale ranging from "mildly depressed" to "severely depressed". The fourth section consists of 24 questions related to the type of pain experienced by patients namely - physiological pain, sensory pain, affective pain, and cognitive pain which are evaluated on a 6-point scale from "Never" to "Always" (Anderson, 2001). The first 33% of the sum range was considered under mild pain category, the next 33% under moderate pain while the last 33% of the sums indicated severe pain.

The last section gathered information about a patient's health outcome and their physical ability to do certain chores on a 4-point Likert-scale with being "without any difficulty" and 3 being "unable to" (Maska et al., 2011). Factor analysis resulted in two main constructs – labelled as 'Lower body Health index' and 'Upper body health index'. Under the lower body health – the ability to get on/off a chair, bed, and toilet, walking on the ground and climbing steps, lifting, and reaching for objects were considered. For the upper body health – the ability to cut vegetables, lifting a cup of water, opening of taps, and latches were considered. The means of these two indices were calculated for all 150 patients and those with a mean of 0-1 were assigned a value 0; mean lying in between 1-1.5 were given 1 and those greater than 1.5 were assigned 2.

4.12.5 Methodology

The data were analysed using logistic regression on IBM SPSS 21. The dependent variable, i.e. RA female patient using CAM for their treatment, was coded as CAM user = 1 and Non-CAM user = 0. Given a binary dependent variable, logistic regression was used to test the previously stated hypotheses. The items within the constructs were summed and all the scores equal and above the median was awarded 1 while the ones below were given 0.

The model leveraged was as follows:

$$P(CAM = 1|\boldsymbol{X}) = G(\alpha + \beta_{10}\, Edu + \beta_2\, POS + \beta_3\, FI + \beta_4\, ComB + \beta_5\, MS + \beta_6\, PP + \beta_7\, AP + \beta_8 CP + \beta_9 DLBA + \beta_{10} DUBA + \beta_{11}\, DEP)$$

Where $\boldsymbol{X}$ = all regressors, $G(x) = \frac{\exp(x)}{1+\exp(x)}$, and

- Edu: 0 if illiterate and 1 otherwise
- POS: 0 if a period of suffering is 6-12 months and 1 otherwise
- FI: 0 if family income <30000/- per month and 1 otherwise
- ComB: 0 if comorbidities were none and 1 otherwise
- MS: 0 if single/divorced /widowed and 1 otherwise
- PP (Physiologial score): score lower than10-mild, 11-20 (moderate), 21-30 (severe)
- AP (affective score): score lower than 8, 9-16 (moderate), 17-24 (severe)
- CP (cognitive score): score lower than 12, 13-24 (moderate), 25-36 (severe)
- SP: score lower than 17, 18-35 (moderate), 36-54 (severe)
- DLBA/DUBA: 0-1 if difficulty in the lower body or upper body related activity is mild, 1-1.5 if moderate and >1.5 if severe
- DEP: 0 if depression score is lower than 15 and 1 otherwise

Annexure-I

Consent form for the concerned clinic/hospital

REQUEST FOR PERMISSION TO CONDUCT RESEARCH IN THE CLINIC/HOSPITAL

Dear Mr./Mrs./Ms. ____________________

My name is ____________________ and I am a student at BITS Pilani Hyderabad.
The research I wish to conduct for my project involves the study of "Determinants and Prevalence of Complementary and Alternative Medicine among Rheumatoid Arthritis (RA) patients". This project will be conducted under the supervision of Dr. Swati Alok, professor at BITS Pilani Hyderabad.
I am hereby seeking your consent to interact with your patients suffering from Rheumatoid Arthritis and are receiving treatment in your reputed clinic.
I have provided you with a copy of my project proposal which includes the copies of the measure and consent forms to be used in the research process, along with an attached copy of the letter I received as an approval from the BITS Pilani Ethics Committee.
Upon completion of my study, I undertake to submit a copy of my research to a couple of reputed journals. Please do not hesitate to contact me on ____________________
Thank you for your time and consideration in this matter.
Yours sincerely,

____

BITS Pilani Hyderabad.

Annexure-II

Determinants and Prevalence of **'Complementary and Alternative Medicine (CAM)'** among Rheumatoid Arthritis patients

Namaste! My name is______________and I am a **student** in BITS Pilani Hyderabad. In this survey, I am studying **"Determinants and Prevalence of Complementary and Alternative Medicine among Rheumatoid Arthritis (RA) patients"**. It would be grateful if you could spare some of your precious time to fill this questionnaire for the same. The survey usually takes about 20 minutes to complete. Whatever information you provide will be kept strictly confidential and will be used for **academic purposes** only. Participation in this survey is **voluntary** and you can choose not to answer any question or all of the questions. However, we hope that you will participate in this survey since your participation is important.

Participant Sign: ------------------------------ Date & Place: ----------------------

I. Socio-Demographic

1.1 Gender	Male		Female		
1.2 Age	< 40	40 - 59	> 60		
1.3 Marital Status	Single	Married	Divorced / Widow		
1.4 Number of members in family					
1.5 Occupation	Unemployed	Employed			
1.6 Education	Illiterate	School Education	University education	Others	
1.7 Household income per month (Rs.)	< 10000	11000 - 30000	31000 - 40000	41000 - 60000	> 60000
1.8 Type of family	Single	Nuclear family	Joint family		
1.9 Do you have Health Insurance	Yes	No			
1.10 How long are you suffering from RA?	6 - 12 months	1 - 3 years	3 - 5 years	> 5 years	
1.11 Prescribed drugs for RA	Homira (Abbott)	Enbrel (Amgen)	Leljanz (Pfizer)		
	Remicade (J2J)	Others			
1.12 Type of formulation	Tablet	Gel	Ointment	Capsule	
	Spray	Oil	Syrup	Injection	
1.13 Type of addiction, if any:	Alcohol	Smoking	Not applicable		
1.14 Comorbidities (presence of one or more diseases that occur along with another disease in the same person at the same time)	Cardiovascular disease	Blood pressure	Diabetes	Thyroid	
	Pulmonary disease	Gastro intestinal	Others		
1.15 Any side effects experienced with prescribed drugs?	Yes	No			
	If yes, mention:				

2. Complementary and Alternative Medicine (CAM) *is referred to all that health related practices that were not based on or not prescribed by medical practitioners affiliated to all allopathic system of medication.*

2.1 Have you ever received treatment for Rheumatoid Arthritis (RA) outside of treatment provided clinic in the past year after being diagnosed?	Yes	No (Please go to Question No. 3.1 directly)	
2.2 If yes, choose all options from below,	Herbal medicines	Acupressure	Unani

<table>
<tr><td>the form of CAM you have used?</td><td colspan="3">Movement therapy - yoga aasan, pranayama, massage</td><td>Others (mention)</td></tr>
<tr><td rowspan="2">2.3 If herbal, preparations:</td><td>Himalaya - Rumalaya</td><td>Patanjali – Peedantak</td><td>Zandu - Romayog Forte</td><td>Dr. Ortho</td></tr>
<tr><td>Dabur - Rheumatil</td><td>Fiziken – Orthofit</td><td>Ortho herb</td><td>Others(mention)</td></tr>
<tr><td rowspan="2">2.4 Form used:</td><td>Tablet</td><td>Gel</td><td>Ointment</td><td>Capsule</td></tr>
<tr><td>Spray</td><td>Oil</td><td>Syrup</td><td>Injection</td></tr>
<tr><td>2.5 How long were you using CAM methods?</td><td colspan="4">__________________(in years)</td></tr>
<tr><td rowspan="2">2.6 What are the common reasons for use of CAM method?</td><td colspan="2">Control of pain</td><td colspan="2">Pressure from family and friends</td></tr>
<tr><td colspan="2">Dissatisfaction with all allopathy medicines</td><td colspan="2">Adverse effect of allopathy medicines</td></tr>
<tr><td>2.7 What was the time period when you started CAM?</td><td>Started before installation of allopathic treatment</td><td colspan="2">Started immediately after diagnosis and before initiation of allopathic treatment.</td><td>Started along with allopathic treatment</td></tr>
<tr><td>2.8 What is the source of information?</td><td>Media</td><td>Trained CAM providers</td><td>Advice of friends/ neighbours/ relatives</td><td>Others</td></tr>
<tr><td>2.9 Do you still continue use of CAM?</td><td colspan="2">Yes</td><td colspan="2">No</td></tr>
<tr><td>2.10 If no, what is the reason for discontinue?</td><td colspan="2">No clinical benefits</td><td colspan="2">Adverse effects</td></tr>
<tr><td>2.11 Do you reveal CAM's use to physicians?</td><td colspan="2">Yes</td><td colspan="2">No (go to Q 2.14)</td></tr>
<tr><td rowspan="2">2.12 If yes, what are the reasons for revealing uses of CAM?</td><td>Physician needs to know everything I am taking</td><td>The physician asked</td><td colspan="2">Physician knows about interactions with prescribed treatment</td></tr>
<tr><td>Physician may know whether CAM works.</td><td colspan="2">To ensure documentation of CAM use in the medical record</td><td></td></tr>
<tr><td>2.13 What are the physicians' reaction to use of CAM?</td><td>Stop using CAM</td><td colspan="2">Continue to use CAM</td><td>Indifference</td></tr>
<tr><td>2.14 If no, what are the reasons for not revealing uses of CAM?</td><td>Physician did not ask</td><td>Forgot to tell the physician</td><td>Used CAM before seeing the physician</td><td>Feared that the physician would disapprove</td></tr>
<tr><td>2.15 Mention the expenditure on CAM,</td><td></td><td colspan="2"></td><td></td></tr>
</table>

whichever is applicable?	--------------- Rs.per month in herbal medicines	---------------- Rs per month in acupressure	------------------- Rs. per month in movement therapy
	-------------------- Rs per month in Unani	Not applicable	

3. Hospital Anxiety / depression	**Select / Tick the suitable responses**			
	(1)	**(2)**	**(3)**	**(4)**
3.1 I feel tense or 'wound up':	Most of the time	A lot of the time	From time to time, occasionally	Not at all
3.2 I still enjoy the things I used to enjoy:	Definitely as much	Not quite so much	Only a little	Hardly at all
3.3 I get a sort of frightened feeling as if something awful is about to happen:	Very definitely and quite badly	Yes, but not too badly	A little, but it doesn't worry me	Not at all
3.4 I can laugh and see the funny side of things:	As much as I always could	Not quite so much now	Definitely not so much now	Not at all
3.5 Worrying thoughts go through my mind:	A great deal of the time	A lot of the time	From time to time but not too often	Only occasionally
3.6 I feel cheerful:	Not at all	Not often	Sometimes	Most of the time
3.7 I can sit at ease and feel relaxed:	Definitely	Usually	Not often	Not at all
3.8 I feel as if I am slowed down:	Nearly all the time	Very often	Sometimes	Not at all
3.9 I get a sort of frightened feeling like 'butterflies' in the stomach:	Not at all	Occasionally	Quite often	Very often
3.10 I have lost interest in my appearance:	Definitely	I don't take so much care as I should	I may not take quite as much care	I take just as much care as ever
3.11 I feel restless as if I have to be on the move:	Very much indeed	Quite a lot	Not very much	Not at all
3.12 I look forward with enjoyment to things:	As much as ever I did	Rather less than I used to	Definitely less than I used to	Hardly at all
3.13 I get sudden feelings of panic:	Very often indeed	Quite often	Not very often	Not at all

3.14 I can enjoy a good book / radio/ TV programme:	Often	Sometimes	Not often	Very seldom

4. Rheumatoid Arthritis Pain	**Select / Tick the suitable responses**								
4.1 I would describe my pain as **gnawing**. (S)	Never	0	1	2	3	4	5	6	Always
4.2 I would describe my pain as **aching**. (S)	Never	0	1	2	3	4	5	6	Always
4.3 I would describe the word **exhausting** to describe my pain(A)	Never	0	1	2	3	4	5	6	Always
4.4 I would describe my pain as **annoying**. (A)	Never	0	1	2	3	4	5	6	Always
4.5 I'm in **constant** pain. (S)	Never	0	1	2	3	4	5	6	Always
4.6 I would describe my pain as **rhythmic**. (S)	Never	0	1	2	3	4	5	6	Always
4.7 I have **swelling** of at least one joint. (P)	Never	0	1	2	3	4	5	6	Always
4.8 I have **morning stiffness** of one hour or more. (P)	Never	0	1	2	3	4	5	6	Always
4.9 I have **pain on motion** of at least one joint. (P)	Never	0	1	2	3	4	5	6	Always
4.10 I cannot perform all the everyday tasks I normally would because of pain. (C)	Never	0	1	2	3	4	5	6	Always
4.11 Pain interfere with my sleep. (C)	Never	0	1	2	3	4	5	6	Always
4.12 I cannot decrease my pain by using methods other than taking extra medication. (C)	Never	0	1	2	3	4	5	6	Always
4.13 I would describe my pain as **burning**. (S)	Never	0	1	2	3	4	5	6	Always
4.14 I find that I guard my joints to **reduce pain**. (A)	Never	0	1	2	3	4	5	6	Always
4.15 I **brace** myself because of pain. (A)	Never	0	1	2	3	4	5	6	Always
4.16 My pain is **throbbing** in nature. (S)	Never	0	1	2	3	4	5	6	Always
4.17 I would describe my pain as **sharp**. (S)	Never	0	1	2	3	4	5	6	Always
4.18 I would say my pain is **severe**. (S)	Never	0	1	2	3	4	5	6	Always
4.19 I feel **stiffness** in my joints after rest. (P)	Never	0	1	2	3	4	5	6	Always
4.20 My joints feel **hot**. (P)	Never	0	1	2	3	4	5	6	Always
4.21 I feel **anxious** because of pain. (C)	Never	0	1	2	3	4	5	6	Always
4.22 I would describe my pain as **tingling**. (S)	Never	0	1	2	3	4	5	6	Always
4.23 I feel my pain is **uncontrollable**. (C)	Never	0	1	2	3	4	5	6	Always
4.24 I feel **helpless** to control my pain. (C)	Never	0	1	2	3	4	5	6	Always

5. Illness Perception	**Select / Tick the suitable responses**												
5.1 How much does your illness affect your life?	no affect at all	0	1	2	3	4	5	6	7	8	9	10	severely affects my life
5.2 How long do you think your illness will continue?	a very short time	0	1	2	3	4	5	6	7	8	9	10	forever
5.3 How much control do you feel you have over your illness?	absolutely no control	0	1	2	3	4	5	6	7	8	9	10	extreme amount of control

5.4 How much do you think your treatment can help your illness?	not at all	0	1	2	3	4	5	6	7	8	9	10	extremely helpful
5.5 How much do you experience symptoms from your illness?	no symptoms at all	0	1	2	3	4	5	6	7	8	9	10	many severe symptoms
5.6 How concerned are you about your illness?	not at all concerned	0	1	2	3	4	5	6	7	8	9	10	extremely concerned
5.7 How well do you feel you understand your illness?	don't understand at all	0	1	2	3	4	5	6	7	8	9	10	understand very clearly
5.8 How much does your illness affect you emotionally? (e.g. does it make you angry, scared, upset or depressed?)	not at all affected emotionally	0	1	2	3	4	5	6	7	8	9	10	extremely affected emotionally

6. Health Assessment	**Select / Tick the suitable responses**			
Are you able to:	**Without any difficulty (0)**	**With some difficulty (1)**	**With much difficulty (2)**	**Unable (3)**
Dressing				
6.1 Dress yourself, plus doing buttons?	0	1	2	3
6.2 Wash your hair?	0	1	2	3
6.3 Comb your hair?	0	1	2	3
Arising				
6.4 Stand up straight from chair?	0	1	2	3
6.5 Get in and out of bed?	0	1	2	3
6.6 Sit cross-legged on floor and get up?	0	1	2	3
Eating				
6.7 Cut vegetables?	0	1	2	3
6.8 Lift a full cup or glass to your mouth?	0	1	2	3
6.9 Break chappati with one hand?	0	1	2	3
Walking				
6.10 Walk outdoors on flat ground?	0	1	2	3
6.11 Climb up five steps?	0	1	2	3
Hygiene				
6.12 Take a bath?	0	1	2	3
6.13 Wash and dry your body?	0	1	2	3
6.14 Get on and off the toilet?	0	1	2	3
Reaching				
6.15 Reach and get down a 2 Kg object (such as bag of sugar) from just above your head?	0	1	2	3

6.16 Bend down to pick up clothing from the floor?	0	1	2	3
Grip				
6.17 Open a bottle previously opened?	0	1	2	3
6.18 Turn taps on and off?	0	1	2	3
6.19 Open door latches?	0	1	2	3
Activities				
6.20 Work in office or house?	0	1	2	3
6.21 Run errands and shop?	0	1	2	3
6.22 Get in and out of bus?	0	1	2	3
6.23 Get in and out of a car or autorickshaw?	0	1	2	3

Any other comments (optional)

Thank you

Chapter 5

Results and Discussion

Many of the clinically used anti-inflammatory drugs are antagonists for natural pro-inflammatory mediators like prostaglandins, leukotrienes, and histamine. Although the known anti-inflammatory treatments are effective, their side effects such as increased risk of serious infections, cost and other serious adverse effects are limiting their use and open a wider range of opportunities for intervention. Exploring new sources of cost-effective herbal medicine with high potency and better safety profiles was taken as one such approach in this present study.

In our previous study, *H. pinifolia* was found to be non-toxic (IC_{50} -699.5 µg/mL) and its anti-inflammatory property was established through attenuation of pan-cytokines (Begum et al., 2021), confirming its potential to be developed as a phytopharmaceutical drug for the treatment of various chronic inflammatory diseases.

5.1 Macroscopy of the selected seagrass *H. pinifolia*

Common names: Needle grass, Bedung korai, Neettu korai, Peria eekku pasi, Kadal korai pasi

Scientific classification

Family: Cymodoceaceae

Genus: *Halodule*

Species: *pinifolia*

Organoleptic characters

Colour: Green to dark green

Odour: Pungent

Taste: Bitter and astringent

Size and shape

The leaf is linear and flat with a length of around 20 cm and a width between 2-10 mm. Three longitudinal leaf veins are present on the leaf sheath with a smooth leaf margin, which is finely serrated at the tip. Stem is erect, short and vertical bearing two leaves. Rhizome is thin and very often covered by leaf scars (Coastal and Marine Integration Network, 2023).

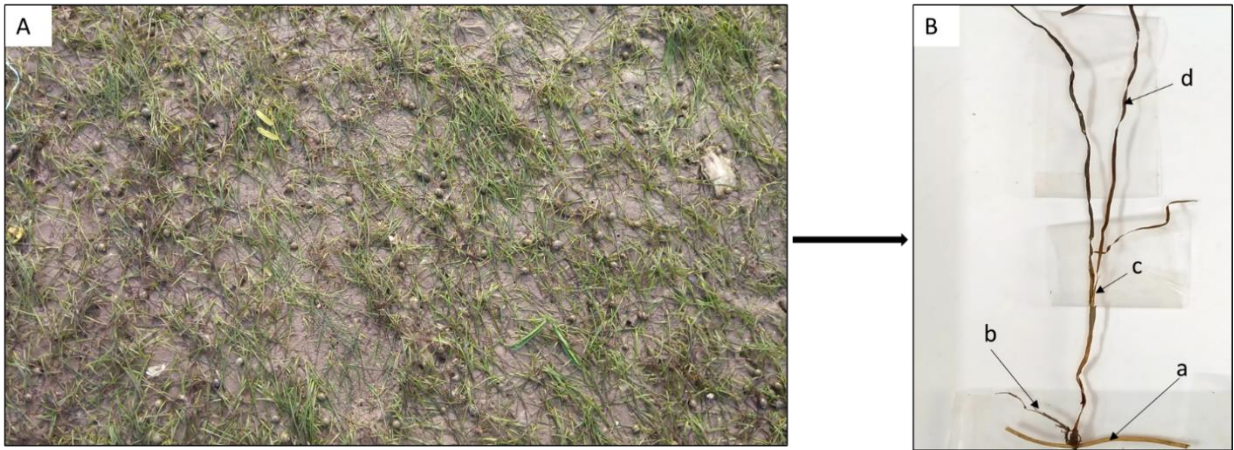


Figure 5.1. (A) Image of *H. pinifolia* found in Vellar Estuary, Tamil Nadu, India (B) Morphology of *H. pinifolia* (a) rhizome (b) root (c) stem and (d) leaf.

5.2 Microscopy of *H. pinifolia*

A transverse section (T. S.) of the leaf, stem and rhizome of *H. pinifolia* was taken to study the microscopic characters

5.2.1. T. S. of Leaf

The T.S of leaf showed a single layer of epidermis that was covered by a cuticle. The epidermal cells were made up of chlorenchymatous cells and a few cells were found to contain tannin. The mesophyll region contained thin-walled spongy parenchymatous cells, some cells contained chloroplast and a large number of air cavities were observed. Three vascular bundles were present which contained proto xylem (Figure 5.2.1).

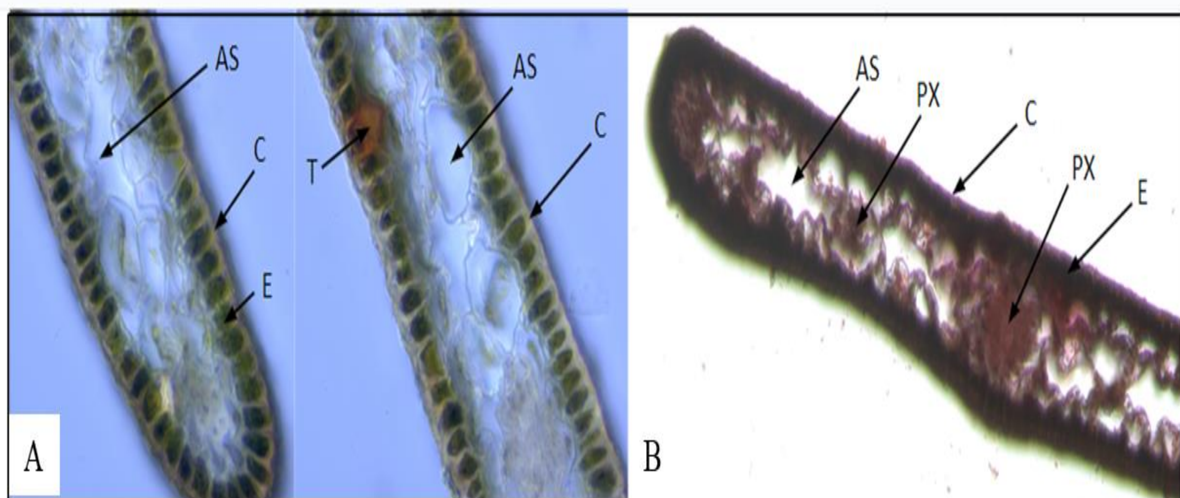


Figure 5.2.1. The transverse section of a leaf of *H. pinifolia* (A) 40X (B) Safranin-stained (10X) C: cuticle E: epidermis, T: tannin, AS: Air space, PX: proto xylem.

5.2.2. T.S. of Stem

In the T. S. of the stem, the presence of thin-walled, single layer of epidermis was observed, followed by a few layers of cortical hypodermal cells. Below the hypodermal cells, spongy parenchymal cells filled with compound starch grains were observed. Also, some air space regions separated by thin-walled septa cells were observed. The centre portion of the stem contained vascular bundle and it constituted proto xylem cells (Figure 5.2.2).

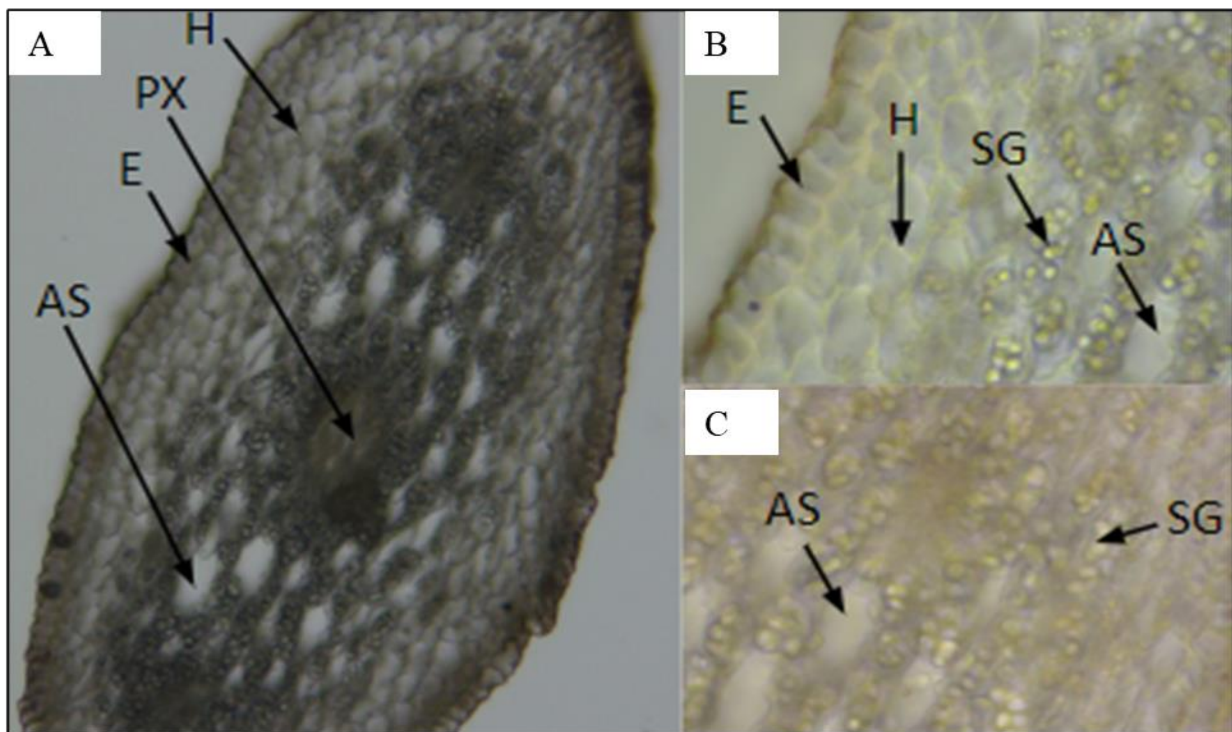


Figure 5.2.2. T. S of the stem of *H. pinifolia*; (A) (10X); (B) (40X) (C) Safranin-stained (40X); E: Epidermis, H: Hypodermis, SG: starch grains, AS: Air space, PX: proto xylem.

5.2.3. T.S. of Rhizome

The microscopic study of the T.S. of rhizome (Figure 5.2.3) revealed the presence of a single layer of outer epidermal cells and the absence of a cuticle. The epidermal cell layer was followed by the cortex region, made up of spongy parenchymatous cells. Many of these cells contained compound starch grains (2 to 6). Also, a number of air cavities were observed. In the central portion of the section of the rhizome, a stele consisting of proto-xylem cells was seen. Further, it was noted that the rhizome showed a greater number of starch grains compared to the stem.

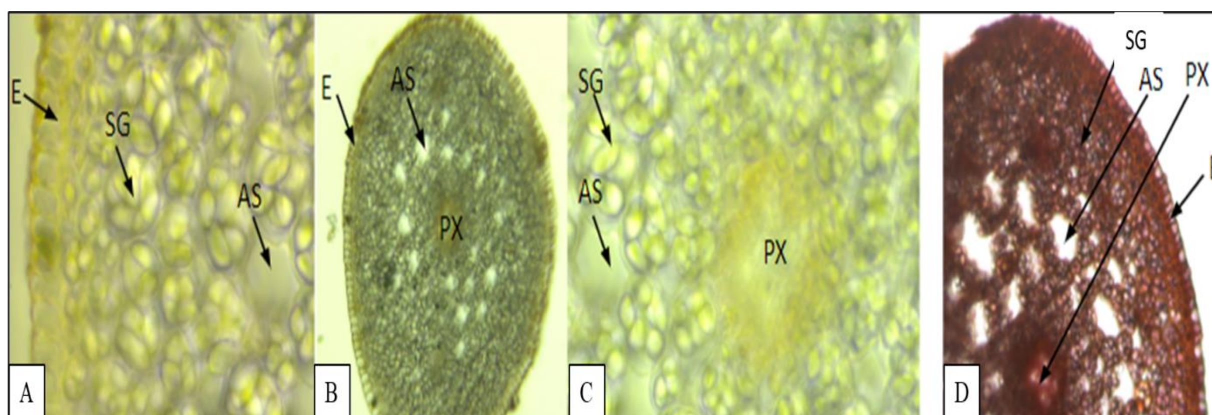


Figure 5.2.3. T.S of rhizome; (A) and (C) 40X: (B) 10X; (D) 10X safranin-stained; E: Epidermis, SG: starch grains, AS: Air space, PX: proto xylem.

5.3 Extraction of plant material *H. pinifolia* and *G. glabra*

The seagrass *H. pinifolia* was collected from Vellar Estuary, Tamil Nadu, India. The plant specimen was authenticated by Dr. Saravana Kumar, Centre for Advanced Studies in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, India. A specimen sample (NPC/2017/HP) is preserved in the Department of Pharmacy, BITS-Pilani Hyderabad Campus. The whole herb material was coarsely powdered and extracted using ethyl acetate at RT for 48 h. The extract was separated by filtration and the marc was added with fresh ethyl acetate solvent and cold extraction was continued for 24 h. The substance was filtered and the filtrate was evaporated under reduced pressure which yielded a dry residue coded as HP.

The second plant material *G. glabra* was supplied by an authenticated plant supplier Dr. V. Chelladurai, Tamil Nadu, India. A specimen sample (NPC/2017/LQ) is preserved in the Department of Pharmacy, BITS-Pilani Hyderabad Campus. The coarsely powdered roots and rhizomes of liquorice was subjected to hot extraction (50 ˚C; 3 h) using aqueous ethanol (H_2O: ethanol 1:3). The substance was filtered and the filtrate was evaporated under reduced pressure which yielded a dry residue (yield 39% w/w) coded as LQ.

5.4 Preparation of compositions 1, 2 and 3

Liquorice extract has been reported to be non-toxic (Wang et al., 2015) and reduce the release of proinflammatory factors IL-1β, IL-6, and TNF-α while increasing the production of anti-inflammatory factor IL-10, hence suppressing the inflammatory response (Huang et al., 2022). In our study, we attempted to understand if there was any incremental (additive/synergistic) effect when both HP and LQ extracts were combined. Therefore, composition 1 was prepared

by mixing HP and LQ. A fixed concentration of HP was blended with varied concentrations of LQ to evaluate the effect and optimise the effective concentration. Therefore, samples having HP and LQ in the ratios of 1:1, 1:0.5, 1:0.25 and 1:0.125, respectively were prepared.

Two other HP-based compositions were also prepared and screened during the study, which involved glycyrrhizin (Gly), the major bioactive constituent of liquorice (composition 2) and its salt monoammonium glycyrrhizinate (MAG) (composition 3). These active constituents were found to be non-toxic (IC_{50} -5mM) (Kim et al., 2015). The expanding use of innovative botanical ingredients in complementary alternative medicine, dietary supplements and foods poses a serious challenge in development and validation of analytical methods for accurate measurement of active ingredients. (Sullivan and Crowley, 2006). Extracts can be complex mixtures containing a large number of active ingredients, impurities and matrix effects. In such a scenario, developing an analytical method for a mixture of two extracts can be a daunting task. Such limitations often cease the further development of a potential formulation. To overcome this limitation to some extent, an exploratory study was taken up by combining HP with the major component of *G. glabra* that is glycyrrhizin - composition 2 and its salt mono ammonium glycyrrhizinate - composition 3. MAG, a salt of glycyrrhizin was incorporated in the exploratory study with an anticipation of improvement in the activity as salts have a better pharmacokinetic profile and bioavailability.

Composition 2 was prepared by mixing HP and Gly, respectively in different ratios 1:0.5, 1:0.25, 1:0.125 and 1:0.0625. Composition 3 was prepared by mixing HP and MAG, respectively in different ratios 1:0.5, 1:0.25, 1:0.125 and 1:0.0625.

5.5 Effect of various extracts, compounds and compositions on pro-inflammatory cytokines under in-vitro model (ELISA)

5.5.1 Effect on the release of TNF-α

Samples HP, LQ, Gly, MAG, composition 1, composition 2, and composition 3 at various concentrations were tested for their inhibition effect on the release of TNF-α in the culture supernatant of RAW 264.7 cells using LPS-stimulated ELISA assay kit. HP and LQ profoundly decreased the TNF-α levels showing IC_{50} values of 90.44 and 20.52 μg/mL, respectively. Composition 1, containing HP and LQ blended in different ratios (1:1, 1:0.5, 1:0.25 and 1:0.125) significantly inhibited the release of TNF-α and the combination 1:1 and 1:0.5 showed excellent inhibitory activity ($P<0.0001$) with 85.91% and 80.35%, respectively. The combinations 100:25 and 100:12.5 μg/mL of composition 1 showed a significant inhibition

(75.67% and 67.82%, respectively) and a CI score of 0.6 against TNF-α featuring HP and LQ as a synergistic combination. Thus, composition 1 showed a synergistic effect at the concentrations where pure HP and LQ (25 µg/mL, 12.5 µg/mL) failed to control the release of TNF-α. Further, both Gly and MAG were found to strongly inhibit the secretions of TNF-α (IC_{50}: 1.22 µM and 5.15 µM, respectively). Out of the different ratios of composition 2 (1:0.5, 1:0.25, 1:0.125 and 1:0.0625 of HP:Gly, respectively), ratio 1:0.5 showed a significant inhibition ($P<0.0001$) with 88.79% and a synergy score of 0.5. Similarly, composition 3 at a ratio of 1:0.5 showed a synergistic effect (CI 0.8) (Figure 5.5.1).

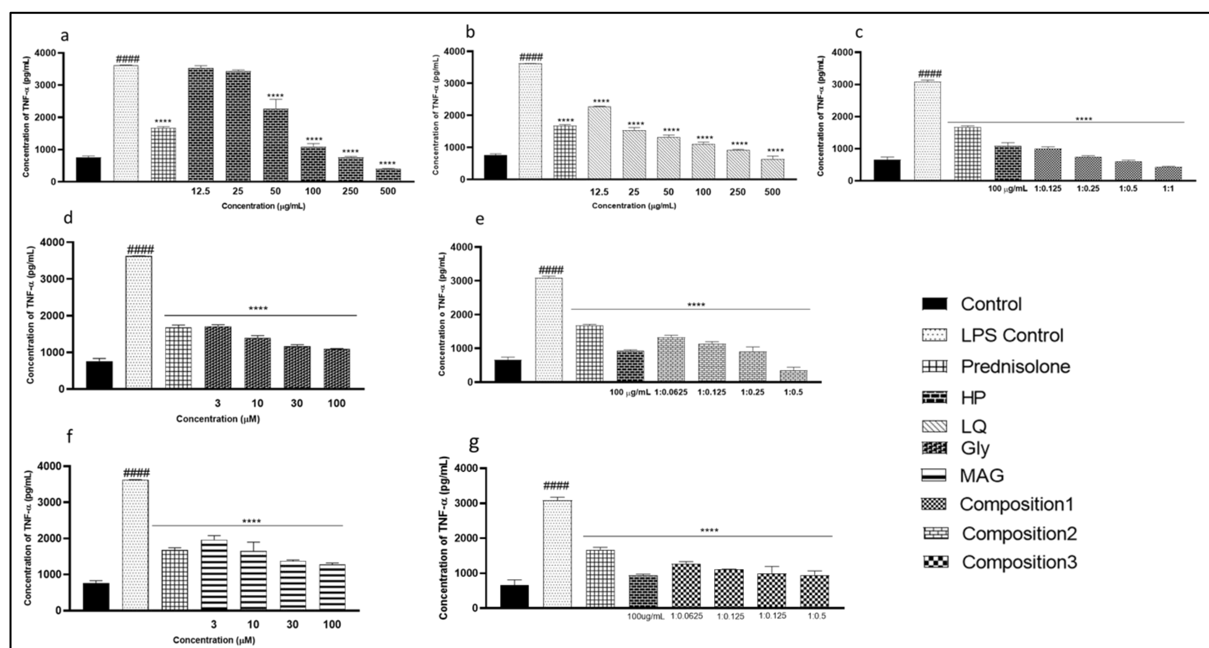


Figure 5.5.1. Effect of various extracts, compounds, and compositions on the expression of TNF-α in LPS-induced RAW 264.7 cells tested using ELISA. Effect of (a) HP, (b) LQ, (c) Composition 1, (d) Gly, (e) Composition 2, (f) MAG, (g) Composition 3, at various concentrations. All values are presented as mean ± SEM (n=3) $^{****}P<0.0001$ versus LPS control group. $^{####}P<0.0001$ versus the control group.

5.5.2 Effect on the release of IL-1β

All the samples were tested for their inhibition effect on the release of IL-1β in the LPS-stimulated culture supernatant of RAW 264.7 cells. HP was found to significantly decrease IL-1β (IC_{50} 78.37 µg/mL) and was more active than LQ (IC_{50} 145.83 µg/mL). All doses of HP and LQ significantly ($P<0.001$) decreased the concentration of IL-1β when compared to LPS control. Composition 1 showed significant inhibition at all ratios (1:1, 1:0.5, 1:0.25 & 1:0.125) and the effect was synergistic with a CI score between 0.3 to 0.5. Similarly, both MAG and Gly showed a more pronounced effect (IC_{50} 10.47 µM and 13.87 µM, respectively) than the extracts. The synergistic effect of compositions 2 and 3 was clearly evident with the CI scores

of less than 1 at all tested ratios. Further, they showed a significant effect (P<0.0001) with inhibition percentages of 67.55% and 67.63%, respectively at the ratio of 1:0.5 (Figure 5.5.2).

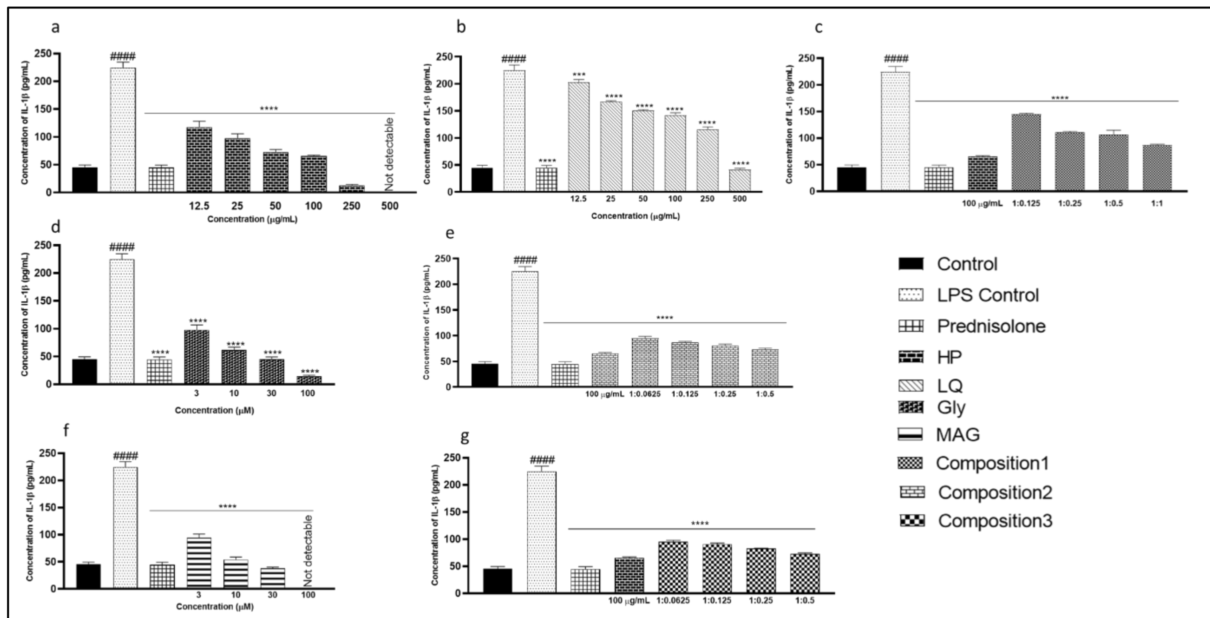


Figure 5.5.2. Effect of various extracts, compounds, and compositions on the expression of IL-1β in LPS-induced RAW 264.7 cells tested using ELISA. Effect of (a) HP, (b) LQ, (c) Composition 1, (d) Gly, (e) Composition 2, (f) MAG, (g) Composition 3, at various concentrations. All values are presented as mean ± SEM (n=3). ***P<0.001, ****P<0.0001 versus LPS control group. $^{####}$P<0.0001 versus the control group.

5.5.3 Effect on the release of IL-6

Expression of IL-6 was significantly (P<0.0001) decreased in the LPS-stimulated culture supernatant of RAW 264.7 cells by HP and LQ. Composition 1 showed an excellent synergistic effect with a CI score of less than 0.5 and percentage inhibition of more than 95% at all tested ratios (1:1, 1:0.5, 1:0.25 and 1:0.125). Compounds, Gly and MAG significantly (P<0.0001) reduced the expression of IL-6 at concentrations 10, 30 and 100 μM. Similar to composition 1, compositions 2 and 3 showed marked inhibition against IL-6 and it was found to be highest (99.53 and 98.86%, respectively) at the ratio of 1:0.5. The other tested ratios 1:0.25 and 1:0.125 showed excellent inhibition of more than 95%. Both compositions 2 and 3 showed a synergistic effect (CI score <0.5) compared to that of the individual treatments (Figure 5.5.3).

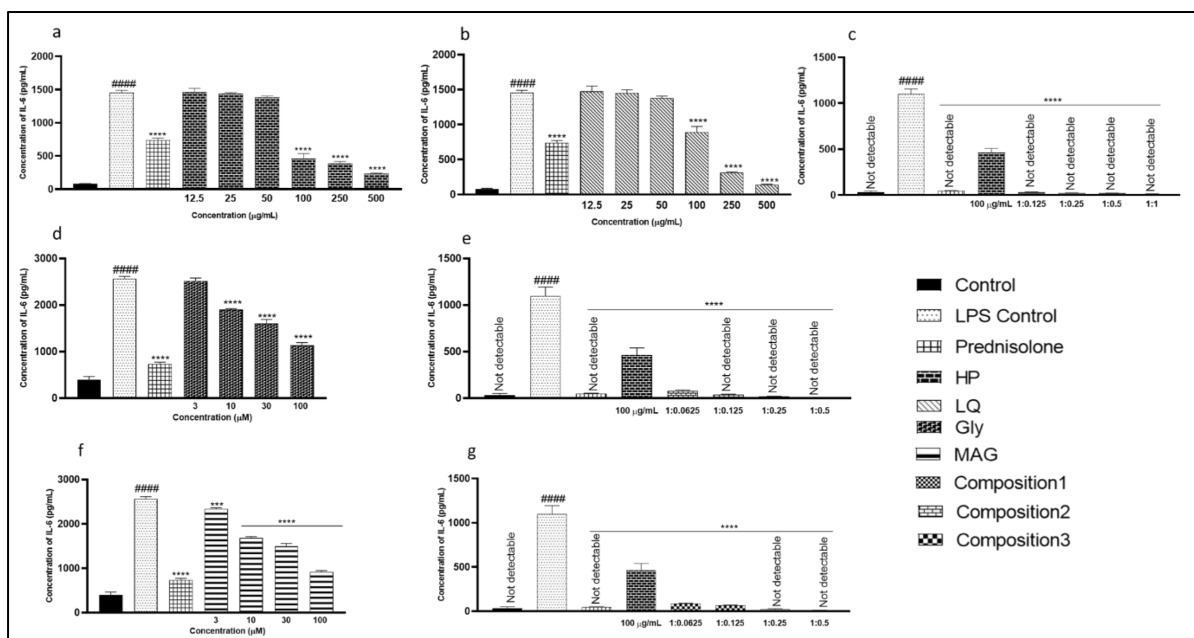


Figure 5.5.3. Effect of various extracts, compounds, and compositions on the expression of IL-6 in LPS-induced RAW 264.7 cells tested using ELISA. Effect of (a) HP, (b) LQ, (c) Composition 1, (d) Gly, (e) Composition 2, (f) MAG, (g) Composition 3, at various concentrations. All values are presented as mean ± SEM (n=3). ***P<0.001, ****P<0.0001 versus LPS control group. $^{\#\#\#\#}$P< 0.0001 versus the control group.

5.6 Effect of various extracts, compounds and compositions on LPS-induced nitric oxide production (In-vitro)

All the samples were tested for their effect on the NO production in the LPS-stimulated RAW 264.7 cell lines. While LQ showed 29.01% inhibition at 50 µg/mL, compounds Gly and MAG showed 32.17 and 37.69%, respectively at 50 µM. Sample HP at 100 µg/mL controlled the production by 33.48% and its effect got increased when LQ was mixed with it and the effect was found to be synergistic (CI<0.5) when the ratios of HP:LQ (composition 1) were 1:0.5, 1:0.25 and 1:0.125, respectively (Fig. 4). Also, it was observed that addition of Gly to HP (composition 2) did not have much change in the activity of HP and vice versa. On the other hand, the addition of MAG to HP (composition 3) showed an improved nitrite inhibition and the effect was concentration-dependent (Figure 5.6).

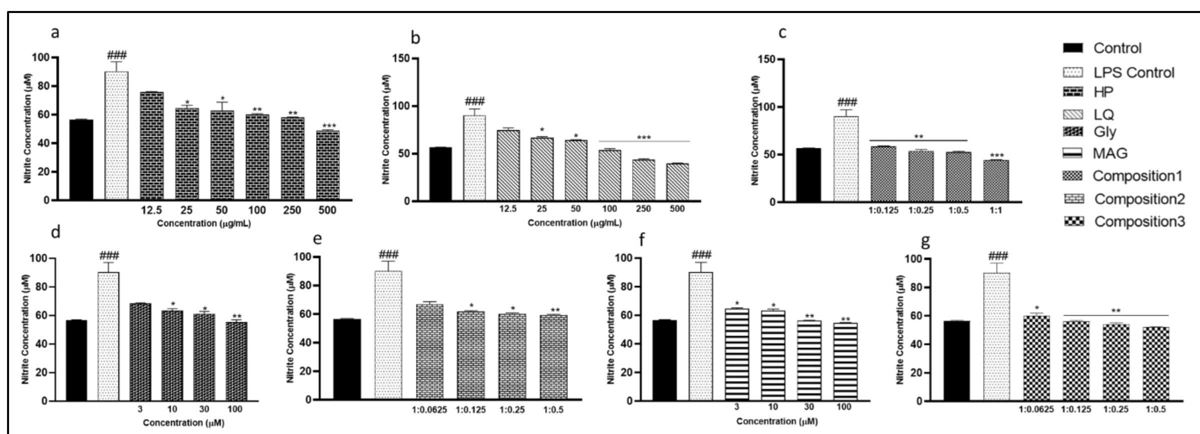


Figure 5.6. Effect of various extracts, compounds, and compositions on LPS-induced nitric oxide (NO) production in RAW 264.7 cells. Effect of (a) HP, (b) LQ, (c) Composition 1, (d) Gly, (e) Composition 2, (f) MAG, (g) Composition 3, at various concentrations. All values are presented as mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001 versus LPS control group. All values are presented as mean ± SEM (n=3) $^{###}$P<0.001 versus the control group.

Seagrasses have been proven incredibly useful to the diverse community as fertilizers, animal feed, food, and medicine. Seagrass ecosystems have always been a resource to sustain the livelihood of the coastal population. Interestingly, seagrass represents the only group of angiosperms that can flourish in the marine environment and therefore, evokes curiosity to explore the possibilities it transpires within itself.

H. pinifolia was identified as an interesting seagrass, reported to have medicinal potential in the literature (Kanan et al., 2013; Sangeetha and Ashokan 2015; Jeyapragash et al., 2015; Begum et al., 2021). Also, *H. pinifolia* has been reported to have high nutritional value with dietary fiber, vitamin content, and high calcium and magnesium content (Jeevitha et al., 2013). A high phenolic, tannins and vitamin E content of *H. pinifolia* have been reported, which adds to its nutraceutical applications (Kannan et al., 2013). In our recent research, the ethyl acetate extract of *H. pinifolia* was discovered to possess pro-inflammatory cytokines inhibition effect, and the extract was identified to contain bioactive compounds such as rosemarinic acid, vanillic acid, ethyl protocatechuate, sitosterol, stigmasterol, campesterol, and dihydrobrassicasterol (Begum et al., 2021). The anti-inflammatory property and mechanism of action of *H. pinifolia* have not been much explored and existing literature suggests its potential to emerge as a hub of new compounds with inherent bioactivities. In view of this fact, an anti-inflammatory composition based on *H. pinifolia* targeting pro-inflammatory cytokines was prepared.

Another medicinal plant selected for developing the oral composition was G. *glabra*, which is widely used in foods, and traditional and herbal medicine since ancient times. The important phytoconstituents of liquorice include flavonoids, triterpenoid saponins, coumarins, phenols

and polysaccharides. Glycyrrhizin is recognized to be the most active ingredient among triterpene saponins (Jiang 2020). Liquorice has been reported for anti-tussive & expectorant activity, antioxidant activity, skin lightening and skin tightening activity, anti-viral effects, anti-fungal activity, anti-bacterial activity, anti-malarial activity, anti-hyperglycemic activity, immune-stimulatory effects, memory enhancing activity, hepatoprotective activity, anticoagulant, hair growth stimulatory activity, etc., (Damle 2014; Pastorino et al., 2018; Bakr et al., 2022). Apart from these therapeutic properties, liquorice is known for its underlining anti-inflammatory and anti-ulcer activity. In Ayurvedic medicine, liquorice has been suggested as a preventive medicine for gastric and duodenal ulcers (Ding et al., 2022). Furthermore, in modern studies, liquorice has been explored for anti-inflammatory and anti-ulcerative properties (Aly et al., 2005; Fu et al., 2013; Yang et al., 2017). The reported anti-ulcer property of liquorice may overcome the significant side effects such as gastric irritation and bleeding caused by general anti-inflammatory drugs (NSAIDS) and therefore formed the rationale for its inclusion in the composition. Its inherent sweetening property constituted the additional possibilities of reducing the artificial sweetening excipients used in formulations. Therefore, the study was taken up to explore the anti-inflammatory potential of a combination of *H. pinifolia* extract and liquorice extract (composition 1). Further, combinations made using *H. pinifolia* and the primary bioactive ingredient of liquorice i.e. glycyrrhizin (composition 2) and its salt mono ammonium glycyrrhizinate (composition 3) were also tested. These ingredients could enhance the activity as well as provide a good taste to the compositions, which can then be formulated into an oral dosage form like granules, tablets, capsules, suspension, etc.

All the individual ingredients (HP, LQ, Gly and MAG) and compositions 1, 2 and 3 were tested under in-vitro and in-vivo models. Under the in-vitro ELISA in RAW 264.7 cell lines, all compositions showed excellent inhibition of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Fig. 1, 2 and 3). While compositions 2 and 3 showed 1:0.5 as a synergistic ratio (CI 0.5), composition 1 showed 1:0.25 and 1:0.125 (CI 0.6) in controlling the secretions of TNF-α. All the compositions were highly active against IL-6 showing >95% inhibition and the ratio 1:0.5 (100:50 µg/mL) was synergistic. Similarly, all the combinations were inhibitory against IL-1β and the ratio 1:0.5 was synergistic. Further, compositions 1 and 3 were effective in ameliorating nitrite production (Figure 5.6). Thus, the ratio 1:0.5 (i.e. 100 µg/mL:50 µg/mL of HP:LQ in the case of composition 1 and 100 µg/mL:50 µM of HP:Gly and HP:MAG in the case of compositions 2 & 3, respectively) was identified as an effective synergistic combination for inhibiting IL-1β, IL-6 and TNF-α in mouse macrophage cell line.

5.7 Standardization of herbal extracts of compositions

The reverse-phase HPLC analysis of HP and LQ was taken as the first step towards standardisation and chemical profiling of the prepared extracts.

5.7.1 Standardisation of HP by HPLC method

Based on a chemical investigation study previously done in our lab, HP was identified to contain sterols and phenolics such as vanillic acid, ethyl protocatechuate and rosemarinic acid (Begum et al., 2021). These phenolics were selected as standard marker compounds for standardisation. A serial dilution of standards resulting to 20, 40, 60, 80 and 100 ppm solutions were used for preparing the calibration curve (Concentration Vs Area under curve). The calibration plots of standards vanillic acid, ethyl protocatechuate and rosemarinic acid (20 ppm – 100 ppm) detected at 254 nm in HPLC is shown in Figure 5.7.1.1. The retention time for vanillic acid was found to be 3.2 min and that of ethyl protocatechuate and rosemarinic acid was found to be 7.097 min and 8.507 min respectively. An overlay HPLC chromatograms of standards vanillic acid, ethyl protocatechuate and rosemarinic acid (20 ppm – 100 ppm) detected at 254 nm has been represented in Figure 5.7.1.2. The amount of vanillic acid (Rt 3.20 min), ethyl protocatechuate (Rt 7.097 min) and rosemarinic acid (Rt 8.507 min) present in HP were quantified from the standard graph. The amounts of vanillic acid, ethyl protocatechuate, and rosmarinic acid present in HP were estimated as 2.92, 0.76, and 4.6 mg/g of dry HP, respectively, using the calibration curve. HPLC chromatogram of HP (2000 ppm) detected at 254 nm is represented in Figure 5.7.1.3.

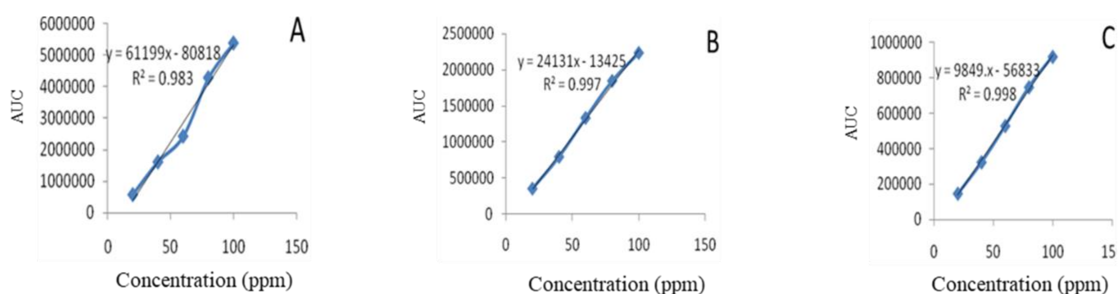


Figure 5.7.1.1. Calibration plots of standards (A)Vanillic acid (B) Ethyl protocatechuate (C) Rosemarinic acid (20 ppm – 100 ppm) detected at 254 nm in HPLC.

Figure 5.7.1.2. Overlay HPLC chromatograms of standards vanillic acid, ethyl protocatechuate and rosmarinic acid (20 ppm – 100 ppm) detected at 254 nm.

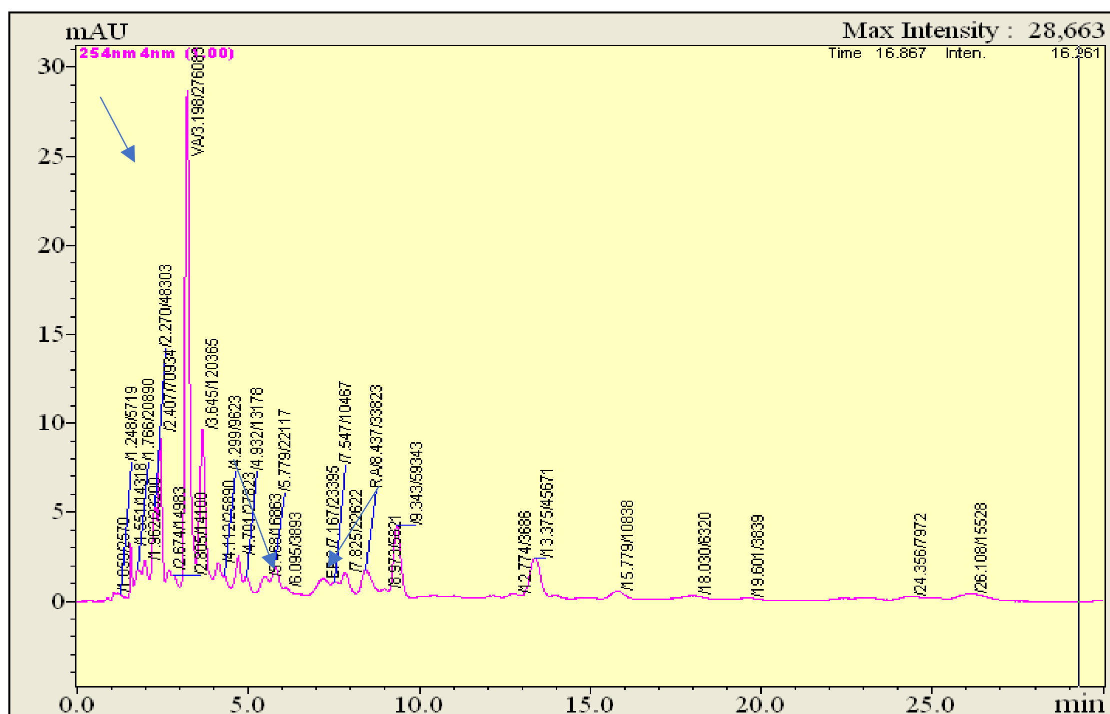


Figure 5.7.1.3. HPLC chromatogram of HP (2000 ppm) detected at 254 nm.

5.7.2 Standardisation of LQ by HPLC method

Glycyrrhizin, the major bioactive constituent of liquorice was selected as a marker compound. A serial dilution of glycyrrhizin resulting to 150, 200, 300, 350 and 400 ppm solutions were used for preparing the calibration curve (Concentration Vs Area under curve). The calibration plot of standard glycyrrhizin (150 ppm – 400 ppm) detected at 254 nm in HPLC is shown in Figure 5.7.2.1. The retention time for glycyrrhizin was found to be 20.235 min. An overlay HPLC chromatograms of standard glycyrrhizin (150 ppm – 400 ppm) detected at 254 nm is presented in Figure 5.7.2.2. The amount of glycyrrhizin present in LQ was quantified from the standard graph and the amount was estimated as 2.752 mg/g of dry LQ. HPLC chromatogram of LQ detected at 254 nm is represented in Figure 5.7.2.3.

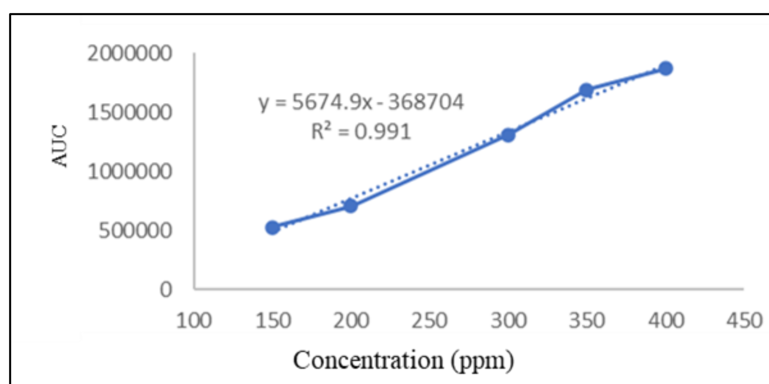


Figure 5.7.2.1. Calibration plot of standard glycyrrhizin (150 ppm – 400 ppm) detected at 254 nm in HPLC.

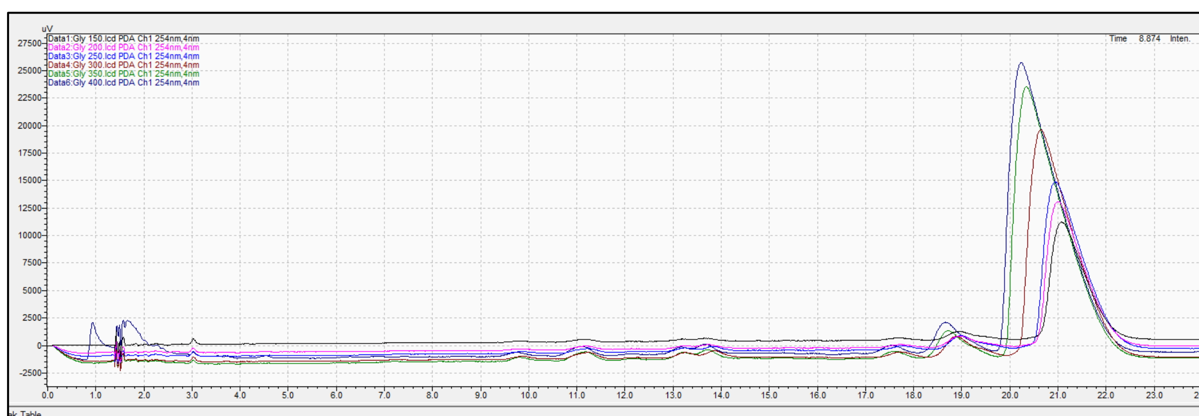


Figure 5.7.2.2. Overlay HPLC chromatograms of standard glycyrrhizin (150 ppm – 400 ppm) detected at 254 nm.

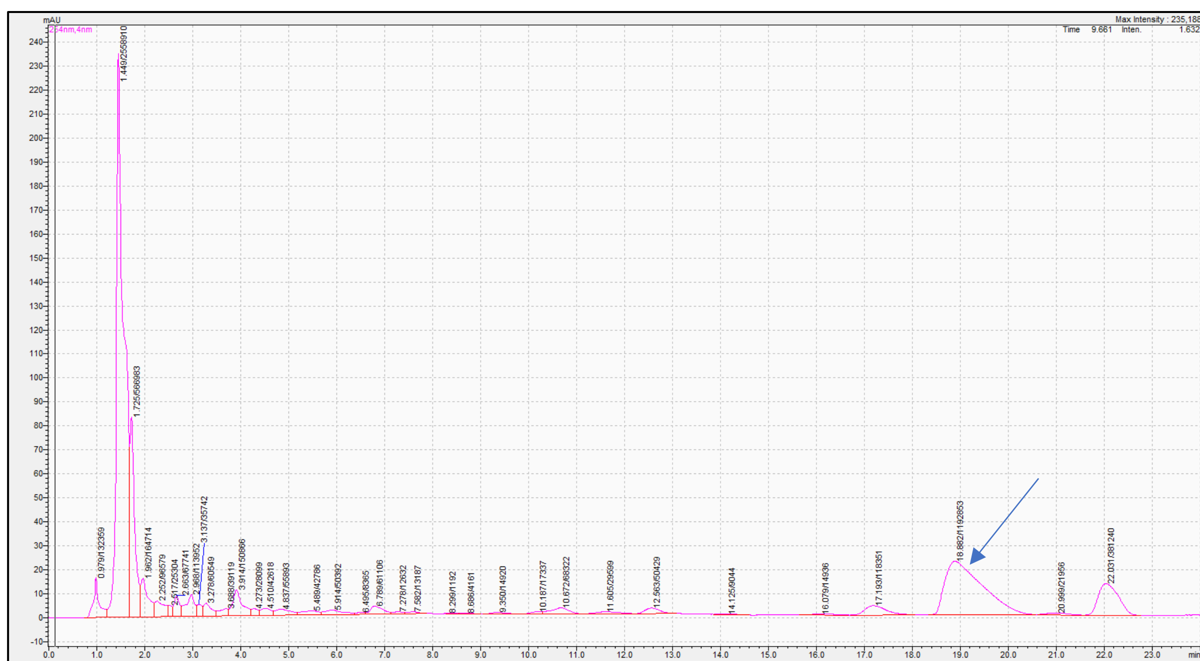


Figure 5.7.2.3. HPLC chromatogram of LQ detected at 254 nm.

The samples HP and LQ used for the study were standardized using HPLC method using their anti-inflammatory constituents as marker compounds. The chromatogram of HP was identified to show peaks for vanillic acid (Rt 3.20 min), ethyl protocatechuate (Rt 7.097), and rosmarinic acid (Rt 8.507 min), and the confirmation was established by retention time match and co-injection analysis using standard marker compounds. Vanillic acid has been reported to attenuate cartilage degradation on human osteoarthritic chondrocytes (Ziadlou et al., 2020) and reduce synovitis and pain mediators (Ma et al., 2021). Protocatechuic acid and its derivatives have been extensively studied for their anti-inflammatory in the literature (Lende et al., 2011). Rosemarinic acid has been proved to have anti-arthritic activity in collagen induced arthritis (Youn et al., 2003) and Freund's complete adjuvant arthritis (Gautam et al., 2019). It has been also reported to downregulate RANKL-induced osteogenesis (Phromnoi et al., 2021) and inhibit the matrix-degrading enzymes in osteoarthritis (Hu et al., 2018).

The chromatogram of LQ was identified to show the peak of glycyrrhizin (Rt 20.235min). The main chemical component in licorice are triterpenoid saponins (glycyrrhizin) and it constitutes around 10% dry weight of licorice (Wahab et al., 2021). It has been reported to dose-dependently inhibit the expression of TNF-α, IL-6, IL-1β and NF-κB (Fu et al., 2014). Its effect in downregulating TRAP osteoclasts and cathepsins B and K in the bone lesions of aged mice has established its anti-arthritic activity (Yamada et al., 2021). Apart from this, many studies in literature have highlighted its anti-arthritic activity (Shi et al., 2010; Marianecci et al., 2012;

Zhou et al., 2021; Luo et al., 2021). Identification of these anti-inflammatory markers in HP in combination with LQ provided evidence to further explore these extracts in in-vivo models of inflammation and arthritis.

5.8 Heavy metal content in HP and LQ

Toxic metals like lead, mercury, arsenic and cadmium are often present as contaminants in herbal drugs (Mazzanti et al., 2008; Sahoo et al., 2010). The contamination of herbal drugs with heavy metals during collection or manufacturing poses a serious threat to health and therefore the prepared extracts HP and LQ were tested for the presence of heavy metals before testing it in in-vivo models. The results revealed that the heavy metal content of HP and LQ extracts was within the limits set by different regulatory bodies like WHO, USFDA and AYUSH (AYUSH, 2018; Zamir et al., 2015; Sahoo et al., 2010) assuring the safety and quality of the prepared extracts (Table 5.8.1).

Table 5.8.1. Heavy metal content of HP and LQ determined by ICP-MS

S. No	Elements	HP (ppm)	LQ (ppm)	Limits (ppm)		
				WHO	USFDA	AYUSH
1.	Arsenic	0.62	0.38	10	10	10
2.	Cadmium	0.06	0.01	0.2	0.3	0.3
3.	Mercury	0.01	ND*	1	1	1
4.	Lead	0.92	0.13	10	10	10

*ND- Not detected

5.9 Acute toxicity study of HP extract

For establishing the safety profile of HP, an acute toxicity study was taken up. Samples, LQ, Gly and MAG were exempted from the toxicity study as they have already been approved for use in foods by the U.S. Food and Drug Administration (FDA), the Council of Europe, and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). They have also been included in the list of substances Generally Recognized as Safe (GRAS) by the Flavor and Extract Manufacturers' Association (FEMA) (Isbrucker and Burdock, 2006). The toxicity study was conducted on Balb/c mice. The various parameters demonstrating the safety/toxicity of HP were measured during the study and the details of the same are explained below.

5.9.1 Alteration in body weight and food-water intake

The body weights of HP administered mice were examined twice weekly and the body weights were found to be comparable to the normal control group in both sexes i.e., male and female mice. The fluctuations observed in body weight during the study were recorded but it was not found significant when compared with the normal control group. This indicates that HP extract treatment has no major impact on body weights (Figure 5.9.1a and b).

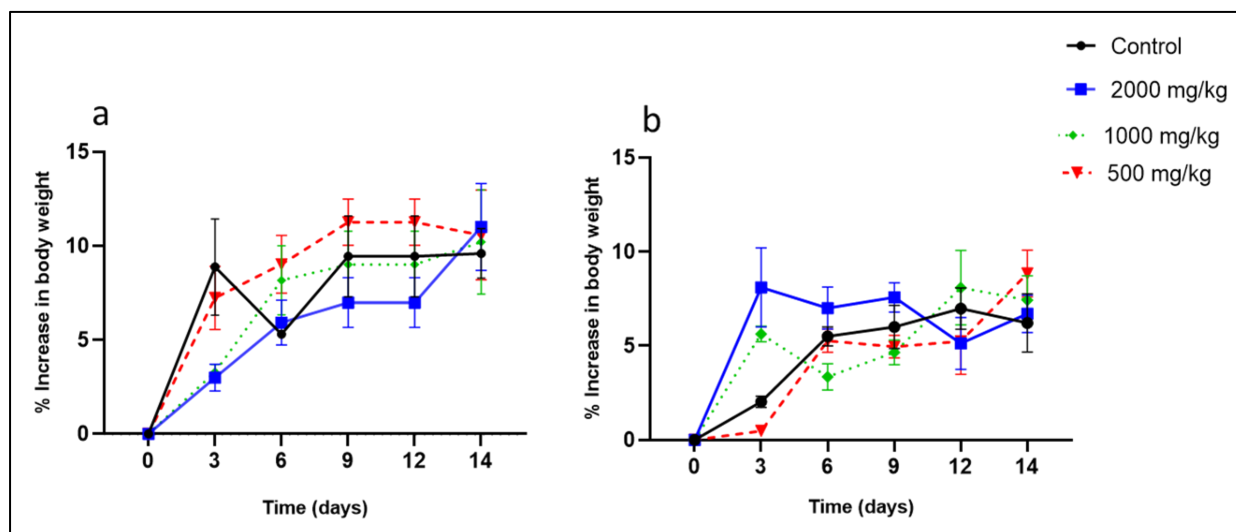


Figure 5.9.1. Body weight of control and *H. pinifolia* extract-administered mice in both sex (a) Male (b) Female. Values expressed as Mean ± SEM, n = 10. Data are considered statistically significant when $P<0.05$.

5.9.2 Morphological alteration

The extract-treated animals did not show any changes in general appearance pertaining to the skin, eyes, fur, mucous membrane, lacrimation, piloerection, respiratory pattern and pupil size. No alteration in posture, gait or any sign of tonic-clonic movements was observed. Typical stereotypies behaviours such as excessive grooming and repetitive circling or any bizarre behaviour like backwards walking and self-mutilation were not observed. Treatment did not alter motor activity in both sexes, which was a positive indication of the absence of any variation in the skeletal system. No other apparent morphological alterations were observed in HP-treated groups.

5.9.3 Haematological and biochemical examination

The haematological and biochemical parameters summarised in Table 5.9.3 were found similar to the control mice. Biochemical estimations for the determination of various body organ functions such as serum creatinine and random blood glucose indicated the absence of any

deviation from the normal range inferring that normal body function regulation was maintained in the extract-administered animals. No significant toxicity was observed even at the highest dose.

Table 5.9.3. Effect of *H. pinifolia* extract (2000 mg/kg dose) on haematological and biochemical profile in mice from the 14 days acute toxicity study

Parameter	Male		Female	
	Control	Sample	Control	Sample
Total RBC count (mL/mm^3)	7.98±1.37	7.41±0.39	7.38±0.81	7.56±1.61
Total WBC count (per mm^3)	8750±926	9750±100	10538±472	11850±1145
Random blood glucose (mg/dL)	61±9.6	65.6±7.7	99.33±1.5	7.8±19.9
Serum creatinine (mg/dL)	1.62±0.01	1.67±0.01	2.43±0.05	2.62±0.05

The treatment group did not show significant alteration in haematological and biochemical parameters in mice. Values are expressed as Mean ± SEM, n = 10. Data are considered statistically significant when $P<0.05$.

5.9.4 Urine analysis

The extract-administered animals when subjected to urine analysis, revealed no observable changes when compared to the normal control animals. The animals portrayed a pale-yellow colouration of urine without pus, glucose, haematocrits or blood cells.

5.9.5 Macroscopic gross necropsy

The extract-administered animals showed normal architecture without any sign of inflammation. The treatment with the extract did not alter organ weights relative to the body weight (Figures 5.9.5a and 5.9.5b) and was found to be comparable with the normal control. Various organs *viz*. kidneys, liver, lungs and heart were subjected to macroscopic examination which revealed the organs were devoid of any lesions or abnormalities corroborating the safety of the administration of extract.

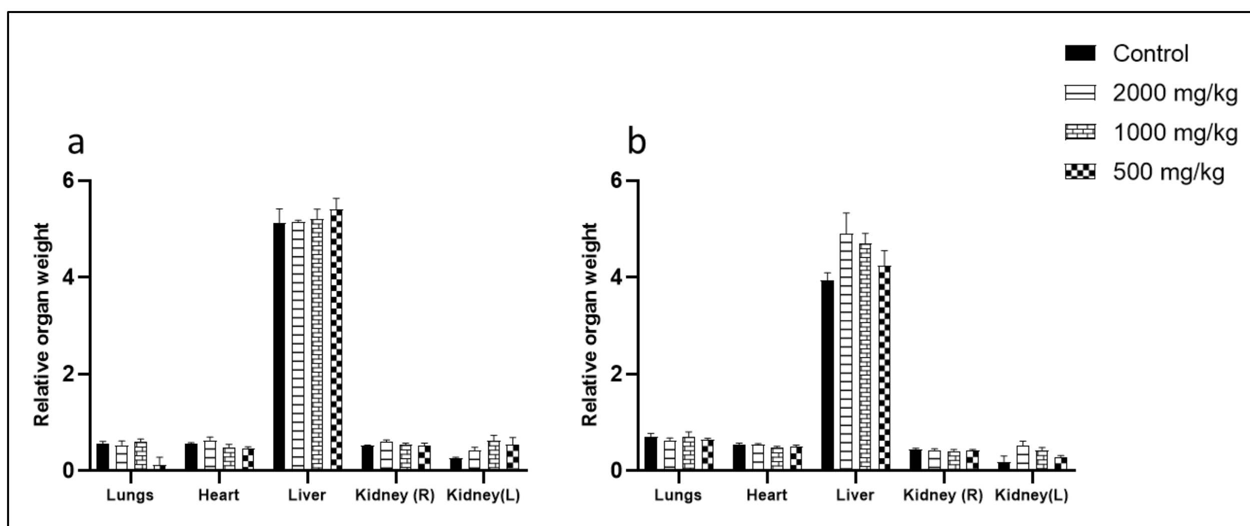


Figure 5.9.5. Effect of *H. pinifolia* extract on the macroscopic alteration in lungs, heart, liver and kidney in both sex (a) Male (b) Female. Values are expressed as Mean ± SEM, n = 10. Data are considered statistically significant when $P<0.05$.

5.9.6 Histopathological studies

No necrosis or morphological cell changes were observed on heart, spleen, liver, lungs and kidney histology of all treated groups after the administration of the extract (Figure 5.9.6). The histology of the heart showed normal architecture of the cardiac with the absence of cardiac myopathy, myofiber degeneration, and mononuclear cell infiltration. Similarly, the kidney sections showed delicate glomerular capillary loops indicating a normal glomerular filtration in the extract and normal control animal groups. In the liver section histology, no incidences of hepato-diaphragmatic nodules, focal inflammation, focal tension, lipid accumulation, etc., were observed. Lung sections showed no evidence of inflammation. However, spleen sections evidently showed normal haematopoiesis which did not differ from the spleen sections of the normal control animals validating the safety of the administration of the extract (Figure 5.9.6).

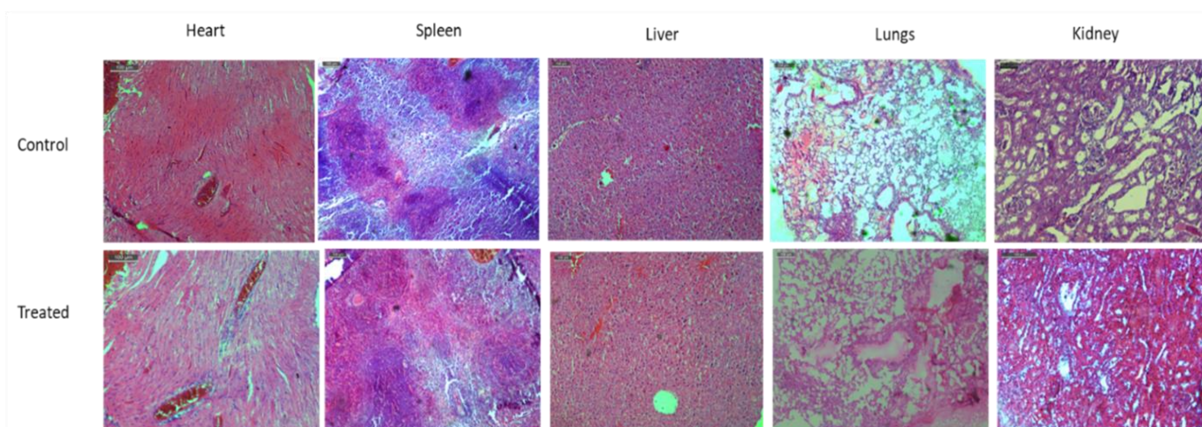


Figure 5.9.6. Histopathological studies of heart, spleen, liver, lungs and kidney in control and *H. Pinifolia* extract-administered mice.

The variations in the behavioural pattern and the mortality rate of mice are indications of toxicity. Toxicity-linked physiological changes were not observed in HP-treated mice during the experimental period of the acute toxicity study. The mice administered with the highest dose of HP (2000 mg/kg) appeared to be normal without any change in their fur, skin and mucous membrane. The investigation of acute and subacute toxicity is a crucial component of translational research to establish safety (Variya et al., 2019). HP did not produce any significant toxicity related to hematological and biochemical parameters. Also, no significant increase in body weight or acute toxicity in mice was observed. The gross necropsy analysis of the various organs revealed no significant alteration in the organ weights compared to the normal control organ weights. Also, the various organs did not portray any lesions, oedema or observable abnormalities. Thus, HP did not expose any treatment-related adverse effects on the organs. During the in-depth microscopic study of the heart, spleen, liver, lungs and kidney through histopathological examination, no pathological findings as evidence of toxicity were observed. The results of this study suggested that ingestion of HP has no adverse action or health-related acute toxicity.

5.10 In-vivo evaluation of anti-inflammatory activity of extracts, compounds and compositions

5.10.1 Effect of extracts, compounds and compositions on cytokines in LPS-challenged mice endotoxaemia model

Bacterial LPS elicits the overproduction of inflammatory mediators, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), nitric oxide, etc. The large number of inflammatory mediators produced in the body is thought to contribute to the LPS-induced

symptoms of various inflammatory diseases (Yoon et al., 2022). The ratio 1:0.5 (i.e. 100 µg/mL:50 µg/mL of HP: LQ in the case of composition 1 and 100 µg/mL:50 µM of HP:Gly and HP:MAG in the case of compositions 2 & 3, respectively) which was identified as an effective synergistic combination for inhibiting IL-1β, IL-6 and TNF-α in in-vitro studies were taken further for testing under in-vivo models.

Mice were pretreated with HP (100 mg/kg), LQ (50 mg/kg), Gly (50 mg/kg), MAG (50 mg/kg) and C1 (HP+LQ; 100+50 mg/kg), C2 (HP+Gly; 100+50 mg/kg) and C3 (HP+MAG; 100+50 mg/kg) as single doses for 1 day and 2 days through an oral route and then challenged with LPS (0.3 mL/kg). The effect of the pretreatment on the secretions of TNF-α, IL-1β, and IL-6 cytokines was understood by estimating the concentration of cytokines in blood plasma using commercially available ELISA kits. The results of the determinations are presented in Tables 5.10.1.1 and 5.10.1.2.

The study revealed that the compositions prepared by combining HP with LQ/Gly/MAG were found to be better active than the individual components in controlling the secretions of pro-inflammatory cytokines and they exhibited a pan-inhibition effect (Table 5.10.1.1). Among the *G. glabra*-derived sample-treated groups, LQ was better active than Gly and MAG, however, the effect was lesser than HP treatment (Table 5.10.1.1 and Figure 5.10.1.1) in the case of TNF-α and IL-6 attenuation and similar in the case of IL-1β. HP at 100 mg/kg dose showed around 50% inhibition of TNF-α and IL-6, and 43% of IL-1β release. HP, when combined with Gly (C2) showed the highest inhibition (60.4%) of TNF-α. Furthermore, when HP was combined with LQ (C1) and MAG (C3), the inhibition effect was higher i.e. 54.37% and 54.91% in controlling the IL-6 secretion. Additionally, HP and MAG combination (C3) exhibited a very high inhibition of IL-1β (65.05%) and nevertheless, HP and Gly combination i.e. C2 also exhibited 62.56% inhibition. Overall, while C3 was found to be more potentially active in attenuating the secretions of IL-1β and IL-6, composition 2 was inhibitory against TNF-α and IL-6. HP and LQ combination (C1) demonstrated >50% inhibition of all three cytokines (Table 5.10.1.1 and Figure 5.10.1.1).

Table 5.10.1.1. The percentage inhibition effect of single oral administration of extracts, compounds and compositions on plasma TNF-α, IL-6 and IL-1β levels under mouse endotoxaemia model

Treatment (Dose mg/kg body weight)	Percentage Inhibition of Cytokines [a]		
	TNF- α	IL-6	IL-1 β
HP (100)	49.16****±2.35	48.4****±3.38	42.58***±0.74
LQ (50)	15.80**±1.02	31.38****±3.06	43.44***±6.24
C1 HP+LQ (100+50)	52.83****±3.78	54.37****±3.69	50.00***±6.74
HP (100)	49.16****±2.35	48.4****±3.38	42.58***±0.74
Gly (50)	9.78****±0.69	28.31****±2.28	32.51**±10.42
C2 HP+Gly (100+50)	60.40****±10.10	40.79***±4.08	62.56***±5.58
HP (100)	49.16****±2.35	48.4****±3.38	42.58***±0.74
MAG (50)	7.07****±2.88	26.62****±3.39	40.03***±10.23
C3 HP+MAG (100+50)	32.03****±5.84	54.91****±3.20	65.05****±5.62
Prednisolone (10)	61.73****±7.55	58.40****±2.78	65.26***±5.21

[a] Each value represents the mean ± SEM (n = 5). **P < 0.01 ***P < 0.001 and ****P< 0.0001 versus disease control group.

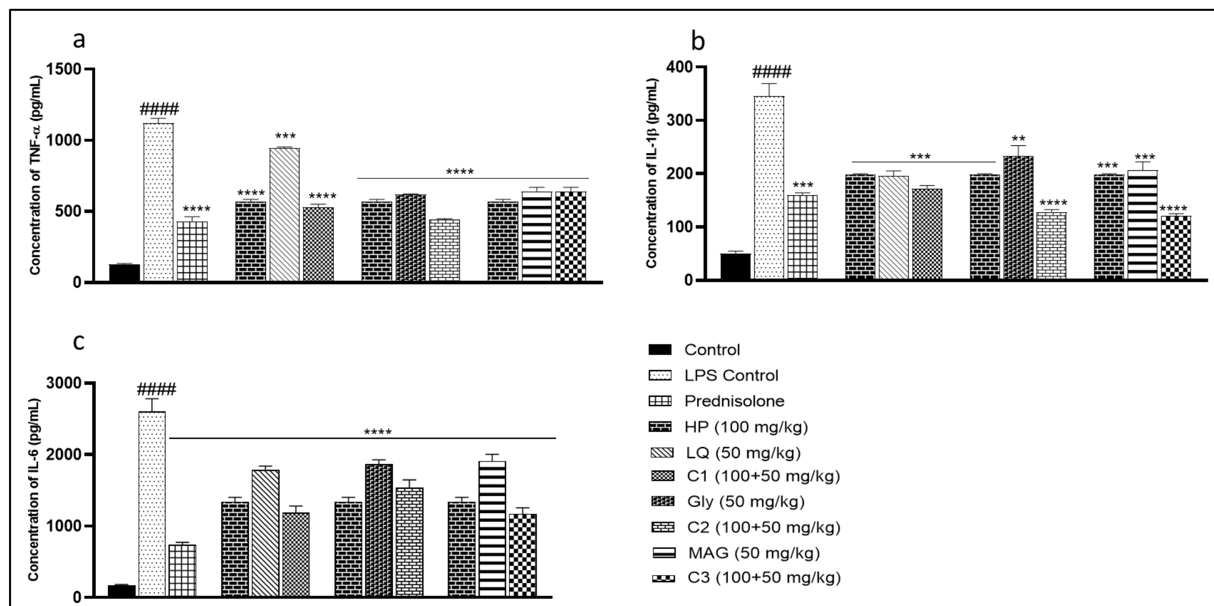


Figure 5.10.1.1 Effect of various extracts, compounds, and compositions on cytokines in LPS-challenged mice endotoxaemia model tested using ELISA. All values are presented as mean ± SEM (n=5). Effect on (a) TNF-α, (b) IL-1β, (c) IL-6. **P < 0.01***P< 0.001, ****P< 0.0001 versus LPS control group. $^{####}$P<0.0001 versus the control group.

Furthermore, all the samples when administered for 2 days showed an increase in inhibition effect compared to single-day administration, which deduces the dose-dependent effect (Table 5.10.1.2 and Figure 5.10.1.2). Samples, HP and C1 showed a higher attenuation effect i.e.,

66.31% and 67.87%, respectively in controlling the secretion of TNF-α. C3 was identified as a potential combination to inhibit IL-1β as it showed the highest inhibition either administered for a single day (65.05%) or two days (79.73%) (Figure 5.10.1.2)

Table 5.10.1.2 The percentage inhibition effect of oral administration of extracts, compounds and compositions for 2 days on plasma TNF-α, IL-6 and IL-1β levels under mouse endotoxaemic model

Treatment (Dose mg/kg body weight) for 2 days	Percentage Inhibition of Cytokines [a]		
	TNF- α	IL-6	IL-1 β
HP (100)	66.31****±1.61	62.61****±1.21	51.87****±3.20
LQ (50)	39.76****±1.56	54.15****±3.41	50.94****±3.46
C1 HP+LQ (100+50)	67.87****±10.10	62.61****±3.20	52.50****±5.56
HP (100)	66.31****±1.61	62.61****±1.21	51.87****±3.20
Gly (50)	14.96****±14.96	37.55****±2.73	55.94****±8.97
C2 HP+Gly (100+50)	47.94****±4.7	51.03****±1.73	57.04****±4.48
HP (100)	66.31****±1.61	62.61****±1.21	51.87****±3.20
MAG (50)	30.12****±3.58	35.44****±4.89	60.60****±14.94
C3 HP+MAG (100+50)	43.81****±2.03	56.08****±7.81	79.73****±4.39

[a] Each value represents the mean ± SEM (n = 5). ****P< 0.001 versus disease control group.

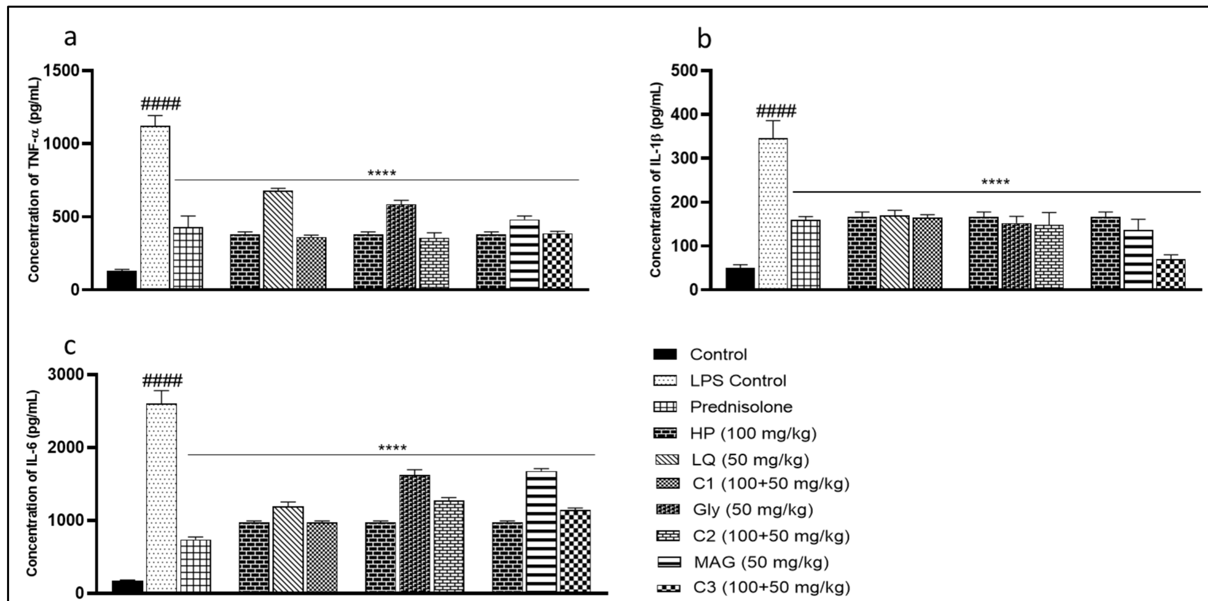


Figure 5.10.1.2. Effect of various extracts, compounds, and compositions on cytokines in LPS-challenged mice endotoxaemia model tested using ELISA. Effect on (a) TNF-α, (b) IL-1β, (c) IL-6 when administered for two days. All values are presented as mean ± SEM (n=5) ****P<0.0001 versus LPS control group. ####P<0.0001 versus the control group.

5.10.2 Effect of oral administration of extracts, compounds and compositions under carrageenan-induced mouse paw oedema model

Carrageenan-induced paw oedema is a well-known acute model of inflammation that is frequently used to screen new anti-inflammatory agents. A biphasic oedema was generated by injecting carrageenan into the sub-plantar surface of a rat paw. The early phase (around 1 h) is associated with the release of histamine, serotonin, bradykinin, and, to a lesser extent, prostaglandins produced by cyclooxygenase enzymes (COX), whereas the delayed phase (after 1 h) is associated with neutrophil infiltration and the continuation of prostaglandin production. The delayed phase of carrageenan-induced acute inflammation is similarly characterised by the release of neutrophil-derived free radicals, nitric oxide (NO), and pro-inflammatory cytokines such as TNF-α and interleukin-1 (Mansouri et al., 2015). Under this model, only C1, C2 and C3 were tested by administering them through the oral route as a single dose containing HP and LQ/Gly/MAG. The difference in mouse paw thickness, mRNA expression levels of TNF-α, IL-1β, and IL-6 in paw tissue homogenates, and the levels of NO and MPO were measured followed by the western blot analysis of NFκB protein expressions in paw tissues.

5.10.2.1 Effect on paw thickness

Injection of carrageenan into the hind paw induced progressive oedema reaching its maximum at 4 h. In the case of normal control animals, the difference in paw thickness was found to remain constant for 4 h. The carrageenan control animals showed an increase in paw thickness at each hour which was significant ($P<0.001$). The standard indomethacin group animals showed a significant decrease in paw thickness at the end of 3rd and 4th h. The difference in paw thickness of this positive control group decreased from 0.504 mm in 1st h to 0.074 mm in 4th h. Among the treatment groups, C2- and C1-treated animals showed better control over the paw oedema. The C1-treated animals showed a significant ($P<0.001$) decrease in paw thickness from 0.446 mm in 1st h to 0.188 mm in 4th h. Similarly, the C2-administered group showed a difference in paw thickness which was statistically significant at 2nd, 3rd and 4th h. The C3 group animals also showed a decrease in paw thickness from 0.579 mm in 1st h to 0.0416 mm at the end of 4th h (Figure 5.10.2.1).

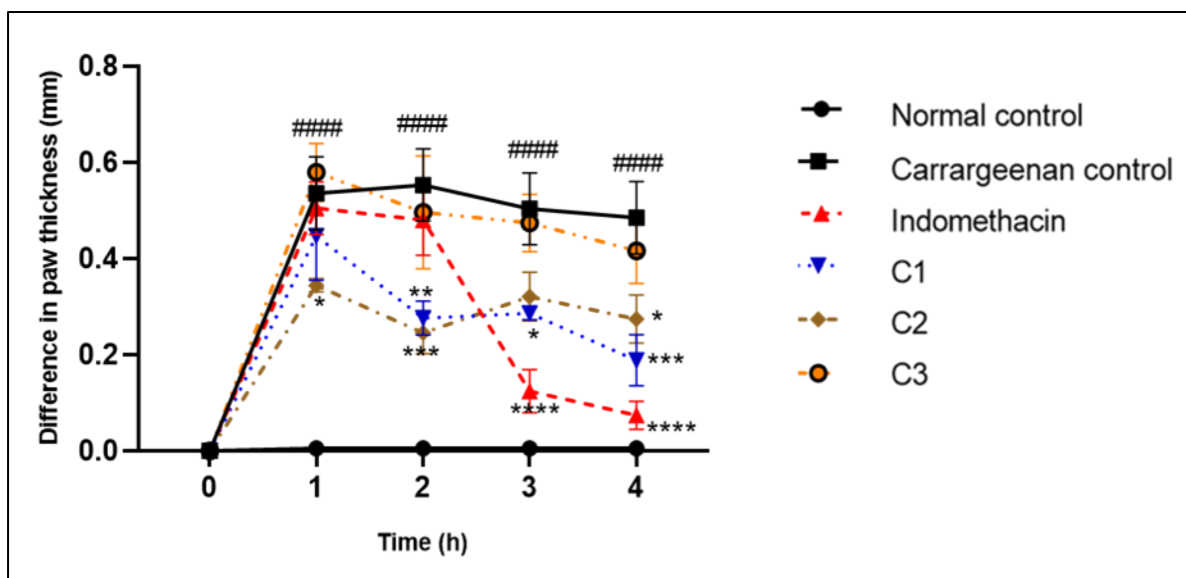


Figure 5.10.2.1. Effect of C1, C2 and C3 (100 HP+50 LQ/Gly/MAG) mg/kg body weight dose and standard Indomethacin (5 mg/kg) on paw thickness in mice. All values were presented as mean ± SEM (n=5). *P<0.05, **P<0.01, ***P<0.001, ****P< 0.0001 versus disease control group. $^{####}$P<0.0001 versus the control group.

5.10.2.2 Effect on the mRNA expression levels of TNF-α, IL-1β, and IL-6

To assess the in-vivo anti-inflammatory effect of C1, C2 and C3, the gene expression of pro-inflammatory cytokines was evaluated in carrageenan-induced paw tissues of mice. As shown in Figure 5.10.2.2, carrageenan injection noticeably elevated the levels of these inflammatory genes. Remarkably, pre-treatment with C1 suppressed the gene expression of IL-1β, IL-6 and TNF-α by 16.2-folds, 8.2-folds, and 4.5-folds, respectively. C2 similar to C1 significantly decreased the expression of IL-1β, IL-6, and TNF-α by 16.7-folds, 4.4-folds and 4.5-folds, respectively. Nevertheless, C3 also displayed significant downregulation of mRNA expression levels of IL-1β (7-folds), IL-6 (5.4-folds), and TNF-α (1.3-folds) (Figure 5.10.2.2).

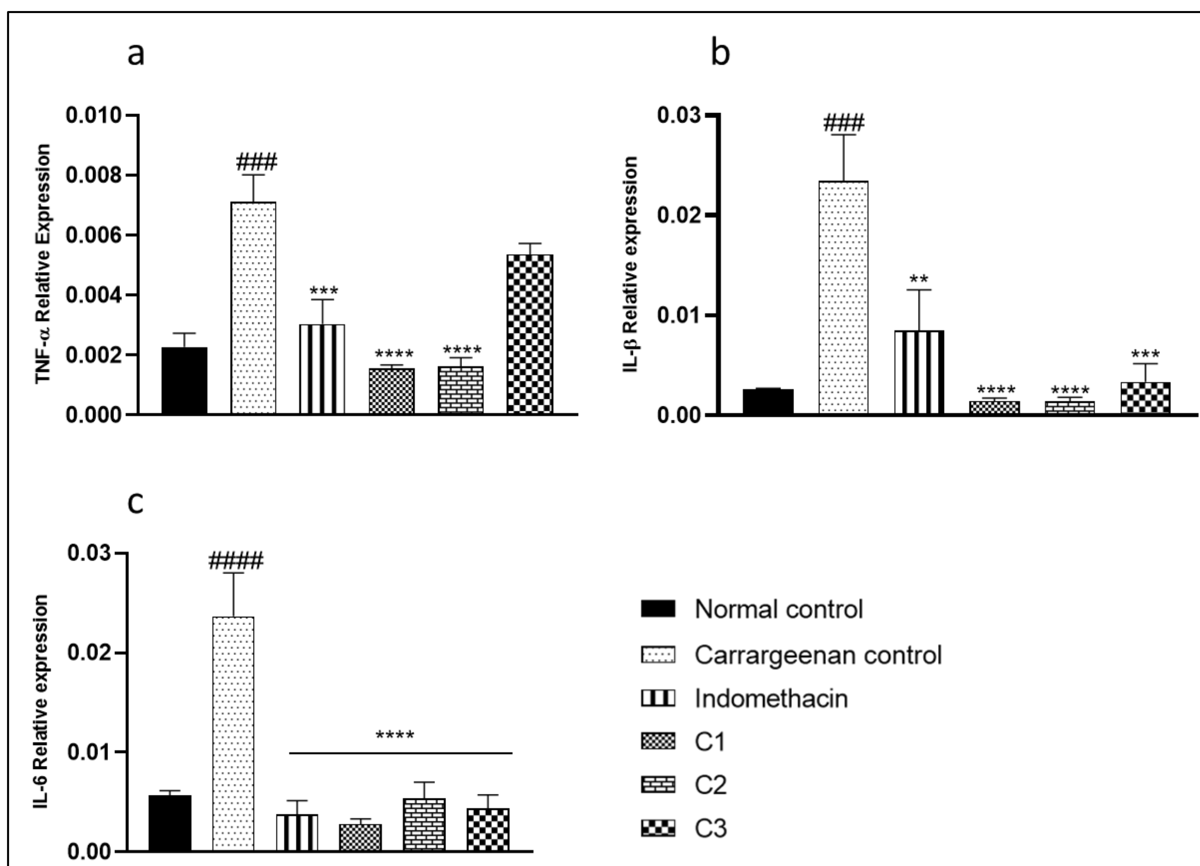


Figure 5.10.2.2. Effect of C1, C2 and C3 (100 HP+50 LQ/Gly/MAG) mg/kg body weight dose and standard Indomethacin (5 mg/kg) on mRNA expressions of (a)TNF-α, (b) IL-1β and (c) IL-6 in mice paw tissues under carrageenan-induced paw edema model. All values were presented as mean ± SEM (n=5). **P<0.01, ***P<0.001, ****P<0.0001 versus disease control group. $^{###}$P<0.001, $^{####}$P<0.0001 versus the control group.

5.10.2.3 Effect on the TNF-α secretions (ELISA)

C1 and C2 significantly (P<0.01) decreased the concentration of TNF-α in paw tissue homogenate when estimated by ELISA (Figure 5.10.2.3). The standard drug indomethacin reduced the stimulated TNF-α by 38.08%. C1 and C2 downregulated the expression of TNF-α by 42.76% and 45.17%, respectively. Nonetheless, composition 3 also displayed an inhibitory effect on the production of TNF-α in the carrageenan-injected paw, however, this effect was not statistically significant (Figure 5.10.2.3).

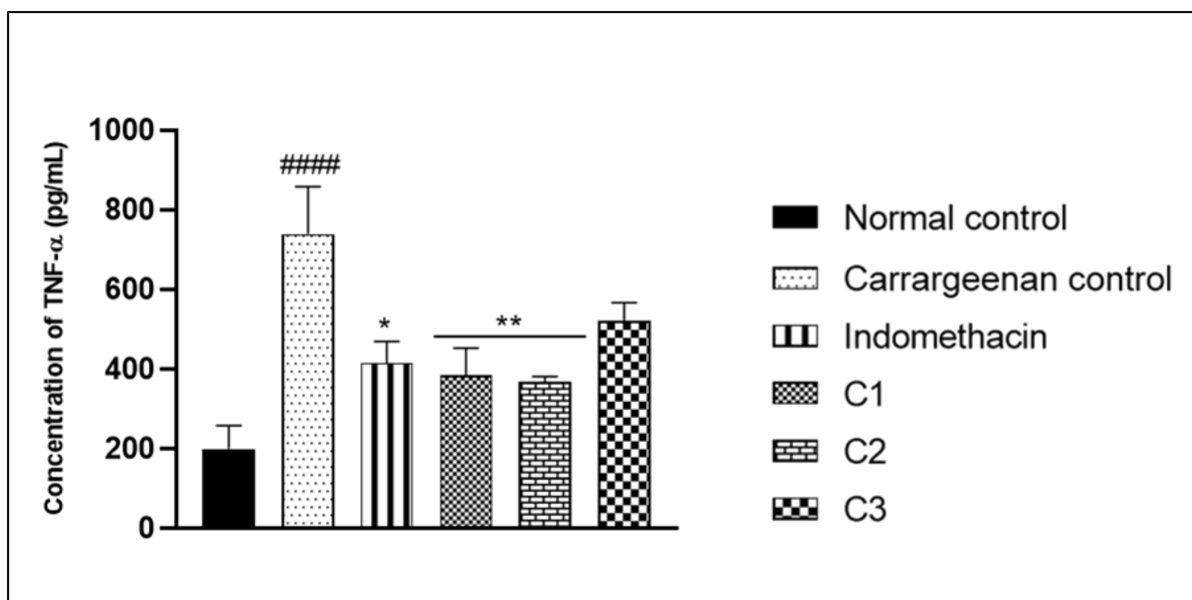


Figure 5.10.2.3. Effect of C1, C2 and C3 (100 HP+50 LQ/Gly/MAG) mg/kg body weight dose and standard Indomethacin (5 mg/kg) on TNF-α levels in mice paw tissues under carrageenan-induced paw oedema model. All values were presented as mean ± SEM (n=5). *P<0.05, **P<0.01versus disease control group. $^{####}$P<0.0001 versus the control group.

5.10.2.4 Effect on the production of NO and MPO

The level of MPO was measured as an index of neutrophil infiltration and the amount of nitrites as an index of nitrosative stress. C1 was most effective (P<0.0001) in attenuating the carrageenan-induced elevation of MPO, compared to carrageenan control. Indomethacin, C2 and C3 also showed a significant reduction in MPO activity (P<0.001). In the case of controlling the nitrite production also, C1 was found to be most effective with 36.39% inhibition. While C2 and C3 showed inhibition effects of 32.31% and 29.17%, respectively, the standard drug indomethacin showed 23.84% (Figure 5.10.2.4).

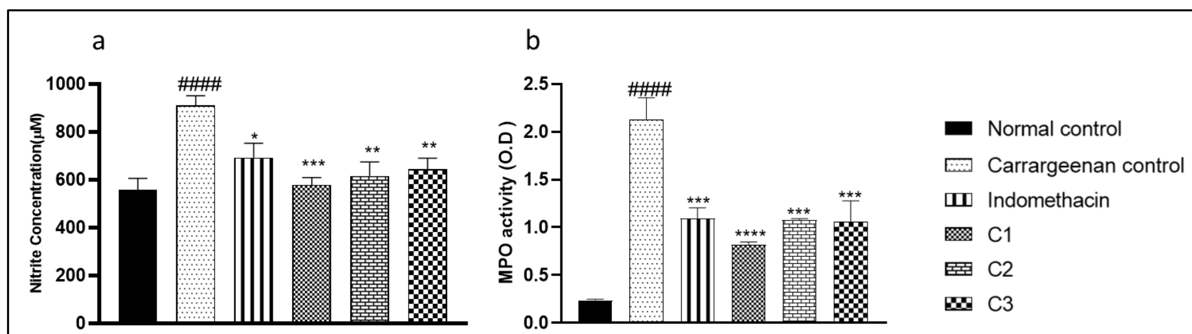


Figure 5.10.2.4. Effect of C1, C2 and C3 (100 HP+50 LQ/Gly/MAG) mg/kg body weight dose and standard Indomethacin (5 mg/kg) on (a) NO levels and (b) MPO levels under carrageenan-induced paw oedema model. All values were presented as mean ± SEM (n=5). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus disease control group. $^{####}$*P*<0.0001 versus the control group.

5.10.2.5 Effect on NF-κB protein expressions in carrageenan-induced paw oedema model under western blot analysis

The protein expression of NF-κB was remarkably enhanced after the induction of inflammation by carrageenan, thus confirming the provoked inflammatory signaling. C1 and C2 significantly (P<0.0001) hindered the elevations in protein expressions of NF-κB. Although the downregulation effect by C3 was moderate, it was still significant at P<0.01. All the proteins were quantified by normalising with β-actin. The analysis and quantification were carried out using ImageJ software and the results are presented in Figure 5.10.2.5.

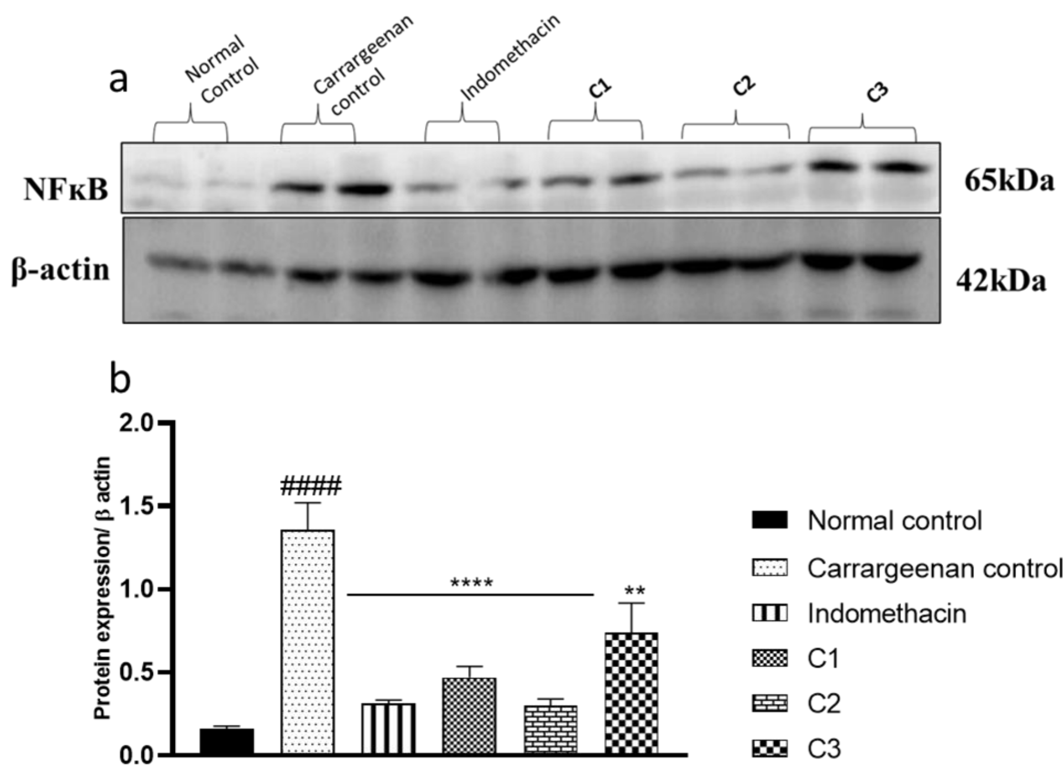


Figure 5.10.2.5. Effect of C1, C2 and C3 (100 HP+50 LQ/Gly/MAG) mg/kg body weight dose and standard Indomethacin (5 mg/kg) on (a) Protein expression of NFκB levels under carrageenan-induced paw oedema model (b) Densitometry analysis. All values were presented as mean ± SEM (n=4). **P<0.01, ****P<0.0001 versus disease control group. $^{\#}$P<0.0001 versus the control group.

The efficacy of compositions and their individual components were evaluated under LPS-induced mouse endotoxaemia model and carrageenan-induced mouse paw oedema model. In the endotoxaemia model, sample HP (100 mg/kg) was found to be more active in attenuating the levels of plasma TNF-α, IL-6 and IL-1β than LQ, Gly and MAG when administered orally as suspension. Further, substantiating the results of in-vitro experiments, all compositions showed excellent inhibition effects, with C3 and C2 showing 65.05% and 62.56% inhibition

of IL-1β, respectively. C2 showed the highest inhibition of TNF-α levels (60.40%) among the tested samples. C1 showed a similar inhibition effect over all the tested cytokines and it was found to be more than 50 % (Table 5.10.1.1 and Figure 5.10.1.1). Interestingly, the pro-inflammatory cytokines inhibition effect got augmented when the samples were administered at the same dose for two days. C3 showed 79.73% inhibition of IL- 1β and C1 showed 62.61% and 67.87% inhibition of plasma IL-6 and TNF-α levels (Table 5.10.1.2 and Figure 5.10.1.2). It could be implicated that the HP mixed with glycyrrhizin and its salt MAG was the right combination which amalgamated well with their characteristics leading to the rise in the activity.

Under the carrageenan-induced oedema model, a significant reduction in paw oedema was shown by C1 and C2 (Figure 5.10.2.1). In this sensitive method, carrageenan produces an acute and local inflammatory response that is useful for detecting orally active anti-inflammatory drugs; thus, it has substantial prognostic value for anti-inflammatory medicines working through acute inflammation mediators (Cordaro et al., 2020). The first 2 h after carrageenan injection, considered an early phase is attributed to the release of pro-inflammatory mediators such as histamine and serotonin. The following 3-5 h after carrageenan injection, considered the second phase is primarily mediated by kinins, prostaglandins, nitric oxide, cyclooxygenase, cytokines, and neutrophil-derived free radicals (Niu et al., 2014, Ishola et al., 2014). As C1 and C2 significantly reduced paw oedema in both phases, the anti-inflammatory activity could be attributed to the inhibition of multiple components and mediators of inflammation (Figure 5.10.2.1). Our observations were found to be in line with the study conducted by Wang et al (2015) where glycyrrhizin was assessed for anti-inflammatory activity at a dose of 160 mg/kg. Further, all the compositions significantly ($P<0.001$) inhibited the mRNA expression of TNF-α, IL-1β and IL-6 and the protein expression of NF-κB, which controls the inflammatory response and the transcription of several pro-inflammatory cytokines and chemokines (Barnabei et al., 2021; Nailwal and Doshi 2021). TNF-α, considered as significant initiator of inflammatory responses (Xu et al., 2014) was significantly downregulated by compositions 1 and 2. Further, all the compositions were identified to play a role in the activation of neutrophils which was corroborated by their ameliorating effect on NO production and MPO activity in paw tissue (Figure 5.10.2.4). Activation of neutrophils is known to generate and release a number of tissue-damaging factors including reactive oxygen species as well as enzymes such as MPO and proteases (Ezzat et al., 2018). Additionally, NO is considered as a target for anti-inflammatory medicines and a critical biomarker, in the pathogenesis of inflammation (Tsai et al., 2015; Pranweerapaiboon et al., 2020).

5.11 In-vivo evaluation of anti-arthritic activity of extracts, compounds and compositions in chronic RA models

The extracts and compositions were evaluated for anti-arthritic activity in two well-established RA models namely: Adjuvant-induced arthritis and Collagen-induced arthritis model. Adjuvant-induced arthritis is an antigen-induced T-cell-mediated aggressive monophasic self-remitting sub-chronic form of arthritis caused by a suspension of heat-killed desiccated mycobacterium powder in oil. Adjuvant-induced arthritis mimics the clinical features of RA namely permanent joint malformations, ankylosis and has been a useful rodent model for evaluating drugs that may be of benefit in treating human RA (De, Kundu and Chatterjee, 2020). Collagen-induced arthritis (CIA) is an autoimmune model that resembles RA in many ways. Immunization with type II collagen (CII) of genetically susceptible strains of rodents and primates results in the development of severe polyarticular arthritis mediated by an autoimmune response (Luross and Williams, 2001; De, Kundu and Chatterjee, 2020).

5.11.1 In-vivo evaluation of anti-arthritic activity of HP, LQ and C1 in Adjuvant-induced arthritis (AIA)

5.11.1.1 Effect of HP, LQ and C1 on Freund's complete adjuvant-induced arthritis in rats

Adjuvant-induced arthritis rodent model is used for evaluating drugs that may be of benefit in treating human RA. Freund's adjuvant induces a series of cellular events leading to T-cell activation and polyarthritis (Billingham et al., 1990). During the study, the immunization of the right hind paw of the rat with FCA caused a significant increase in paw volume in the arthritis control group ($P<0.0001$). The difference in paw volume (DPV) was calculated for all the animals and is presented in Figure 1. On the 14th day, when the treatment was started, the control group showed a DPV of 0.1 mL and the arthritic control animals showed 1.328 mL ($P<0.0001$). Treatment with HP (DPV 0.623 mL), LQ (DPV 0.645 mL) and C1 (DPV 0.628 mL) significantly decreased ($P<0.0001$) the paw volume by around 60% on the 28th day, compared to the arthritic control group (1.580 mL). The standard drug-treated group exhibited 81.9% reduction in the paw volume (0.325 mL) compared to the arthritic control group (1.580 mL) (Figure 5.11.1.1). Treatment with test samples showed a quick action of oedema control compared to dexamethasone and HP was more active than dexamethasone during the initial four (14-18) days (Figure 5.11.1.1).

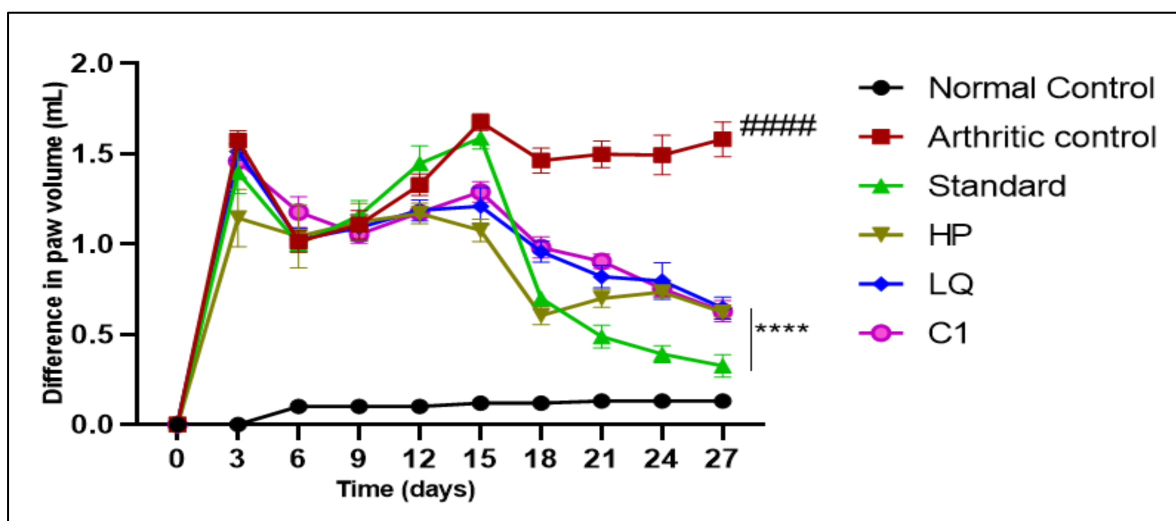


Figure 5.11.1.1. Effect of HP, LQ and C1 at an oral dose of 100 mg/kg, 50 mg/kg and (100 mg HP+50 mg LQ)/kg body weight, respectively and standard drug dexamethasone (0.1 mg/kg), on paw volume in rats in FCA model. All values were presented as mean ± SEM (n=8). ****P<0.0001 versus the arthritic control group. $^{####}$P<0.0001 versus the control group.

5.11.1.2 Effect of HP, LQ and C1 on spleen index

The relative weight of the spleen got significantly (P<0.0001) increased in the arthritic control group (3.6) compared to the control group (2.51) (Figure 5.11.1.2)). A marked decrease in the relative weight of the spleen (spleen index) was found in the animals of all treatment groups [standard - 2.4, HP - 2.7, LQ - 2.7 and C1 - 2.6] compared to the arthritic control group (3.6) (Figure 5.11.1.2).

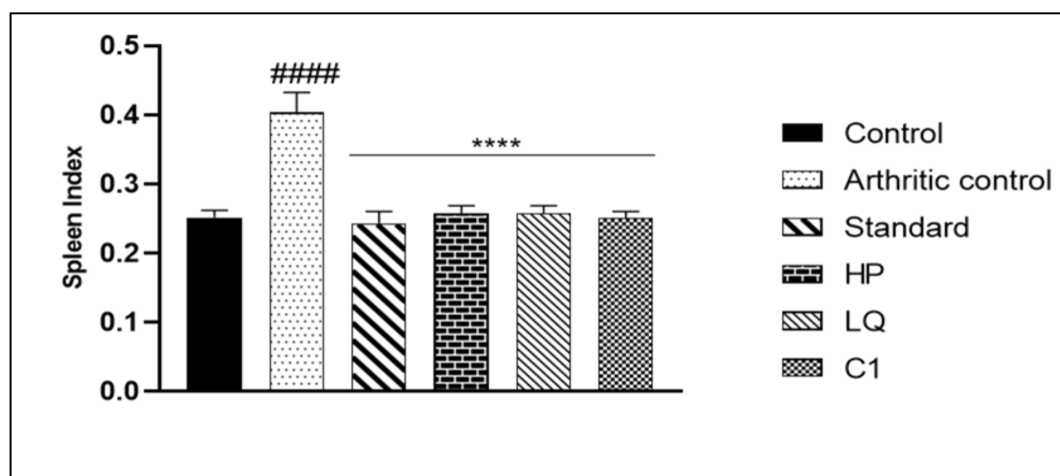


Figure 5.11.1.2. Effect of HP, LQ and C1 at an oral dose of 100 mg/kg, 50 mg/kg and (100 mg HP+50 mg LQ)/kg body weight, respectively and standard drug dexamethasone (0.1 mg/kg), on spleen index in rats in FCA model. All values were presented as mean ± SEM (n=8). ****P<0.0001 versus the arthritic control group. $^{####}$P<0.0001 versus the control group.

5.11.1.3 Effect of HP, LQ and C1 on haematological parameters

A significant elevation of platelet count, white blood cells and ESR in the arthritic control group (1021×10^3 cells/µL, 13.3×10^3 cells/µL and 5×10^3 mm/h, respectively) compared to the normal control group (785.3×10^3 cells/µL, 7.6×10^3 cells/µL and 3.66×10^3mm/h, respectively) was observed during the experiment. Treatment with standard, HP, LQ and C1 resulted in a significant (P<0.05) reduction in the number of white blood cells, (8.37×10^3, 7.7×10^3, 8.05×10^3 and 8.6×10^3 cells/µL, respectively) compared to the arthritic control group. However, only in HP-treated animals, a significant restoration of platelet count (704×10^3 cells/µL) was found. ESR was significantly reduced to 3.0×10^3 mm/h in both HP and LQ groups compared to arthritic control. The C1-treated group exhibited an overall reduction in ESR and platelet count compared to arthritic control, however, it was statistically not significant (Figure 5.11.1.3).

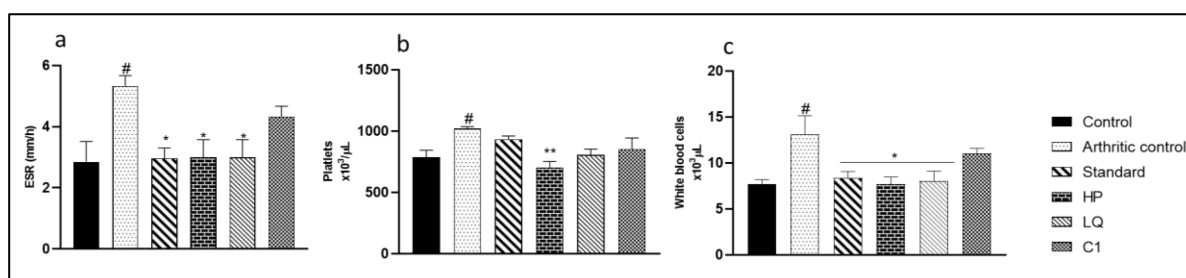


Figure 5.11.1.3. Effect of HP, LQ and C1 at an oral dose of 100 mg/kg, 50 mg/kg and (100 mg HP+50 mg LQ)/kg body weight, respectively and standard drug dexamethasone (0.1 mg/kg), on haematological parameters (a) ESR (b) Platelets (c) White Blood Cells of all animal groups on 28th day in FCA model. All values were presented as mean ± SEM (n=5). *P<0.05, **P< 0.01 versus arthritic control group. #P<0.05 versus the control group

5.11.1.4 Effect of HP, LQ and C1 on pro-inflammatory cytokines and TRAP levels in paw tissues

The concentrations of pro-inflammatory cytokines, TNF-α (23920 pg/mL), IL-6 (16426.7 pg/mL) and TRAP (2267.41 pg/mL) were significantly (P<0.0001) increased in the paw tissues of FCA alone administered group (disease control) in comparison with the normal control group (TNF-α 8286 pg/mL, IL-6 2411 pg/mL and TRAP 505 pg/mL). HP, LQ and C1 groups significantly (P<0.0001) decreased the concentration of TNF-α, IL-6 and TRAP in paw tissue homogenate (Figure 5.11.1.4). The standard drug dexamethasone reduced the levels of TNF-α, IL-6 and TRAP by 56.43%, 81.30% and 79.14%, respectively. HP showed a significant inhibitory effect on the release of TNF-α (67.64%), IL-6 (75.69%) and TRAP (48.9%). Similarly, LQ and C1 downregulated the expression of TRAP (40.41% and 38.75%, respectively) and IL-6 (83.08% and 74.82%, respectively) (Figure 5.11.1.4).

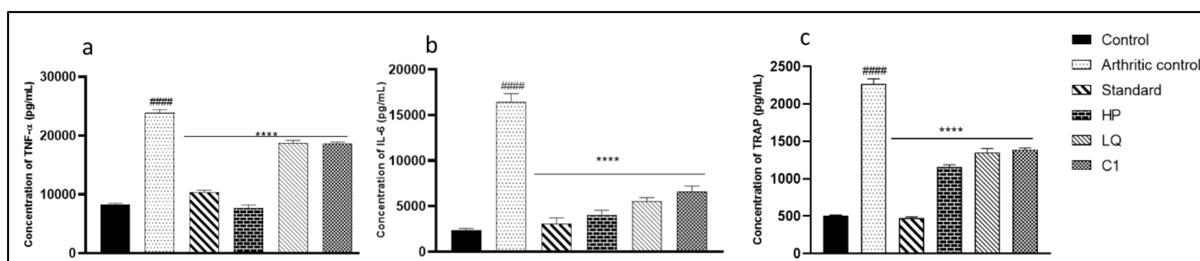


Figure 5.11.1.4. Effect of HP, LQ and C1 at an oral dose of 100 mg/kg, 50 mg/kg and (100 mg HP+50 mg LQ)/kg body weight, respectively and standard drug dexamethasone (0.1 mg/kg), on (a) TNF-α; (b) IL-6 and (c) TRAP in rat paw tissues on 28th day in FCA model. All values were presented as mean ± SEM (n=8). ****P<0.0001 versus arthritic control group. $^{####}$P< 0.0001 versus the control group.

5.11.1.5 Effect on the mRNA expression of pro-inflammatory cytokine and osteoclast markers

Injection of FCA noticeably elevated the gene expression of pro-inflammatory cytokines as tested in the paw tissue homogenate. Interestingly, more than 5-fold suppression of the gene expression of IL-1β, IL-6 and TNF-α was observed after the administration of HP, LQ and C1 (Figure 5.11.1.5). Similarly, the expression of osteoclast markers RANK, TRAP and cathepsin K were significantly downregulated (P<0.001) by HP, LQ, C1 and standard. HP showed a significant reduction of mRNA expression levels of TNF-α (8-fold) and RANK (20-fold), which was found to be more effective than the standard (5-fold and 21-fold, respectively). C1 showed a better effect in suppressing the expression of IL-6 (8.9-fold) and TRAP (4.9-fold) compared to the individual effect [HP: 6.2-fold (IL-6) & 2.5-fold (TRAP) and LQ: 4.5-fold (IL-6) & 4.2-fold (TRAP)] (Figure 5.11.1.5).

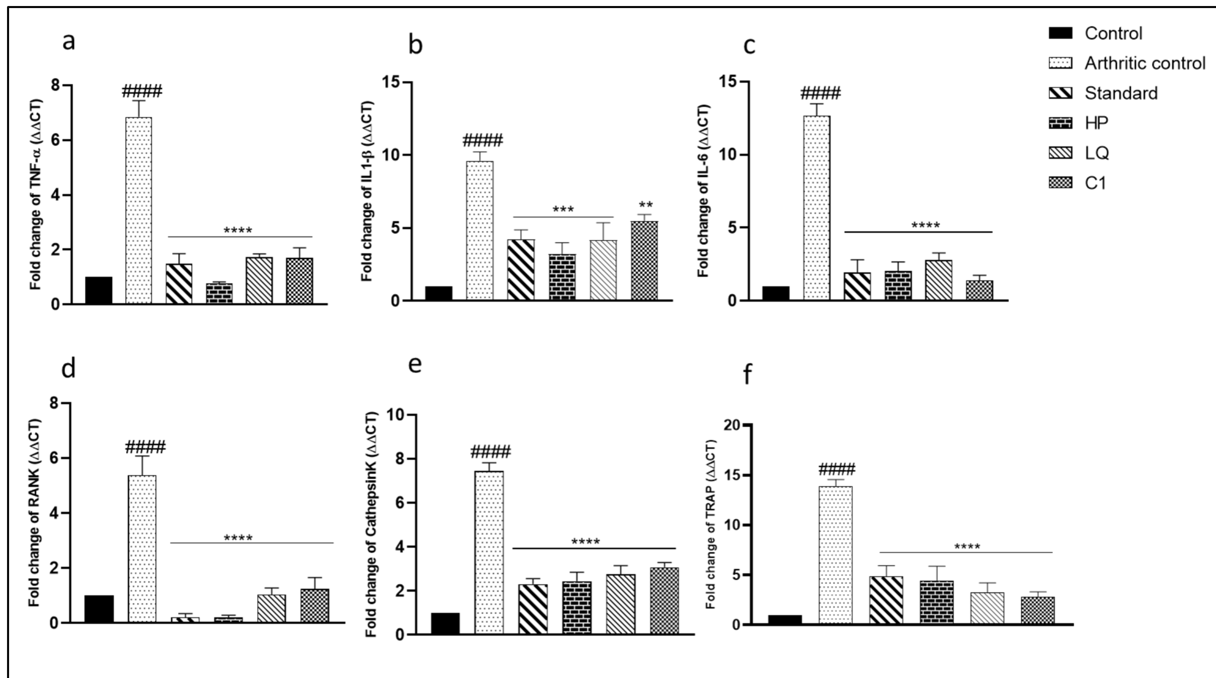


Figure 5.11.1.5. Effect of HP, LQ and C1 at an oral dose of 100 mg/kg, 50 mg/kg and (100 mg HP+50 mg LQ)/kg body weight, respectively and standard drug dexamethasone (0.1 mg/kg), on mRNA expression of (a) TNF-α (b) IL-1β (c) IL-6 (d) RANK (e) Cathepsin K (f) TRAP on 28th day in FCA model. All values were presented as mean ± SEM (n=8). ****P<0.0001 versus the arthritic control group. $^{####}$P<0.0001 versus the control group.

5.11.1.6 Effect of HP, LQ and C1 on ankle-joint histo-architecture

Pathological staining with haematoxylin and eosin sections is shown in Figure 5.11.1.6. The arthritic group displayed significant hyperplasia, with increased layer and thickening of synovial lining cells, accompanied by a large number of inflammatory cells infiltrating into the ankle joint cavity and cartilage destruction at the joint compared to the normal control group (Figure 5.11.1.6). The treatment with HP significantly (P<0.01) reduced the inflammation of ankle joints with improved synovial lining cell stratification and reduced inflammatory cell infiltration. The treatment groups LQ and C1 also showed reduced (P<0.05) synovial hyperplasia, cell infiltration and cartilage destruction.

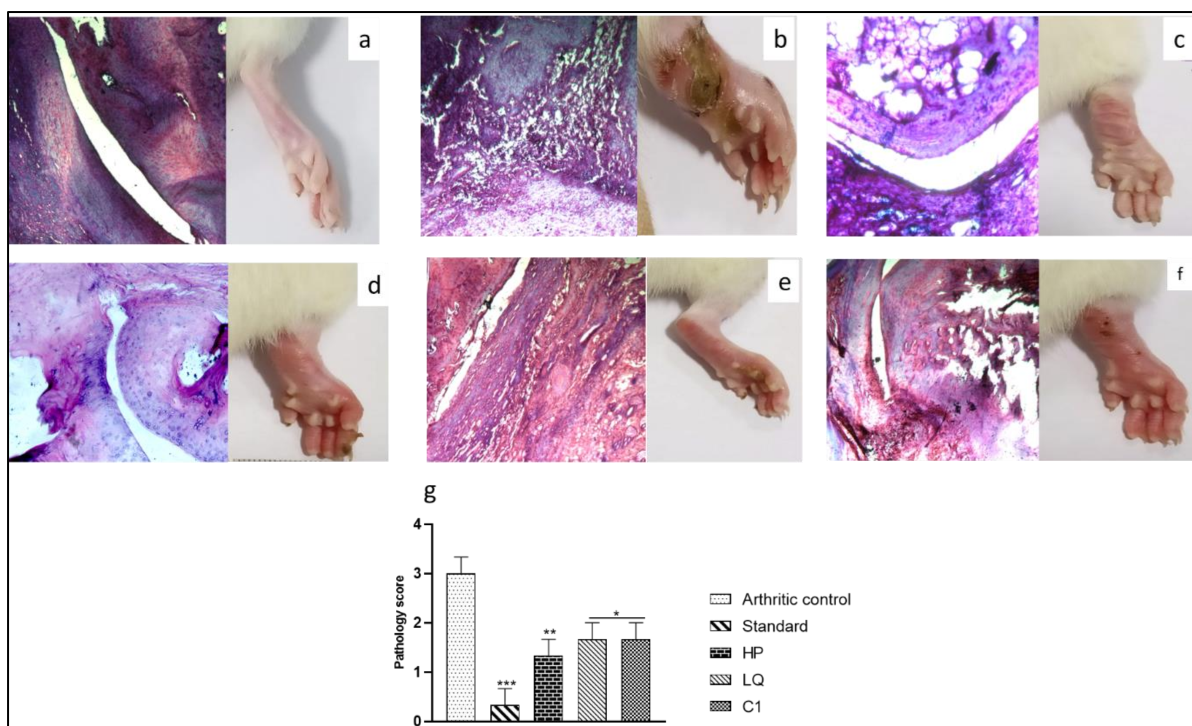


Figure 5.11.1.6. Haematoxylin-Eosin staining of arthritic ankle joints of FCA-induced rats belonging to (a) Control (b) Arthritic control (c) Standard (d) HP (e) LQ and (f) C1. Graph (g) presents the pathology score. All values were presented as mean ± SEM (n=5). $^{*}P<0.05$, $^{**}P<0.01$ $^{***}P<0.001$ versus arthritic control group. Scoring was done on the basis of narrowing of joint space and neutrophil infiltration 0) No change 1) Mild 2) Moderate and 3) Severe.

5.11.1.7 Radiological interpretation

During the study, while the normal bone matrix with no pathological changes was observed in healthy control rats, a marked soft tissue swelling and diminished joint spaces with periarticular erosive changes along with bone matrix resorption were seen in adjuvant-induced arthritic group (Figure 5.11.1.7a & 5.11.1.7b). In the case of the standard group, residual minimal osteoarthritic changes with no evidence of bone erosion and significant soft tissue swelling with minimal residual joint space narrowing were observed (Figure 5.11.1.7c). HP (Figure 5.11.1.7d) and C1 treated groups (Figure 5.11.1.7f) showed mild inflammation with less reduction in joint space and less soft tissue swelling. LQ-treated animals showed signs of osteoarthritis with bone resorption, moderate soft tissue swelling and moderately narrowed joint spaces as well as mild periarticular erosive changes (Figure 5.11.1.7e).

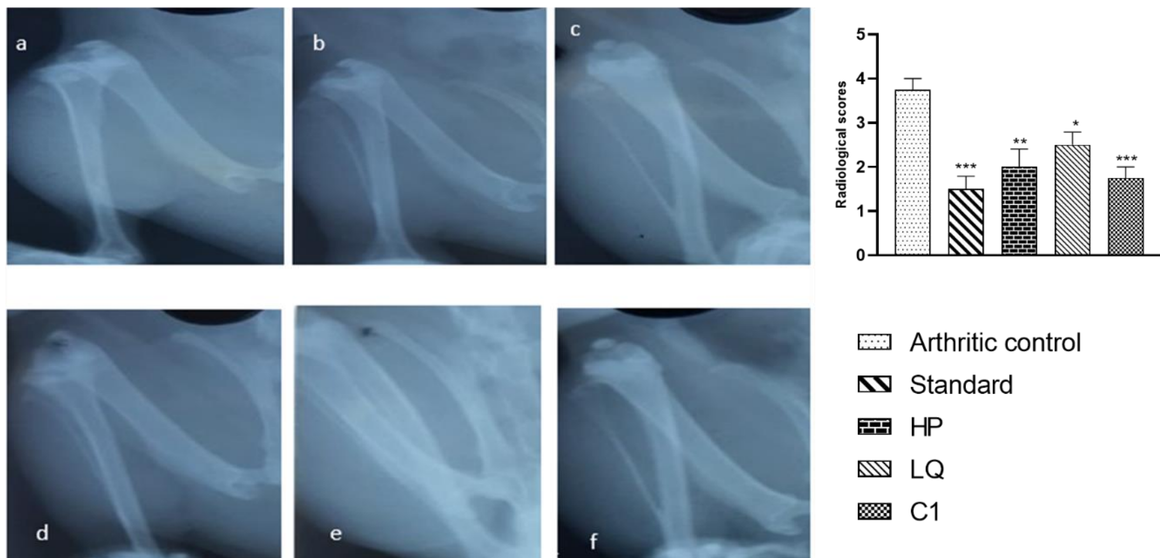


Figure 5.11.1.7. Radiographic changes in joints of (a) Control (b) Arthritic control (c) Standard (d) HP (e) LQ and (f) C1. Graph (g) presents the radiological scores. All values were presented as mean ± SEM (n=5). *P<0.05, **P<0.01, ***P<0.001 versus the arthritic control group. Scoring was done based on the degree of (i) soft tissue swelling (0 = normal with no soft tissue swelling, 1 = minimal soft tissue swelling, 2 = mild to moderate soft tissue swelling, 3 = moderate soft tissue swelling, 4 = marked to severe soft tissue swelling) and (ii) degree of joint destruction and periarticular erosive changes (0 = no joint destruction, 1 = minimal joint destruction, 2 = mild to moderate joint destruction, 3 = moderate joint destruction, 4 = marked to severe joint destruction with evident periarticular erosive changes). All scores were assessed by an observer blinded to the experimental groups.

5.11.1.8 Effect of HP, LQ and C1 on CD51 and MMP-3

CD51, the CD marker respective to osteoclasts, was assessed by using a western blot for the samples of all groups (Figure 5.11.1.8). The relative expression of osteoclasts marker with respect to β-actin signified that the treatment with HP, LQ and C1 inhibited or restricted the expression of CD51. However, the expression of CD51 was elevated significantly (P<0.001) in the arthritic control group. Simultaneously, pre-osteoclastic protein marker MMP-3 was analysed and it was observed that the FCA alone administered group exhibited a significant (P<0.0001) increase in protein expression when compared to the normal control group. The groups treated with HP, LQ and C1 significantly lowered the expression levels of MMP-3 compared to the arthritic control group (Figure 5.11.1.8).

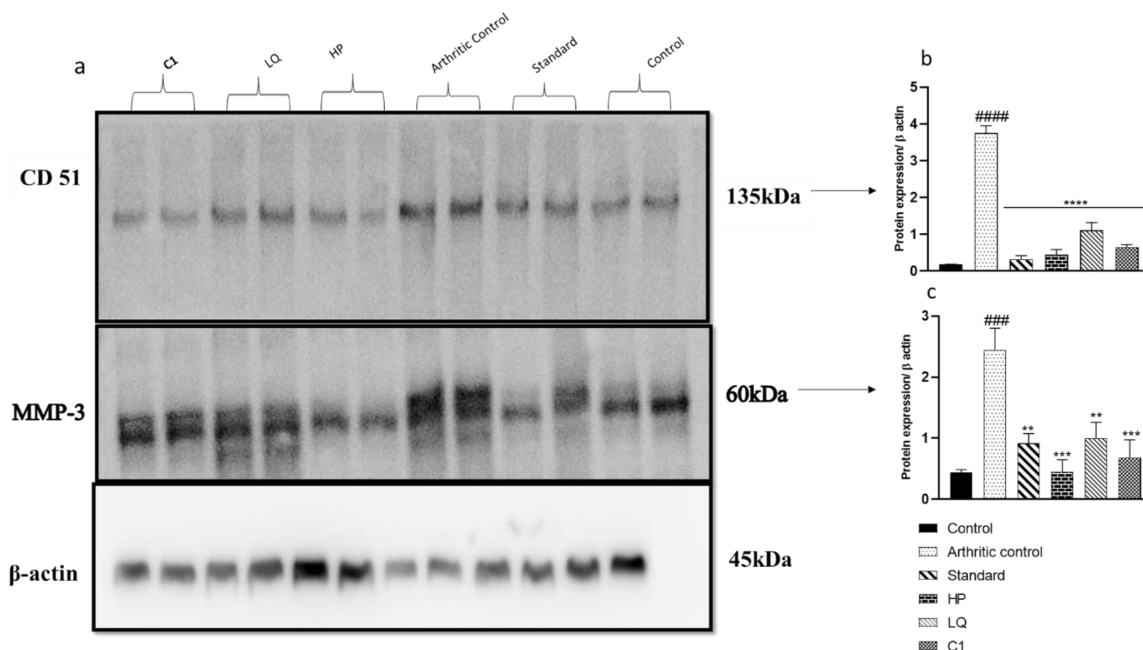


Figure 5.11.1.8. Effect of HP, LQ and C1 at an oral dose of 100 mg/kg, 50 mg/kg and (100 mg HP+50 mg LQ)/kg body weight, respectively and standard drug dexamethasone (0.1 mg/kg), on tissue expression of proteins (a) CD51 and MMP-3, (b) Equal loading of protein was confirmed by the levels of β-actin and (c) Densitometry analysis. Results are presented as mean ±SEM of three independent experiments (n=4). Results were expressed as mean ±SEM (n=4). ***P< 0.001 versus the arthritic control group. $^{####}$P< 0.0001 versus the control group.

5.11.2 In-vivo evaluation of anti-arthritic activity of C1 and lipid nano-emulsion C1-N in Collagen Induced Arthritis (CIA)

5.11.2.1 Preparation and characterization of lipid nano-emulsion C1-N

A lipid nano-emulsion C1-N containing the same amount of HP and LQ as that of C1 was prepared with the objective to develop a proof of concept that nano formulation would exhibit better anti-arthritic activity compared to the conventional suspension C1 prepared using a mortar and pestle. Both C1 and C1-N were characterized by analysing various parameters such as particle size, polydispersity index, zeta potential, stability and total phenolics.

5.11.2.2 Particle size, polydispersity index (PDI) and zeta potential

The particle size, PDI and zeta potential of formulations C1 and C1-N were determined. The average particle size of C1 was found to be 819 nm showing a PDI of 0.630. It exhibited a zeta potential of -61.1 mV. However, it was observed that C1 formed sediment and two separate layers within 1h of its preparation. On the other hand, the average particle size of C1-N on the day of preparation of formulation was found to be 292 nm and PDI was 0.322. The zeta

potential was found to be -36.1 mV (Figure 5.11.2.2). C1-N was analysed twice a week for 30 days and was found to exhibit particle size in the range of 292-400 nm, PDI 0.322-0.357 and zeta potential between -36 to -51.7 mV (Figure 5.11.2.2).

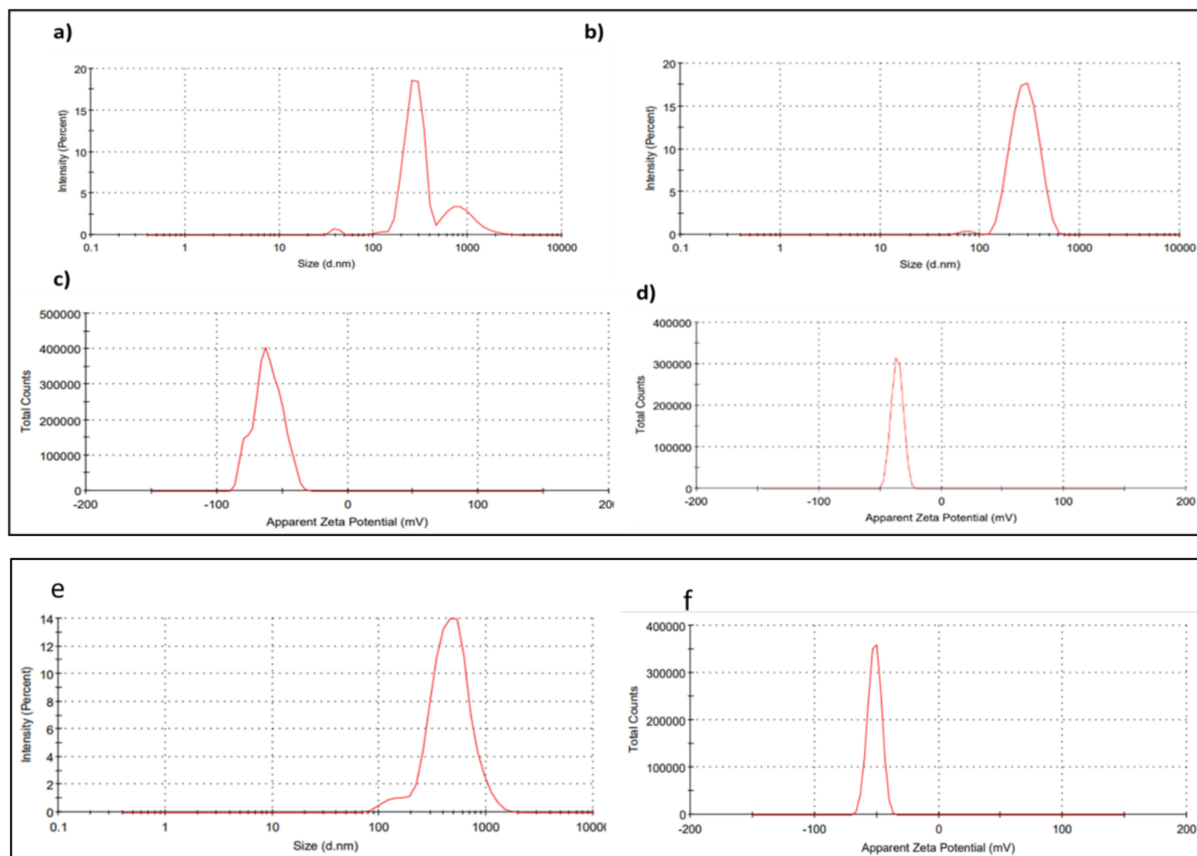


Figure 5.11.2.2. (a) & (b) Particle size and zeta potential of C1 on day 0; (c) & (d) particle size and zeta potential of C1-N on day 0 and (e) & (f) particle size and zeta potential of C1-N on day 30.

5.11.2.3 Morphological characters using FESEM

Figures 5.11.2.3 (a, b) and (c, d) shows the SEM micrographs of the C1 and C1-N formulations, respectively taken at different magnifications. It was observed that both the samples exhibited spherical shapes and smooth topology. However, the SEM images of freshly prepared samples of C1 showed some agglomeration regions, indicating that these samples are not stable even for one day. In contrast, the SEM images of C1-N showed no such agglomeration even after 30 days. Thus, C1-N was found to be stable. Moreover, it was evident from these micrographs, that the particle size of C1-N was comparatively smaller and uniform, unlike C1 (Figure 5.11.2.3).

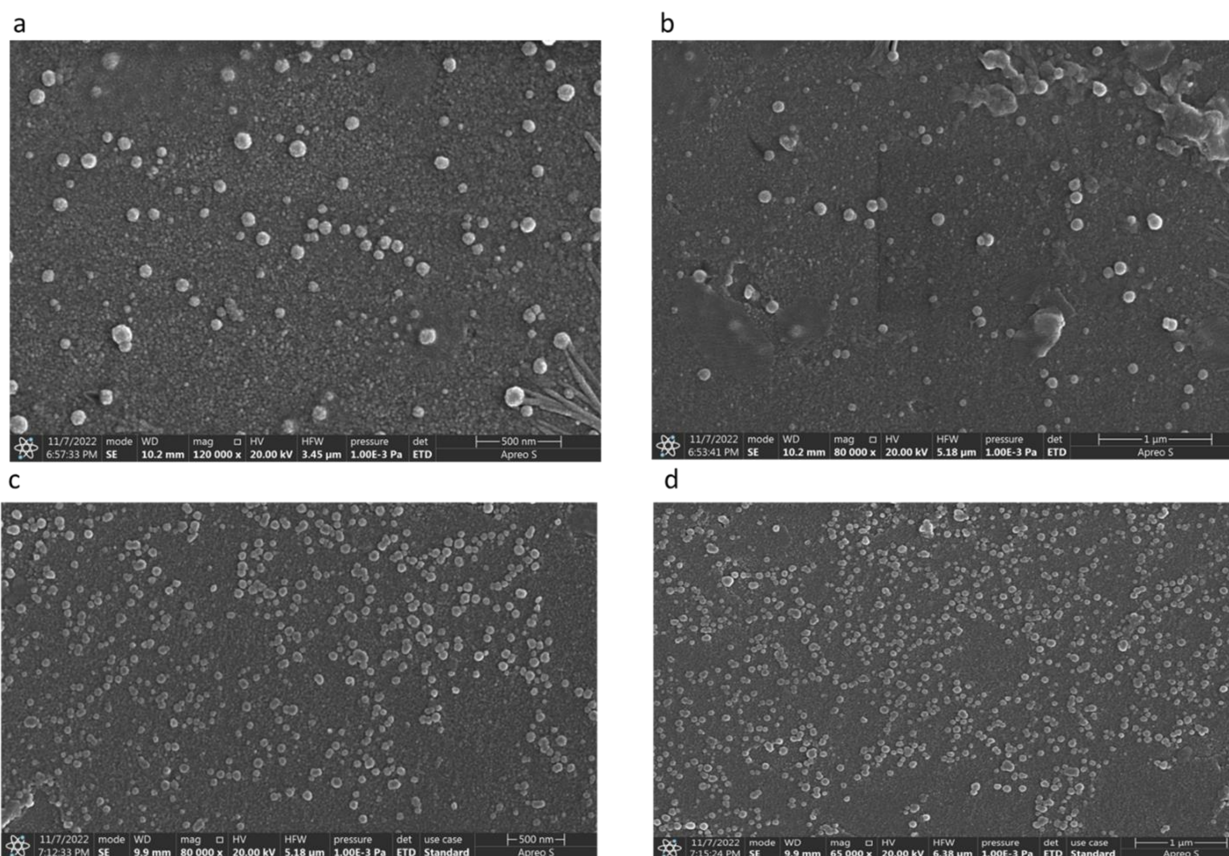


Figure 5.11.2.3. SEM images of (a) C1 @ 120000 x, (b) C1 @ 60000 x; (c) C1-N @ 120000 x, and (d) C1-N @ 60000 x.

5.11.2.4 Total phenolic content of C1 and C1-N

The total phenolic content of C1 and C1-N was estimated using Folin–Ciocalteu reagent (Singleton et al., 1999 and Mahibalan et al., 2013). C1-N was found to contain 34.13 mg/g equivalent of gallic acid. This result was comparable to the estimated phenolic content of C1 (32.33 mg/g equivalent of gallic acid) indicating there was no incompatibility between the active ingredients (HP and LQ) and the excipients used for the preparation of nano-formulation C1-N.

5.11.2.5 Stability studies

The stability of C1-N after 3 months of storage at 5° C, 25° C /60% RH, and 40° C /75% RH is shown in Table 5.11.2.5. In terms of particle size, size distribution, zeta potential and total phenolic content, C1-N was stable at 5° C and 25° C /60% RH. Although at 40° C /75% RH, an increase in PDI and a decrease in the total phenolic content were observed, the difference was minor proving the stability of C1-N (Table 5.11.2.5).

Table 5.11.2.5. Stability studies on C1-N after 3 months

Condition	Size (nm)	PDI	Zeta potential (mV)	Total phenolic content (mg/g equivalent of gallic acid)
5±2 ° C	354±5.08	0.485	-50.1±3.08	32.561
25±2 ° C 60±5% RH	250.6 ±3.07	0.425	-44.5±5.91	34.13
40±2 ° C 75±5% RH	298.2 ±3.08	0.413	-39.6±6.26	24.245

Sample C1, a conventional suspension containing 100+50 mg/kg of HP and LQ, respectively and C1-N, a lipid nano-emulsion containing 100+50 mg/kg of HP and LQ, respectively were administered orally to SD rats and tested under the CIA model. The effect of the formulations was determined by measuring various parameters such as paw volume, haematological parameters, inflammatory and osteoclast markers, ankle-joint histo-architecture, radiography, etc.

5.11.2.6 Effect of C1 and C1-N on paw swelling under collagen-induced arthritis (CIA) model

The difference in paw volume was evaluated using a digital Plethysmometer. A significant (P<0.0001) increase in paw volume was observed at the end of 7th week in disease-induced arthritic rats (1.26±0.17 mL) as compared with rats in the normal control group (0.26±0.04 mL). The increased swelling in the paw could be attributed to fluid accumulation in the synovium of joints of the CIA rats which is due to increased inflammatory cells and synthesis of pro-inflammatory cytokines (Khan et al., 2020). Treatment with C1 and C1-N eventually lead to a significant decrease in paw swelling. Further, it was noticed that C1-N showed a better effect in decreasing paw volume (1.05±0.09 mL) when compared to C1 (1.16±0.17 mL). Interestingly, the effect of C1-N was found to be comparable to that of dexamethasone group (0.95±0.14 mL) (Figure 5.11.2.6).

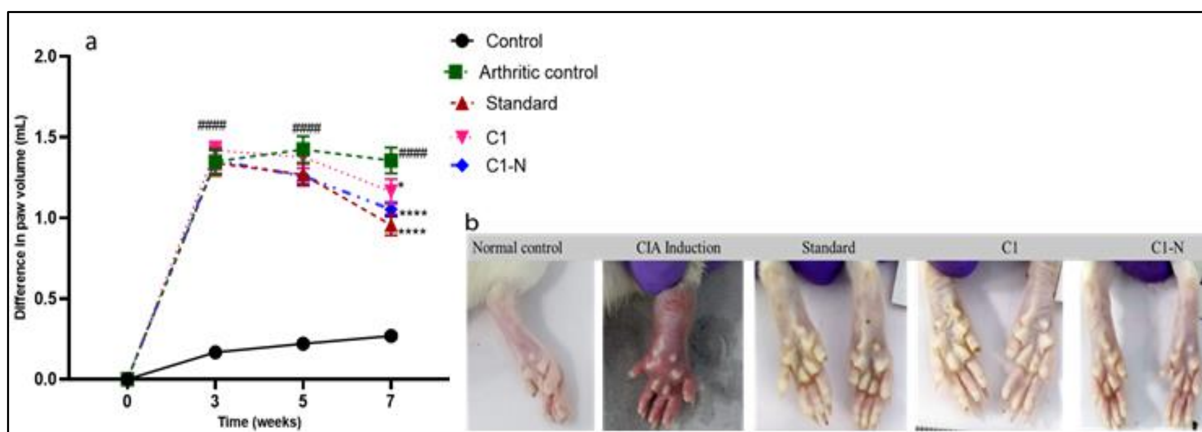


Figure 5.11.2.6. (a) Effect of oral administration of C1, C1-N and standard (0.1 mg/kg) on paw swelling of rats under CIA model. (b) representative images of various groups under the study. All values were presented as mean ± SEM (n=5). *P<0.05, ****P<0.0001 versus the arthritic control group. $^{####}$P<0.0001 versus the control group.

5.11.2.7 Effect of C1 and C1-N on spleen and liver index

Enlargement of liver and spleen is associated with inflammatory disease like RA (Bedoya et al., 2015, Alavala et al., 2020). Thus, for evaluation of pathology of RA spleen index and liver index was done. In our study, we found arthritic rats exhibited an increase in spleen (P<0.0001) and liver index (P<0.01) as compared to control rats which was significantly restored after the treatment of standard C1 and C1-N.

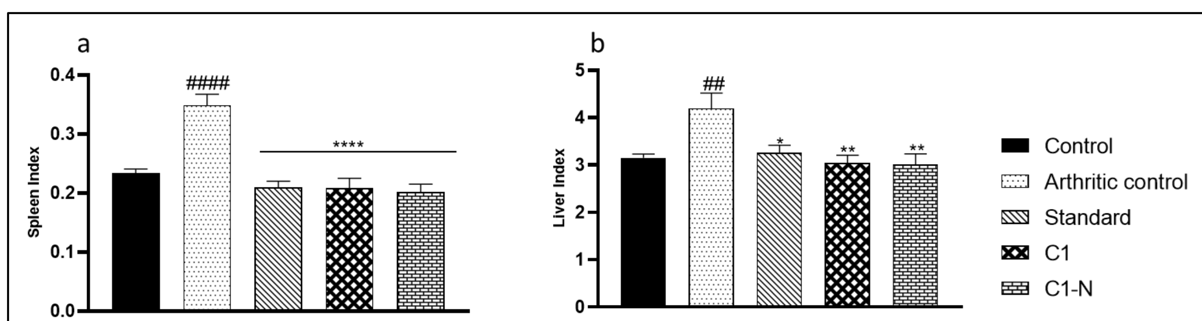


Figure 5.11.2.7. Effect of oral administration of C1, C1-N and standard (0.1 mg/kg) on (a) spleen index (b) Liver index of rats under CIA model. All values were presented as mean ± SEM (n=5). *P<0.05, **P<0.01, ****P<0.0001 versus the arthritic control group. $^{##}$P<0.01, $^{####}$P<0.0001 versus the control group.

5.11.2.8 Effect of C1 and C1-N on haematological parameters

A significant elevation in platelet count, white blood cells and ESR was observed in the blood samples of arthritic control group (1097±148.7×10^3 cells/µL, 11.3±2.68×10^3 cells/µL and 10.9±0.84×10^3 mm/h, respectively) compared to the normal control group (212±83.46×10^3 cells/µL, 10.1±2.36×10^3 cells/µL and 7.8±0.44×10^3 mm/h, respectively) which corroborated

the disease induction. Treatment with standard, C1 and C1-N resulted in a significant (P<0.0001) reduction in white blood cells and ESR, compared to arthritic control group. RF, an important marker in RA was found to be significantly reduced in both C1 and C1-N groups (P<0.01). ESR was significantly reduced in both C1 (P<0.001) and C1-N (P<0.0001) groups compared to arthritic control. However, a significant elevation in haemoglobin (P<0.01) and RBC (P<0.0001) and reduction in platelets was observed only in C1-N group (P<0.01) signifying its better pharmacological effect compared to C1 (Figure 5.11.2.8).

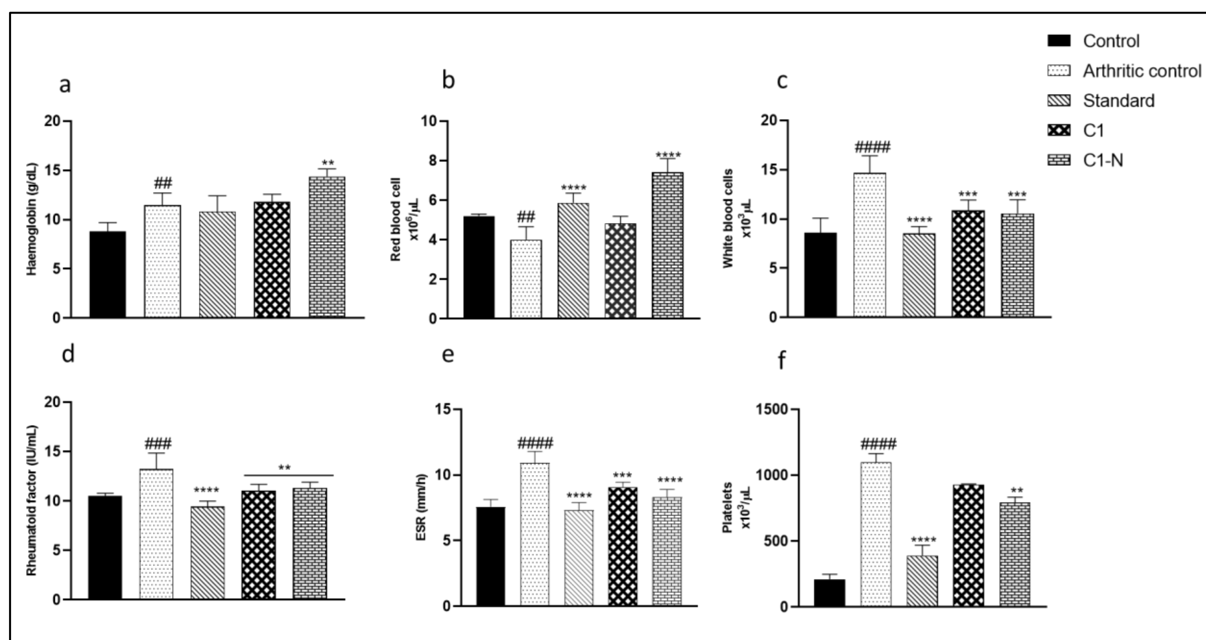


Figure 5.11.2.8. Effect of oral administration of C1, C1-N and standard (0.1 mg/kg) on haematological parameters of all animals of the CIA model on the 7th week. (a) Haemoglobin, (b) Red blood cells, (c) White blood cells (d) Rheumatoid factor, (e) ESR, (f) Platelets. All values were presented as mean ± SEM (n=5). **P<0.01 ***P<0.001, ****P<0.0001 versus arthritic control group. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 versus the control group.

5.11.2.9 Effect of C1 and C1-N on IL-6 and TRAP levels in paw tissues

The concentrations of IL-6 (72766.67± 542.11 pg/mL) and TRAP (2154.04±424 pg/mL) were significantly (P<0.0001) increased in the paw tissues of the disease control group in comparison with the normal control group (IL-6 24044.01 ± 1930.76 pg/mL and TRAP 1255.16± 89.18 pg/mL). C1 and C1-N significantly (P<0.0001) decreased the concentration of IL-6 and TRAP in paw tissue homogenate (Figure 5.11.2.9). The standard drug dexamethasone reduced IL-6 by 54.73% and TRAP by 40.46%. Sample C1 showed a significant inhibitory effect on the release of IL-6 (56.58%) and TRAP (36.63%). C1-N showed excellent inhibition of IL-6 (58.41%) and TRAP (62.21%) (Figure 5.11.2.9).

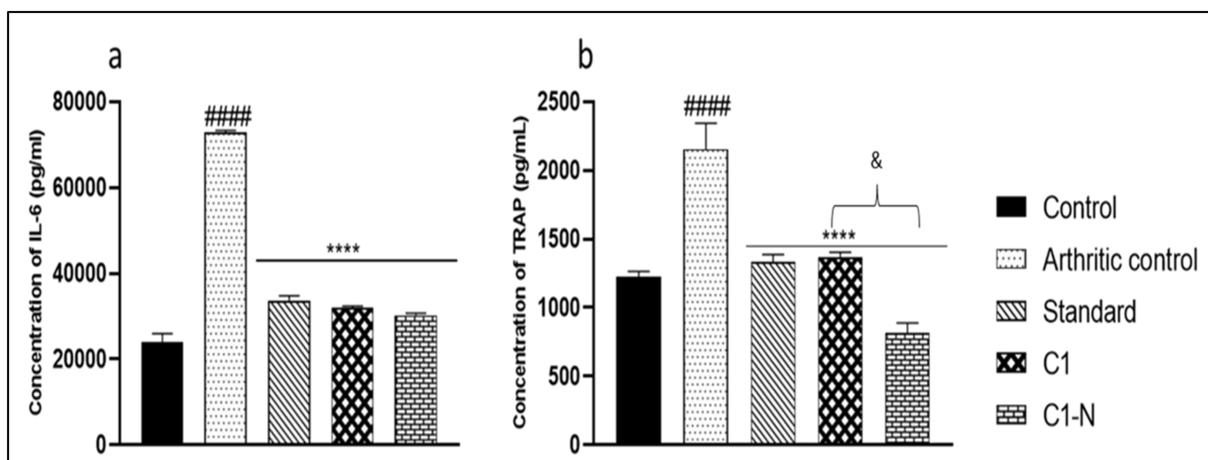


Figure 5.11.2.9. Effect of oral administration of C1, C1-N and standard (0.1 mg/kg) on (a) IL-6 and (b) TRAP levels in paw tissues collected on the 7th week in CIA model. All values were presented as mean ± SEM (n=5). ****P<0.0001 versus the arthritic control group. $^{\&}$P<0.05 versus C1group. $^{\#\#\#\#}$P<0.0001 versus the control group.

5.11.2.10 Effect of C1 and C1-N on the mRNA expression levels of pro-inflammatory cytokines and osteoclast markers

Under RT-PCR analysis, samples of the disease control group showed elevated expression of pro-inflammatory cytokines. C1-N significantly suppressed the gene expression of IL-1β (2-fold) and TNF-α (9-fold) as anticipated. Similarly, the expressions of osteoclast markers like RANK (2-fold) and Cathepsin K (6-fold) were significantly downregulated (P<0.0001) in C1-N treatment. The C1 also showed significant downregulation of mRNA expression levels of TNF-α (3-fold), IL-1β (2-fold), RANK (2-fold) and cathepsin K (2-fold) (Figure 14). C1-N exhibited a better effect than C1 in downregulating the gene expression of cathepsin K and TNF-α which was found to be significant at P<0.001 (Figure 5.11.2.10).

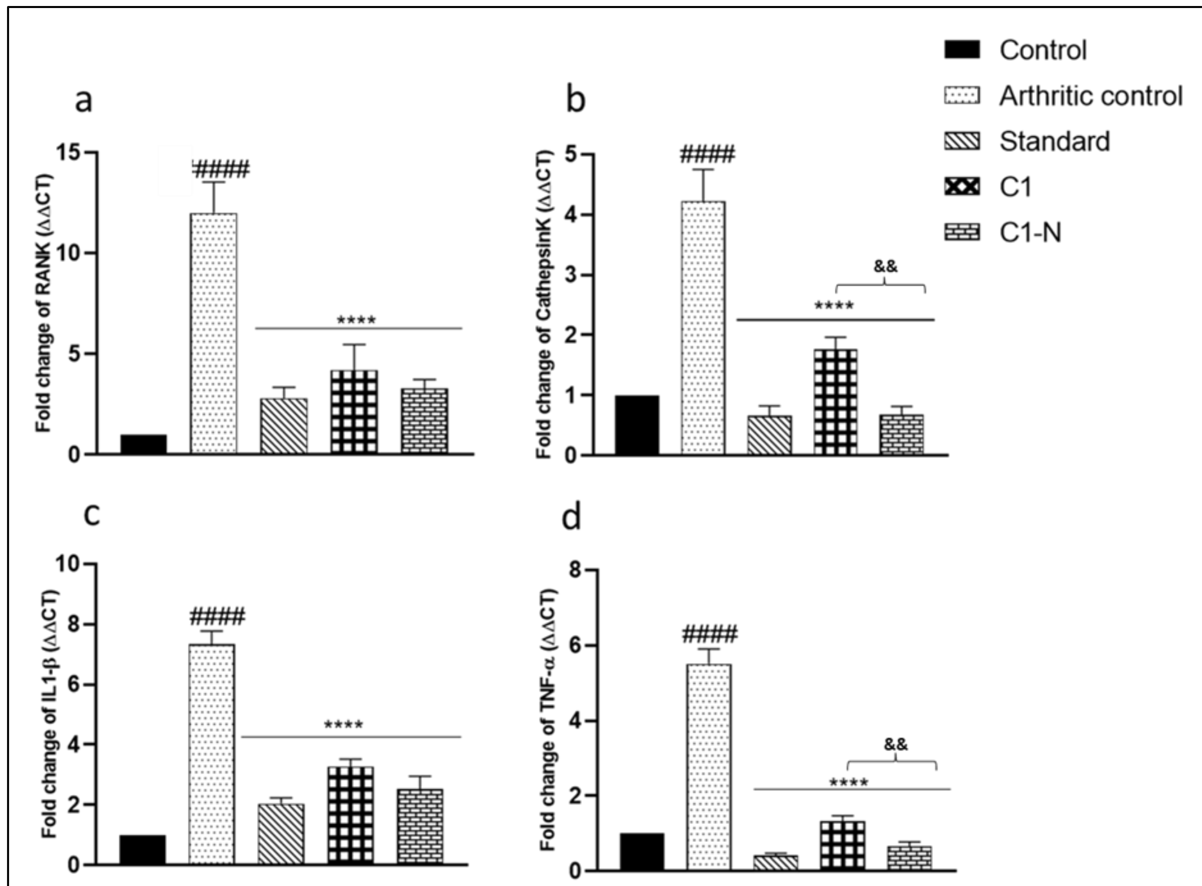


Figure 5.11.2.10. Effect of oral administration of C1, C1-N and standard (0.1 mg/kg) on mRNA expression of (a) RANK (b) Cathepsin K (c) IL-1β and (d) TNF-α in CIA model. All values were presented as mean ± SEM (n=5). ****P<0.0001 versus the arthritic control group. $^{\&}$P<0.05 versus C1group. $^{\#\#\#\#}$P<0.0001 versus the control group.

5.11.2.11 Effect of C1 and C1-N on the ankle-joint histo-architecture

Pathological staining with haematoxylin and eosin revealed the major tissue abnormalities found in the CIA-induced arthritic group. Examination displayed significant hyperplasia, with increased layer and thickening of synovial lining cells, accompanied by a large number of inflammatory cells infiltrating into the ankle joint cavity and cartilage destruction at the joint. A significant reduction in the inflammation of ankle joints with improved synovial lining cell stratification and reduced inflammatory cell infiltration was observed in the samples of C1-administered group. Further, the treatment with C1-N showed better effects in terms of synovial hyperplasia, cell infiltration and cartilage destruction (Figure 5.11.2.11).

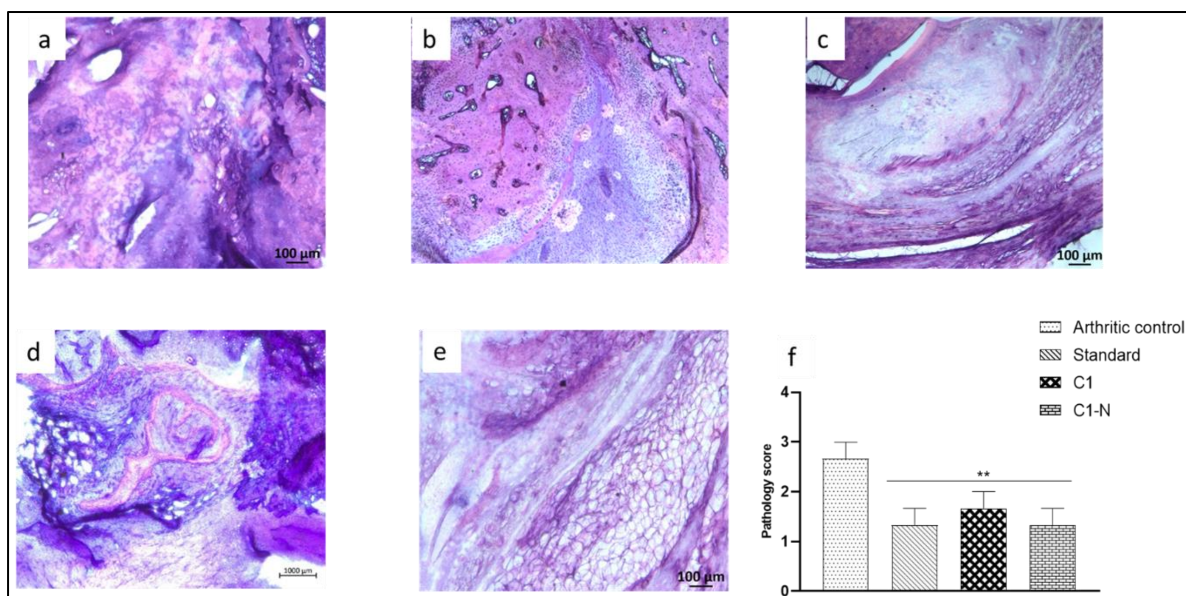


Figure 5.11.2.11. Haematoxylin-Eosin staining of arthritic ankle joints of rats belonging to (a) Control (b) Arthritic control (c) Standard (d) C1 (e) C1-N (f) Pathology score in CIA model. All values were presented as mean ± SEM (n=5). **P<0.01 versus the arthritic control group. Scoring was done on the basis of narrowing of joint space and neutrophil infiltration 0) No change 1) Mild 2) Moderate and 3) Severe.

5.11.2.12 Radiological interpretation

The X-ray images were taken digitally at three different regions namely: paw, ankle joint and knee. The normal control group showed a regular bone matrix with no soft tissue swelling or inflammation whereas the disease control group showed marked soft tissue swelling and markedly diminished joint spaces with periarticular erosive changes along with bone matrix resorption. The dexamethasone group showed residual minimal osteoarthritic changes with no evidence of bone erosion and mild soft tissue swelling with minimal residual joint space narrowing. The C1-administered group resulted in mild soft tissue swelling observed in few animals claiming a mild inflammation. C1-N showed no evidence of bone erosion or soft tissue swelling and no signs of inflammation (Figure 5.11.2.12).

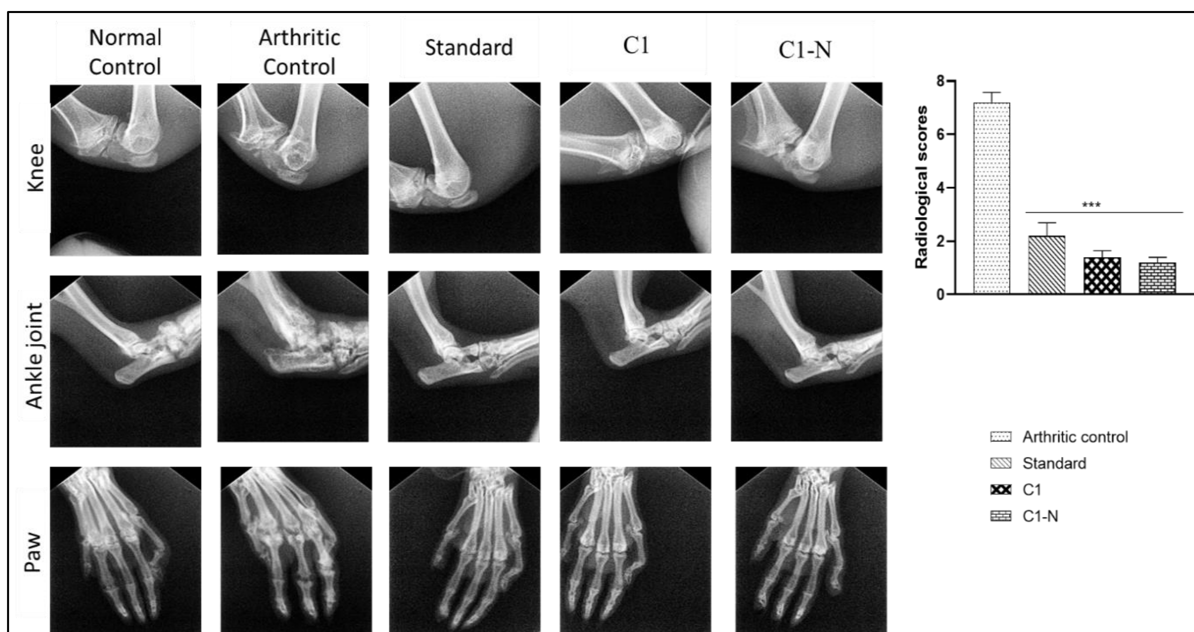


Figure 5.11.2.12. Radiographic changes in joints of rats of all groups of CIA model. All values were presented as mean ± SEM (n=5). ***P<0.001 versus the arthritic control group. Scoring was done based on the (i) degree of soft tissue swelling (0 = normal with no soft tissue swelling, 1 = minimal soft tissue swelling, 2 = mild to moderate soft tissue swelling, 3 = moderate soft tissue swelling, 4 = marked to severe soft tissue swelling) and (ii) degree of joint destruction and periarticular erosive changes (0 = no joint destruction, 1 = minimal joint destruction, 2 = mild to moderate joint destruction, 3 = moderate joint destruction, 4 = marked to severe joint destruction with evident periarticular erosive changes). All scores were assessed by an observer blinded to the experimental groups.

5.11.2.13 Impact of C1 and C1-N on MPO activity and nitric oxide levels in paw tissue

The activity of MPO was measured as an index of neutrophil infiltration and the amount of nitrites was measured as an index of nitrosative stress. C1-N was most effective (50% inhibition) in attenuating the collagen-induced elevation of MPO. The standard drug and C1 also showed significant (P<0.001) control with 48% and 36% inhibition in MPO activity, respectively. In the case of nitrite production also, C1-N was found to be most effective with 54% attenuation compared to standard and C1, which showed 49% and 46%, respectively. C1-N was highly active in inhibiting both NO and MPO activity (Figure 5.11.2.13).

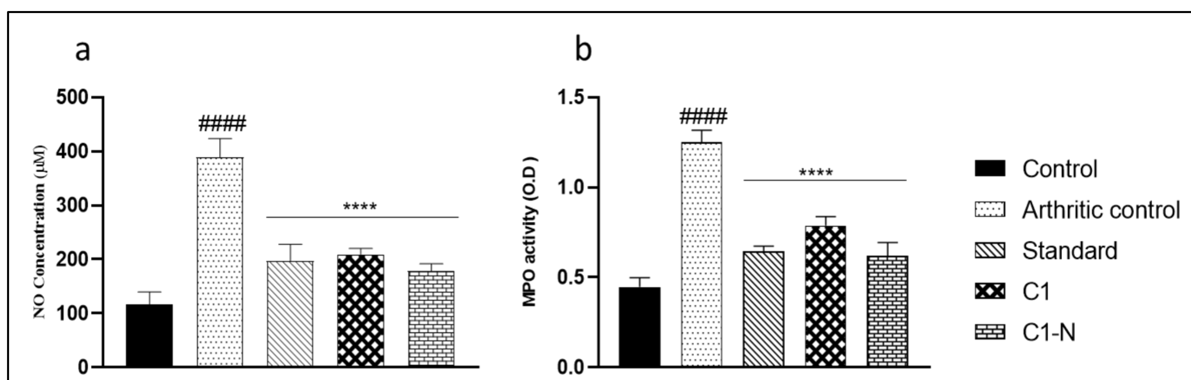


Figure 5.11.2.13. Effect of oral administration of C1, C1-N and standard (0.1 mg/kg) on (a) NO activity and (b) MPO activity in CIA model. All values were presented as mean ± SEM (n=5). ****P<0.0001 versus the arthritic control group. ####P<0.0001 versus the control group.

5.11.2.14 Effect of C1 and C1-N on CD51 and MMP-3

The osteoclast marker CD51 was assessed using a western blot. The relative expression of CD51 with respect to β-actin signified that the treatment with C1 and C1-N inhibited or restricted the expression of CD51. The expression of CD51 was significantly higher (P<0.0001) in arthritic control group. Also, pre-osteoclastic protein marker MMP-3 was analysed and it was found to be elevated in the disease control group. The groups treated with C1 and C1-N significantly lowered the elevated expression levels of MMP-3 (Figure 5.11.2.14).

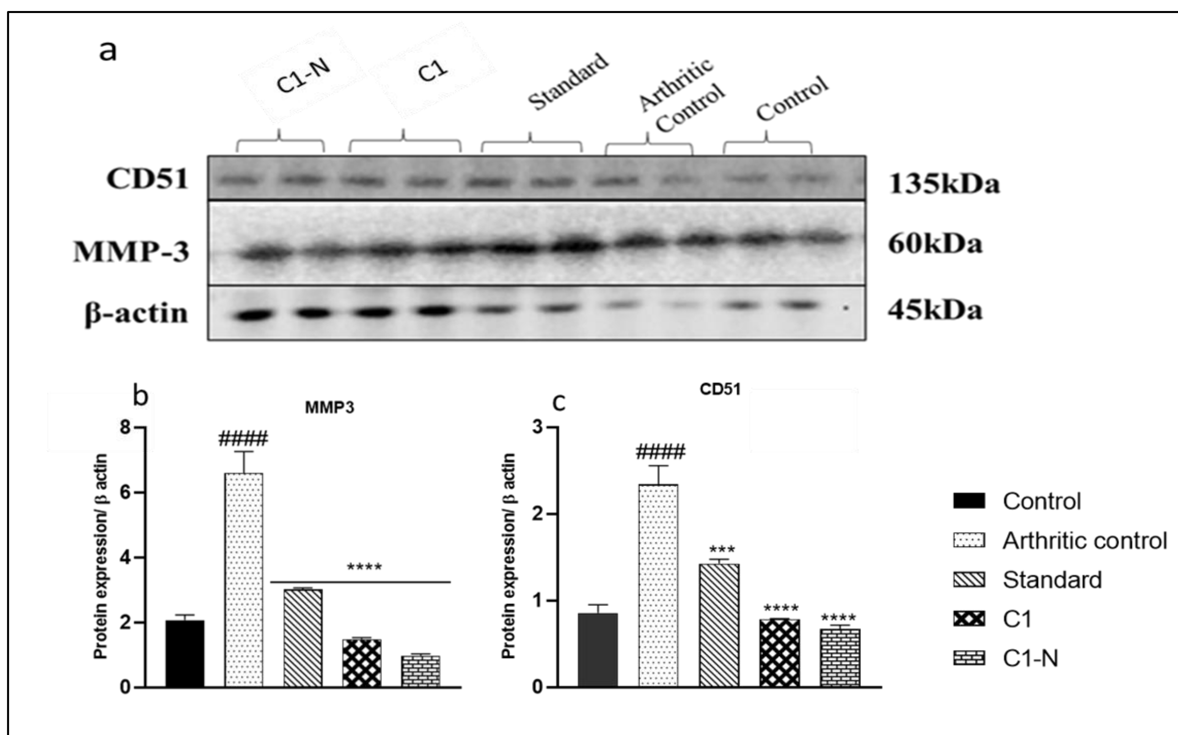


Figure 5.11.2.14. Effect of oral administration of C1, C1-N and standard (0.1 mg/kg) on tissue expression of proteins in CIA model (a) CD51 and MMP-3 (b) Equal loading of protein was confirmed by the levels of β-Actin (c) Densitometry results. All values are presented as mean ±SEM of three independent experiments (n=4). ***P<0.001, ****P<0.001 versus the arthritic control group. $^{####}$P< 0.0001 versus the control group.

The long-term drug regimen of arthritis brings severe side effects by NSAIDS, corticosteroids and DMARDs which have proved an incessant challenge in treating arthritis (Lin et al., 2020). Given these challenges, patients frequently consider herbal medicines to be more efficient and safer for the treatment of RA (Akram et al., 2021). In view of this, the present study evaluated the anti-arthritic efficacy of HP and a combination of it with LQ, considering cartilage and bone degeneration, inflammation and associated stress in adjuvant-induced arthritis. Liquorice extract and glycyrrhizin have been reported to possess immunomodulatory, anti-inflammatory (Yang et al., 2017; Bisht et al., 2022), anti-arthritic activity (Kim et al., 2010; Yamada et al., 2021; Luo et al., 2021) and antiulcer activity (Asha et al., 2013). Apart from these pharmacological activities, liquorice has also been reported to have a sweetening property and its potency is about 50 times that of sucrose (Gupta et al., 2018, Eccles, 2020). Liquorice extract has also been used as a flavouring agent in many cough syrups (Eccles, 2020). Studies on the anti-ulcer activity of liquorice extract have proven its protective effect on the stomach lining from the damage caused by NSAIDs (Kulkarni, 2017). Considering the beneficial medicinal effects and the excellent inherent excipient properties of liquorice extract, C1, a combination of HP and LQ was prepared and tested. This is the first study that has evaluated the anti-arthritic

efficacy of *H. pinifolia*, a widely distributed seagrass in the backwaters of the Bay of Bengal. The evaluation was done by administering (oral) standardized HP (containing 2.92 mg of vanillic acid, 0.76 mg of ethyl protocatechuate, and 4.6 mg of rosemarinic acid/g of dry HP), LQ (containing 2.752 mg/g of glycyrrhyzinic acid), and their combination prepared as suspension (C1) and lipid nano-emulsion (C1-N) in two arthritis models (FCA and CIA).

FCA is an antigen-induced T-cell-mediated aggressive monophasic self-remitting sub-chronic form of arthritis which is commonly acknowledged as one of the most widely used models for studying the anti-inflammatory and anti-rheumatic effects of compounds (De, Kundu and Chatterjee, 2020). On the other hand, the immunopathogenesis of CIA involves both a T-cell and B-cell-specific response to type II collagen (Brand et al., 2007). CIA model shares autoimmunity against self-antigens like RA and results in severe inflammatory arthritis, destruction of cartilage, and bone erosion (De et al., 2020).

Samples HP, LQ and C1 showed remarkable efficacy against arthritis in the FCA-induced arthritis model. All samples essentially normalized the physical changes like hind paw oedema, preventing the associated impairment in mobility, tapering of the bone joint and reduced inflammation. The spleen is an important organ in the formation of immune cells and antibodies (Vigneshwaran et al., 2023). The increased spleen weight in arthritic rats due to the infiltration of inflammatory cells was reduced in treatment groups demonstrating the anti-arthritic effect of oral administration of HP, LQ and C1. Previous research suggests that during the arthritic condition, the level of platelets was reduced and WBC and ESR levels were increased (Aiyalu et al., 2016; Mahdi et al., 2018). Treatment with HP significantly restored the platelets, ESR and WBC levels suggesting the protective effect of HP against adjuvant arthritis-related inflammation. LQ and C1 could significantly restore the levels of ESR and WBC, respectively indicating their potential anti-arthritic effect. These observations were found in line with the recent study conducted by Alavala et al. (2020).

Studies have proven the important role of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the pathogenesis of RA (Liu et al., 2021). The overexpression of these cytokines develops inflammatory and destructive peripheral arthritis characterized by many hallmarks of RA such as joint inflammation, bone erosion, cartilage destruction, etc., (Aletaha and Smolen, 2018). TNF-α, IL-1β and IL-6 stimulate synoviocyte proliferation and activate RANKL expression, which promotes osteoclastogenesis and MMP synthesis, which contributes to cartilage breakdown (Mrid et al., 2022). The crucial proof of concept for a pivotal role for TNF-

α, IL-1β and IL-6 in RA pathogenesis is provided by a clinical trial study in which the TNF inhibitor (infliximab), anti-IL-6R antibody (Tocilizumab) and anti-IL-1 therapy (anakinra) suppressed disease activity and erosive progression in RA patients. (Noack and Miossec, 2017). In the current investigation, the results of ELISA and RT-PCR assay revealed that the treatment with HP, LQ and C1 significantly reduced the cytokine levels in paw tissue homogenate. The osteoclast markers for bone and cartilage destruction like RANK - produced by cytokine-activated fibroblasts in combination with TNF-α and IL-6 (Aletaha and Smolen, 2018), TRAP - released upon chondrocyte death caused by cartilage degradation (Seol et al., 2009) and cathepsin K - a proteolytic enzyme which gets activated at acidic pH and can potentially lead to bone and cartilage destruction (Behl et al., 2022) were evaluated in treatment groups. The results of radiological scanning exhibited a significant reduction in the joint deterioration by treating the animals with HP, LQ and C1 in FCA-induced arthritic rats. Histological examinations revealed the FCA-induced arthritic rats' ankle joints were aberrant, with considerable synovial hyperplasia and inflammation, as well as widespread erosive alterations in the bone and cartilage. Rats treated with C1 notably showed a better effect than the individual treatments with HP and LQ.

The overexpression of CD51 has been associated with the aggressive behaviour of the synovial fibroblasts, leading to the destruction of cartilage and bone in the joints (Treese et al., 2008). Synoviocytes in RA produce MMP-3 and result in the irreversible destruction of cartilage, tendon and bone. The downregulation of both CD51 and MMP-3 suggested an ameliorative effect of HP, LQ and C1. A recent study by Chuang et al, (2023) found that reducing the levels of macrophage-related inflammatory cytokines and MMPs alleviates bone erosion in the ankle joint, constituting a potential therapeutic strategy for RA.

Thus, the results of the FCA experiment proved the strong anti-arthritic effect of HP, LQ and C1. Further, it was observed that sample C1 showed a better effect in downregulating mRNA expression of IL-6 and TRAP compared to the individual administration of HP and LQ, which widened the scope for further exploration.

Nanonisation of herbal extracts often increases their bioavailability by improving the dissolving velocity, wetting, particle surface area, and saturation solubility (Gupta, Kesarla, and Omri, A, 2013; Jahan et al., 2016). Hence, in order to improve the efficacy of the combination of HP and LQ (which was initially prepared as a conventional suspension C1), attempts were made to prepare a lipid nano-emulsion (C1-N). C1-N was characterized by

testing various properties in the aspect of formulation development. The particle size is an essential parameter in quality assurance as the physical stability of nanoparticle dispersion is dependent on particle size and distribution. SEM analysis was utilized to assess the uniformity of size, shape, and physical stability features of aggregation or irregularity. The size of C1-N ranged from 292 to 400 nm, and the PDI was 0.3 with more than 90% of particles being in the nanometre range and having a characteristic spherical form, indicating a homogenous nano-formulation which was reproducible. The total phenolic content of C1-N was similar to that of C1 indicating there was no incompatibility in the formulation. Further, C1-N was found to have better physical properties in terms of size, PDI and morphology when compared to the conventionally prepared suspension C1. The formulation C1-N was found to be stable for 3 months when subjected to accelerated temperature and humidity in the stability chamber.

Further, investigation of the anti-rheumatoid arthritis effect of C1 and C1-N through oral administration to rats under CIA model proved the positive effect i.e., both the formulations reduced the progression of arthritis by alleviating the paw swelling. However, there was a significant difference between the arthritis-mitigating ability of C1 and C1-N. Sample C1-N was more effective in reducing the paw swelling and the results were comparable to that of the standard. The spleen and liver index of both C1 and C1-N groups were significantly reduced compared to the arthritic control group. During the arthritic condition, the haematological levels of red blood cells, white blood cells, haemoglobin and platelets were found to be altered (Li, Kakkar and Wang, 2018) which were resumed to the normal level when treated with C1 and C1-N treatment. The significantly low level of ESR in the C1-, C1-N- and standard drug-treated animals, showed their anti-inflammatory potential. The ESR is an indirect means of measuring inflammation in the body. Because of protein production in inflammation, erythrocytes move together, stack up in a clump, become denser, and settle faster, increasing the erythrocyte sedimentation rate. ESR is raised in a variety of stressful situations, cell necrosis, and inflammation (Mbiantcha et al., 2017). The amount of RF in the serum correlates with the amount of serum IgM, and the amount of RF in the serum has a direct link with the development of inflammation (Aloke et al., 2021). RF is a significant serological clinical biomarker of inflammatory arthritis (Kaur et al., 2023). The considerable increase in the concentration of RF in the arthritic rats was significantly reduced by C1 and C1-N treatment in this study. This demonstrated that the formulations had the anti-arthritic effect, and the mechanism might be linked to the production of autoantibodies against the Fc fragment, thereby protecting cartilage from degradation.

Furthermore, C1-N showed remarkable inhibition of TRAP (62.21%) compared to C1 (36.63%) highlighting the better effect of the nano-formulation. C1-N showed a similar trend in downregulating the mRNA expression of cathepsin K (6-fold) and TNF-α (9-fold) establishing the improved efficacy of the formulation. A noteworthy feature was that the activity of C1-N appeared very similar to that of dexamethasone-treated animals. The histopathological examination of the ankle joint sections of arthritis-induced rats stained with H&E demonstrated them to be abnormal, as evidenced by pronounced synovial hyperplasia and inflammation as well as extensive erosive changes in the bone and cartilage. Administration of C1 and C1-N reduced the joint bone and cartilage erosion with C1-N showing a better ameliorating effect over the synovial inflammation. The radiological results claimed a significant reduction of joint destruction in treatment groups, further corroborating the previous discussions.

The release of MPO is one of the earliest consequences of neutrophil infiltration at sites of inflammation such as joints. This could be attributed to the interaction between neutrophils and macrophages that encourages a chronic inflammatory status. Thus, in the inflamed synovium of patients with RA, MPO in circulation and/or MPO released from the locally damaged neutrophils might recruit more leukocytes and thus aggravate inflammatory infiltration and tissue destruction (Wang et al., 2014). Another recently published study supported the role of MPO in mediating joint inflammation and damage in an experimental model of arthritis (Odobasic et al., 2014). In our present study, it was found that both C1 and C1-N significantly reduced the levels of NO and MPO establishing the protective effect of treatment. Besides, C1-N showed a better effect in both CD51 and MMP-3. The study disclosed HP, C1 and C1-N as potentially active compositions useful for the treatment of RA. This was validated based on the positive effect exhibited by them on various inflammatory and arthritic parameters under the FCA and CIA rat models.

5.11.3 In-vivo evaluation of anti-arthritic activity of DEX-H, C2 and C3 in Adjuvant-induced arthritis (AIA)

Glucocorticoids (GCs) having potent immunosuppressive effects are widely used in the management of RA and are also classified as disease-modifying anti-rheumatic drugs. GCs such as prednisone, hydrocortisone, prednisolone, and dexamethasone have greater potency and efficacy than NSAIDs and are recommended for treatment by EULAR and ACR from the beginning of the diagnosis of RA (American College of Rheumatology, 2002). They delay the

radiologic progression in early disease stages by general suppression of gene expression (Littlejohn and Monard, 2018). GCs increase the expression of lipocortin-1, which inhibits the enzymatic activity of phospholipase A2 and the production of arachidonic acid. The transcriptional products of glucocorticoids inhibit the activities of NF-kB and activator protein 1 that are responsible for the upregulation of various proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1β) (Maity et al., 2021; Paolino et al., 2017). The long-term usage of these drugs can cause the development of a number of undesirable illnesses, including diabetes, weight gain, cushingoid symptoms, hypertension, peptic ulcers, and avascular necrosis of the bones as side effects (Ingawaleet al., 2020; Sarzi-Puttini et al., 2019). Since the side effects of glucocorticoids are dose-dependent, the combination of a less-dose of glucocorticoid and an immunosuppressive herb/natural substance could serve as a better therapeutic agent with significantly fewer side effects resulting in steroid-sparing effects. This hypothesis has been proved in the present study taking dexamethasone (DEX) as a prototype. DEX is a well-established GC prescribed for the treatment of RA, particularly to delay the progression of the disease. The established anti-inflammatory activity of *H. pinifolia*, compositions C2 and C3 formed the basis of its selection and evaluation for anti-arthritic activity.

5.11.3.1 Effect of DEX, DEX-H, C2 and C3 on the paw volume on FCA-induced arthritic rats

After immunization of the rat's right hind paw with FCA, a significant ($P<0.0001$) increase in paw volume in the arthritis control group was observed. On the day of the start of treatment i.e. the 14th day, the difference in the paw volume (DPV) of the arthritic control animals was 1.35 mL and that of the control group was 0.1 mL. After 14 days of treatment with test samples, the paw volume was recorded and analysed (Figure 5.11.3.1). The steroid DEX-treated group exhibited a significant reduction in the paw volume (0.189 mL) compared to the arthritic control group (1.890 mL). Treatment with DEX-H, C2 and C3 also showed a significant decrease in paw oedema, showing DPV of 0.26, 0.20 and 0.31 mL, respectively.

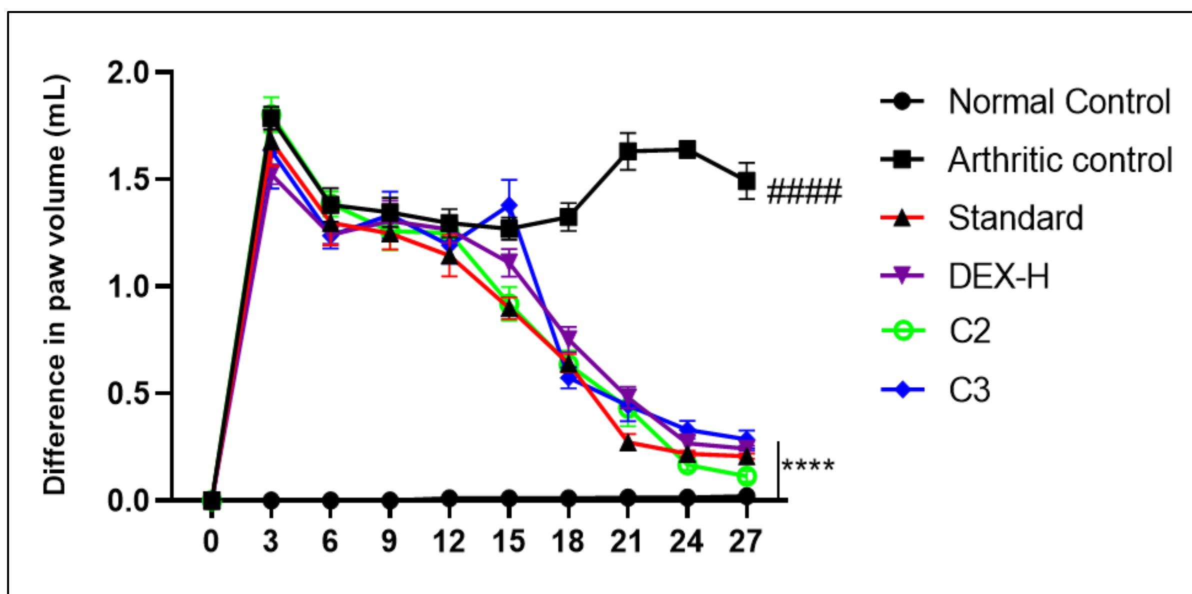


Figure 5.11.3.1. Effect of DEX-H, C2 and C3 and standard DEX (0.1mg/kg body weight) on paw volume in FCA model. All values were presented as mean ± SEM (n=8). ****P<0.0001 versus arthritic control group versus arthritic control group. ####P<0.0001 versus the normal control group.

5.11.3.2 Effect of DEX, DEX-H, C2 and C3 on spleen index on FCA-induced arthritic rats

The relative weight of the spleen per 100 g body weight was found to be significantly (P < 0.0001) increased in the arthritic control group compared to the control group (Figure 5.11.3.2.). The arthritic rats which were administered with DEX, DEX-H and C2 showed a significant decrease in the spleen index (P<0.0001). C3 also caused a decrease in spleen index (P<0.001), however, the effect was lesser than that of other groups. Interestingly, the effect shown by DEX-H and C2 were higher than DEX.

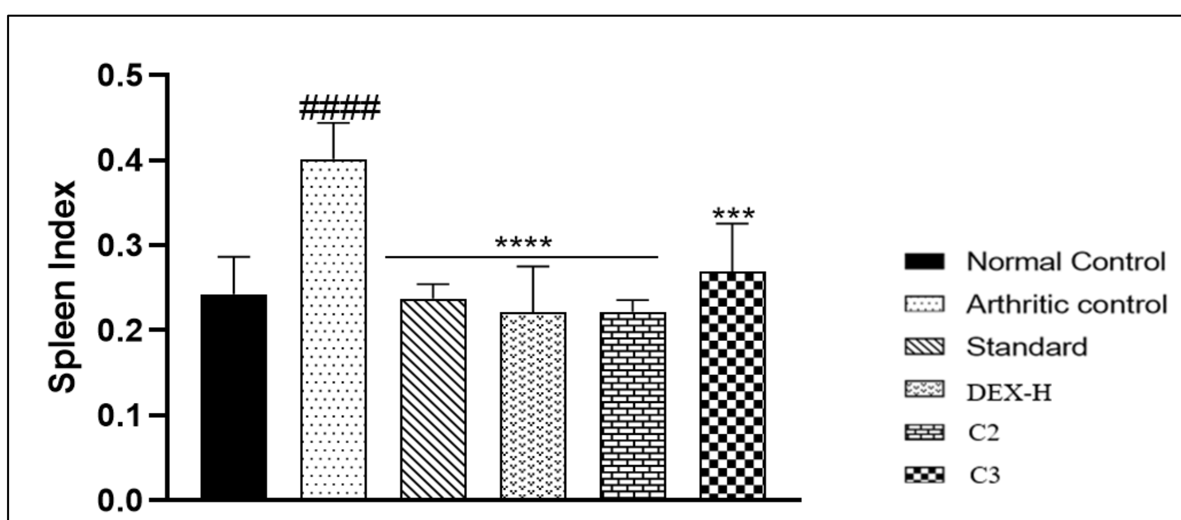


Figure 5.11.3.2. Effect of DEX-H, C2 and C3 and standard DEX (0.1mg/kg body weight) on spleen index in FCA model. All values were presented as mean ± SEM (n=8). ****P<0.0001 versus arthritic control group, ***P<0.001 versus arthritic control group. ####P<0.0001 versus the normal control group.

5.11.3.3 Effect of DEX, DEX-H, C2 and C3 on IL-6 and TRAP levels in paw tissues

Treatment with DEX-H, C2 and C3 significantly (P<0.0001) decreased the concentration of IL-6 and TRAP in paw tissue homogenate (Figure 5.11.3.3). The DEX-H and C3 showed a similar effect showing a reduction of IL-6 levels by 48.6% and 48.8%, respectively. The ameliorating effect of C2 (51.86%) was more than all other treatments including DEX (50.6%). Further, DEX and DEX-H treated groups showed a marked decrease in the levels of TRAP (49.02% and 39.03%, respectively). Both C2 and C3 could significantly inhibit (P<0.0001) TRAP levels by 35.81 and 37.74%, respectively.

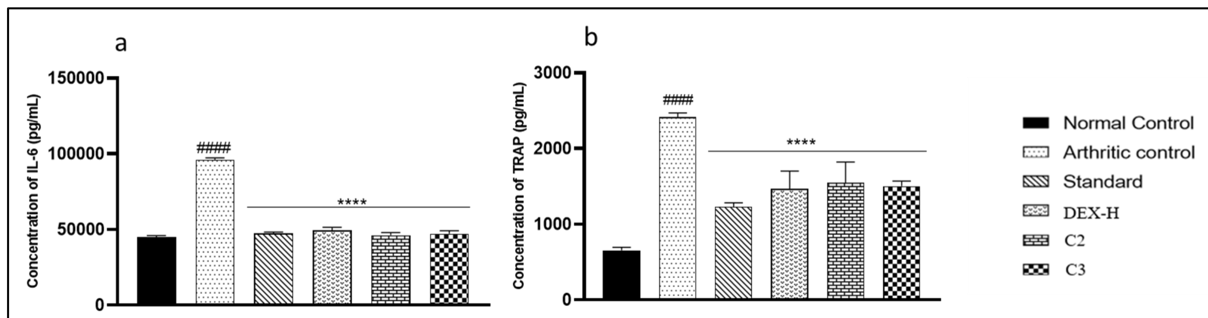


Figure 5.11.3.3. Effect of DEX-H, C2 and C3 and standard DEX (0.1mg/kg body weight) on IL-6 (a) and TRAP levels (b) in paw tissues on day 28th under FCA model. All values were presented as mean ± SEM (n=8). ****P<0.0001 versus arthritic control group. $^{####}$P<0.0001 versus the control group.

5.11.3.4 Effect of DEX-H, C2 and C3 on the mRNA expression levels of pro-inflammatory cytokines and osteoclast markers

All three treatment groups significantly reduced the mRNA expression of cytokines and osteoclast markers. However, a marked downregulation of TNF-α and IL-1β was recorded with rats treated with DEX and C2. The treatment with DEX downregulated mRNA expression of TNF-α by 5.5-folds and C2 showed marked downregulation of IL-1β (5.8-folds) which was higher than DEX-H (4.9-fold) and DEX (3.7-fold) group. Similarly, the expression of osteoclast markers like RANK, and Cathepsin K was significantly (P<0.0001) reduced by all the treatment groups. DEX downregulated mRNA expression of RANK by 6.3-fold and DEX-H downregulated cathepsin K by 6.3-fold. While C2 and C3 significantly reduced cathepsin K expression by 3.3 and 4.2-fold, respectively, they downregulated RANK by 2.4 and 2.7-fold, respectively.

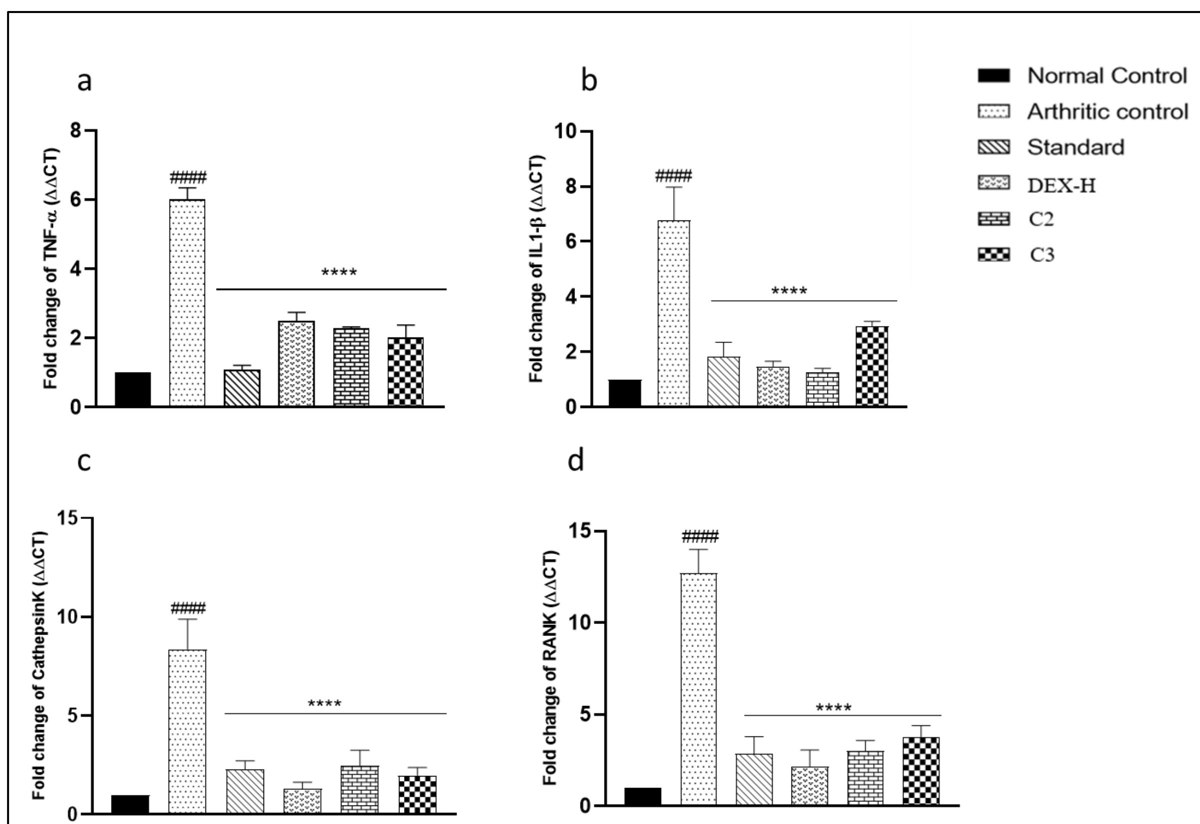


Figure 5.11.3.4. Effect of DEX-H, C2 and C3 and standard DEX (0.1mg/kg body weight) on mRNA expression levels of (a) TNF-α (b) IL-1β (c) Cathepsin K (d) RANK in rat paw tissues collected on day 28 of study under FCA model. All values were presented as mean ± SEM (n=8). ****P< 0.0001 versus arthritic control group. $^{\#\#\#\#}$P < 0.0001 versus the control group.

5.11.3.5 Effect DEX-H, C2 and C3 on ankle-joint histo-architecture

Microscopical observation of sections revealed a normal joint structure and space, without erosion of cartilage and bone in the animals of the normal group (Figure 5.11.3.5). In the arthritic control group, most of the cartilage and bone tissues were damaged by erosion and a large number of inflammatory cells infiltrating into the ankle joint cavity were seen. It was interesting to note that the treatment with DEX-H significantly reduced the inflammation of ankle joints with improved synovial lining cell stratification and reduced inflammatory cell infiltration. The treatment with C2 showed better effects in terms of synovial hyperplasia, cell infiltration and cartilage destruction. In the case of samples of C3 group, the joint inflammation was found to be reduced compared with that of the arthritic group (Figure 5.11.3.5).

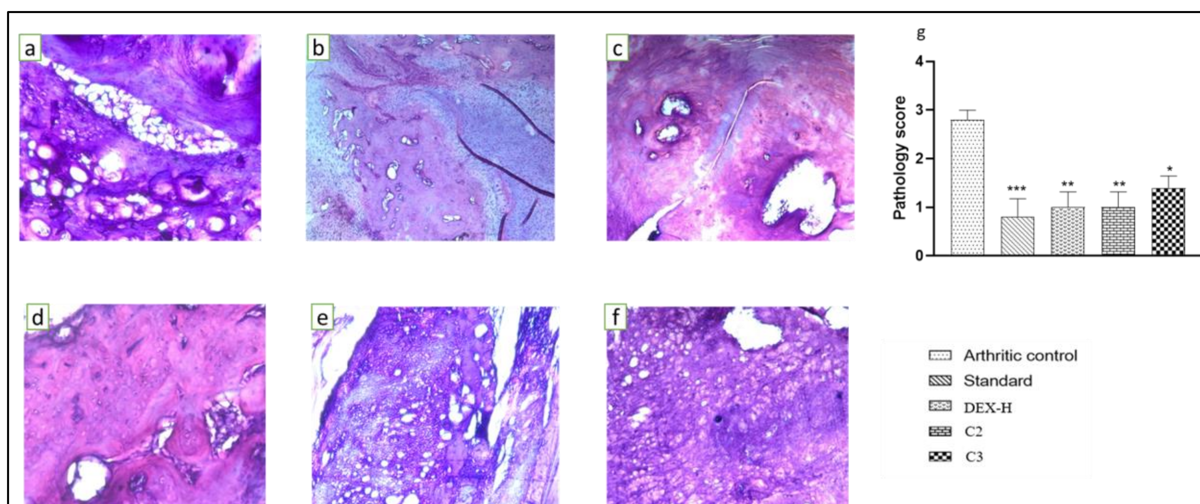
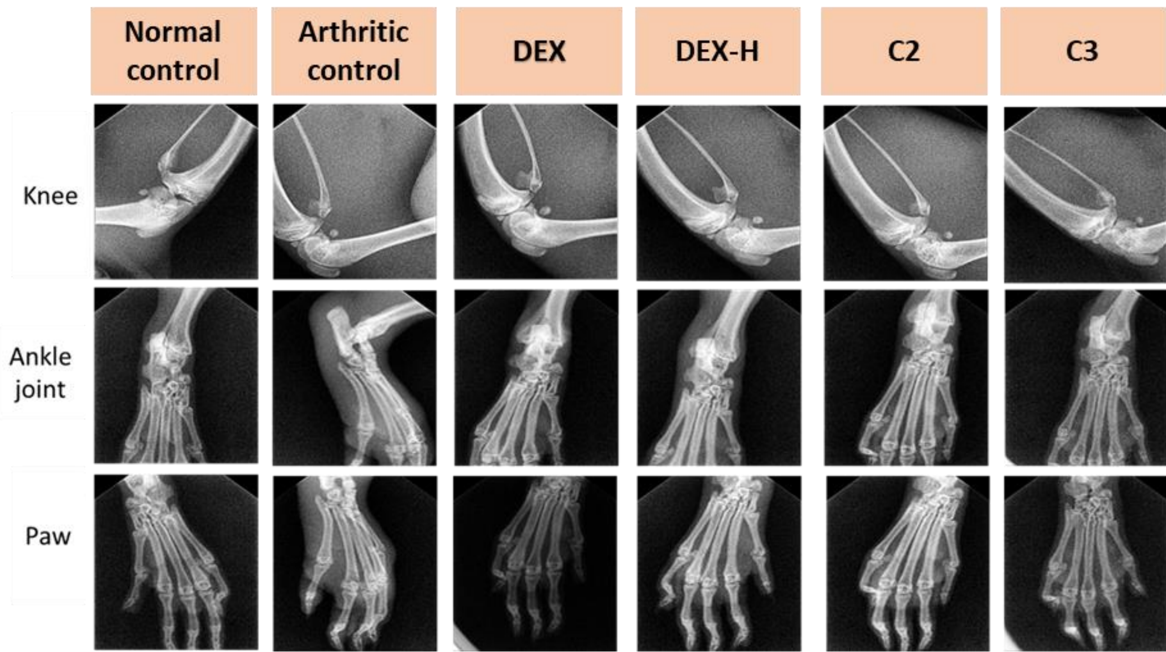


Figure 5.11.3.5. Haematoxylin-Eosin staining of arthritic ankle joints of rats belonging to (a) Control (b) Arthritic control (c) DEX (d) DEX-H (e) C2 (f) C3 groups under FCA model. Graph (g) presents the pathology score. All values were presented as mean ± SEM (n=5). *P<0.05, **P<0.01 ***P<0.001 versus arthritic control group. Scoring was done on the basis of narrowing of joint space and neutrophil infiltration 0) No change 1) Mild 2) Moderate and 3) Severe.

5.11.3.6 Radiological interpretation

The radiological results showed normal bone matrix in healthy control rats with no pathological changes whereas the adjuvant-induced arthritic group showed persistent soft tissue swelling mostly in the region of the ankle joint, with loss of joint space at the ankle and development of erosive/cystic changes of ankle joints. Early osteophyte formation was also present at this time. Periarticular erosive changes were noted along with bone matrix resorption. DEX administration resulted in residual minimal osteoarthritic changes with no evidence of bone erosion, nor significant soft tissue swelling with minimal residual joint space narrowing. DEX-H, C2 and C3 treated groups showed a mild inflammation with less reduction in joint space and less soft tissue swelling (Figure 5.11.3.6).



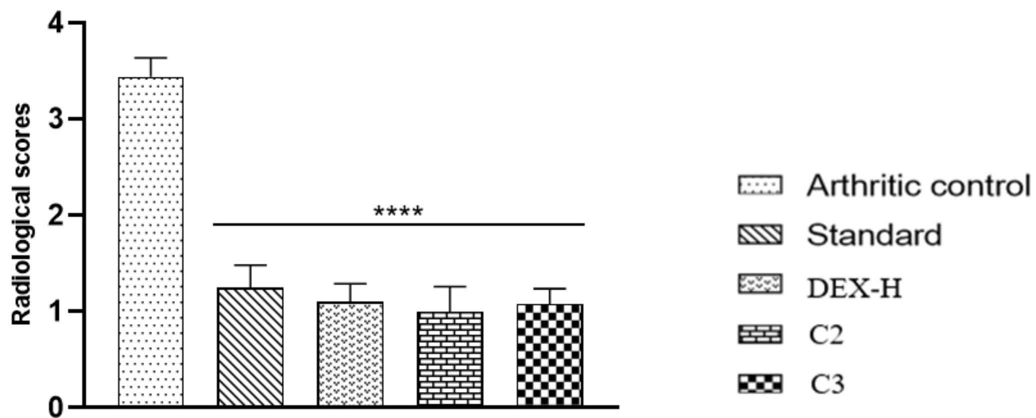


Figure 5.11.3.6. Radiographic changes in the joints of rats of all groups under FCA model. All values were presented as mean ± SEM (n=5). ****P< 0.0001 versus the arthritic control group. Scoring was done based on the (i) degree of soft tissue swelling (0 = normal with no soft tissue swelling, 1 = minimal soft tissue swelling, 2 = mild to moderate soft tissue swelling, 3 = moderate soft tissue swelling, 4 = marked to severe soft tissue swelling) and (ii) degree of joint destruction and periarticular erosive changes (0 = no joint destruction, 1 = minimal joint destruction, 2 = mild to moderate joint destruction, 3 = moderate joint destruction, 4 = marked to severe joint destruction with evident periarticular erosive changes). All scores were assessed by an observer blinded to the experimental groups.

5.11.3.7 Effect of DEX, DEX-H, C2 and C3 on myeloperoxidase activity and nitric oxide levels in paw tissue

A significant attenuation ($P<0.0001$) of the FCA-induced MPO and NO levels was observed in the samples of treatment groups. C2 exhibited 36.06% inhibition of paw tissue NO levels, which was comparable to the DEX group (35.7%). The DEX-H and C3 treatment showed similar effects in inhibiting NO production (26% and 25.20%, respectively). C2 showed a notable inhibition effect (58.68%) against MPO activity compared to DEX (50.99%) and DEX-H (38.26%) (Figure 5.11.3.7).

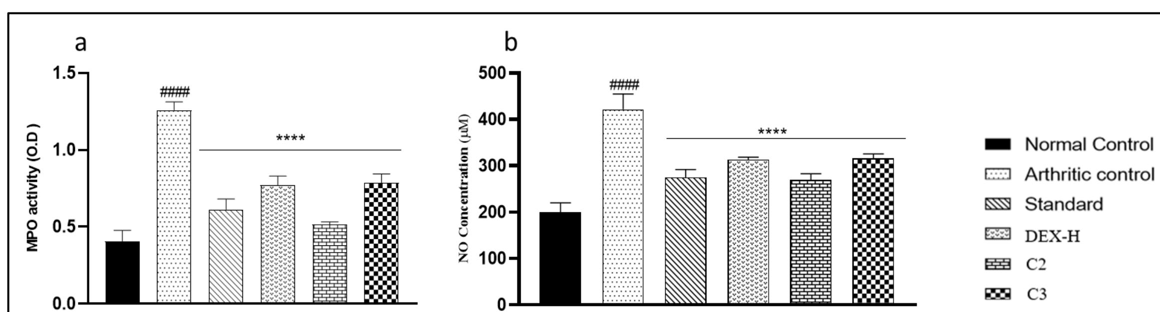


Figure 5.11.3.7. Effect of DEX-H, C2 and C3 and standard DEX (0.1mg/kg body weight) in attenuating NO and MPO levels in paw tissues under FCA model. All values were presented as mean ± SEM (n=8). ****$P< 0.0001$ versus the arthritic control group. ####$P < 0.0001$ versus the control group.

5.11.3.8 Rota rod test

The functional assessments of experimental rats were performed by rota rod analysis. The functional recovery was measured in arthritic control, DEX, DEX-H, C2 and C3 treated arthritic rats on the 27th day of arthritis induction. A significant reduction ($P<0.0001$) in rota rod activity was observed in arthritic control rats (19.6 sec) when compared with their normal control counterparts (125.6 sec). A significant difference ($P<0.0001$) in rota rod activity was recorded in DEX-H treated animals (89 sec), C2 (74.6 sec) and C3 (55.4 sec). DEX (0.1 mg/kg) treated arthritic rats, however, showed a significant difference in their rota rod activity (129 sec) when compared with that of their arthritic control counterparts.

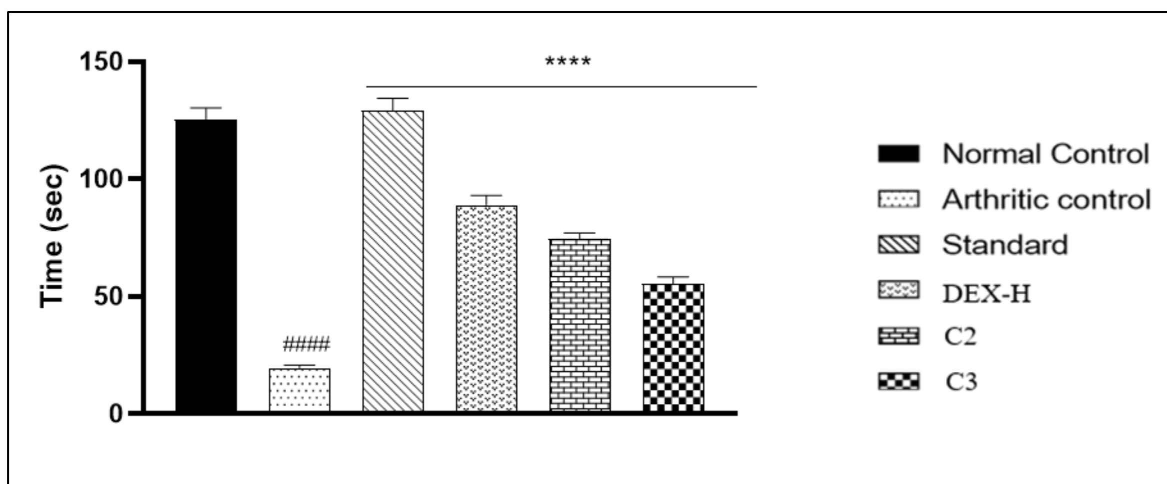


Figure 5.11.3.8. Effect of DEX-H, C2 and C3 and standard DEX (0.1mg/kg body weight) on motor activity using rota rod test under FCA model. All values were presented as mean ± SEM (n=5). ****P< 0.0001 versus arthritic control group. $^{\#\#\#\#}$P < 0.0001 versus the control group.

5.11.3.9 Western Blot analysis

The osteoclast marker CD51 was assessed in the paw tissue samples of all groups using a western blot. The relative expression of CD51 with respect to β-actin signified that the treatment with DEX, DEX-H and C2 inhibited the expression of CD51 and the effect was higher than C3. The expression of CD51 was significantly higher (P<0.0001) in the arthritic control group. Also, the pre-osteoclastic protein marker MMP-3 was analysed and it was found to be elevated in the disease control group (P<0.0001). The groups treated with DEX, DEX-H, C2 and C3 significantly (P<0.0001) lowered the elevated expression levels of MMP-3.

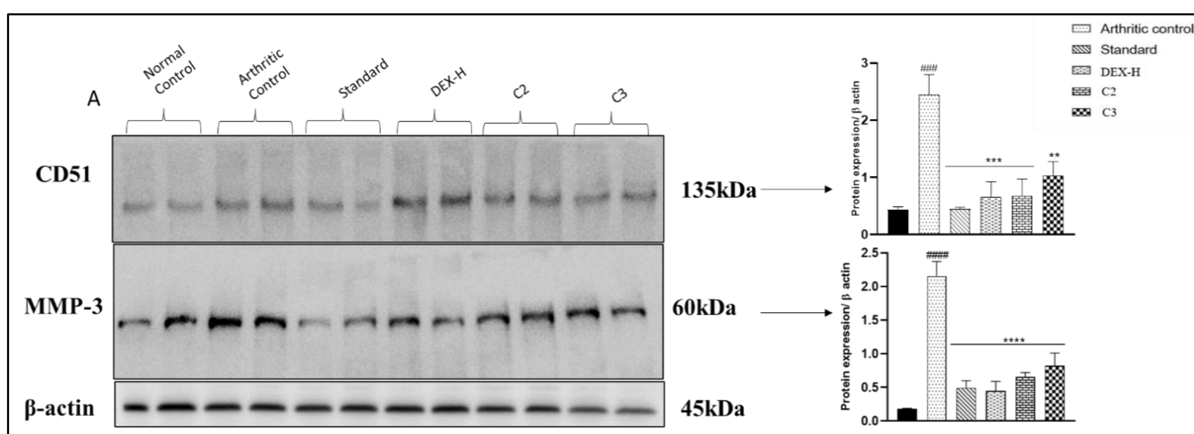


Figure 5.11.3.9. Effect of DEX-H, C2 and C3 and standard DEX (0.1mg/kg body weight) on the tissue expression of proteins such as CD51 and MMP-3 in experimental FCA animals. Equal loading of protein was confirmed by the levels of β-Actin. All values were presented as mean ± SEM (n=4). **P< 0.01 ***P< 0.001, ****P< 0.0001 versus arthritic control group.

This study was performed to explore natural herbs/compounds that have a steroid-sparing effect or can act as an alternative medicine for the treatment of arthritis by either reducing the dosage of steroids or replacing them. The evaluation of the anti-arthritic activity of dexamethasone at a half-dose combined with HP (DEX-H) along with two novel compositions C2 and C3 was endeavoured.

The three novel combinations DEX-H, C2 and C3 containing the standardised extract of HP were prepared as oral suspensions and evaluated for their anti-arthritic effect by administering them to arthritis induced rats in FCA model. The results revealed interesting effects exhibited by these novel compositions and the activity was found to be comparable to that of dexamethasone (0.1 mg/kg body weight) and therefore have the potential to circumvent the side effects associated with the long-term usage of dexamethasone. Treatment with DEX-H (0.05 mg of DEX + 100 mg of extract) and C2 suppressed the arthritic progression to the extent of only DEX (0.1 mg/kg body weight) and showed pronounced pro-inflammatory cytokine inhibition and downregulation of osteoclast markers.

Paw swelling was significantly ($P<0.0001$) inhibited with a reduced dose of DEX (0.05 mg/kg) in combination with HP (85.85%) and the novel composition C2 (89%) and the effect was comparable to that of 0.1 mg/kg DEX treatment alone (89.85%) indicating that both anti-inflammatory and immune response attenuation can be achieved by either lowering DEX dose or replacing DEX with new novel compositions. The decrement in plasma concentration of IL-6 and TRAP by DEX-H, C2 and C3 in the ELISA assay was found similar to that of DEX. The downregulation of mRNA expression of IL-1β by C2 (5.8-fold), Cathepsin K by DEX-H (6.3-fold) and MAG (4.2-fold) was found to be higher than DEX alone treated group (IL-1β :3.7-fold; Cathepsin K: 3.6-fold). These results reflected the potential of the new combinations as an alternative therapy for rheumatoid arthritis.

The anti-arthritic action of DEX vs new compositions was further validated by the radiographic and histological study of the ankle joint in arthritis. Changes in articular cartilage, synovium, bone destruction, and other symptoms were successfully controlled by the treatment groups. The NO and MPO levels which are the earliest signs of neutrophil infiltration in inflammation were significantly reduced in all treatment groups. The DEX-H group showed a better effect when functional assessment using the rota-rod was done. The expressions of CD51 and MMP-3 in western blot analysis were found to be downregulated by all the treatment groups with DEX-H and C2 showing a better effect.

A study conducted by Roy et al. (2013) established that co-administration of dexamethasone at a reduced dose and epigallocatechin suppressed the arthritic progression in rats and the suppression was higher than dexamethasone monotherapy at a higher dose. Gly has been reported to dose-dependently inhibit the expression of TNF-α, IL-6, IL-1β and NF-κB (Fu et al., 2014). It has also been reported to significantly diminish positive TRAP osteoclasts and cathepsins B and K in the bone lesions of aged mice (Yamada et al., 2021). In yet another study, Gly was reported to suppress the upregulation of HMGB1 and inflammatory mediators, NO, proinflammatory cytokines and MMPs highlighting glycyrrhizin as a potential therapy for arthritis (Zhou et al., 2021). Treatment with 10 mg/kg glycyrrhizin has been shown to suppress MMP-1, MMP-3, IL-1β, IL-6, TNF-α, and iNOS in serum and cartilage (Luo et al., 2021). MAG has also been reported to up-regulate the IL-10 level and down-regulate the TNF-α, MPO activity and neutrophil percent (Shi et al., 2010; Marianecci et al., 2012). The combined administration of dexamethasone at a reduced dose with *H. pinifolia* and novel compositions C2 and C3 suppressed arthritic progression more compared to dexamethasone monotherapy. This finding has the potential to widen the scope and future perspectives of pharmacotherapy of rheumatoid arthritis.

5.12 Marketing Strategies for proposed herbal product used by RA patients

This objective was taken to understand the journey of our product from laboratory to market. HP has been discovered to have anti-arthritic activity based on the studies conducted on animal arthritis models (discussed in previous sections). To ensure the commercial success of the herbal product prepared using HP among the target consumers (RA patients), a comprehensive marketing plan needs to be implemented. This plan involves the following key steps:

1. Identifying Market Opportunities: Thoroughly analyse the existing RA drug market to identify potential opportunities for the proposed product. This includes evaluating competitors' offerings, understanding their strengths and weaknesses and identifying any gaps in the market that the product can fulfil.
2. Studying Consumer Buying Behaviour: Gain a deep understanding of the purchasing patterns and preferences of RA patients. This involves researching their needs, concerns, and decision-making factors when it comes to choosing RA treatments. By understanding their motivations and barriers, we can tailor marketing strategies to effectively reach and engage this target audience.

3. Developing Effective Marketing Strategies: Based on the findings from the market analysis and consumer behaviour study, develop appropriate marketing strategies for a successful prelaunch of the developed product. These strategies should encompass various aspects such as product positioning, branding, pricing, distribution channels, and promotional activities. By aligning the product's unique selling points with the identified market opportunities and addressing the specific needs of RA patients, we can create a compelling marketing campaign that resonates with the target audience.

A thorough market analysis is important to ensure a successful commercial launch of the developed herbal product. This market scan involves assessing the status of the global RA drug market, as well as understanding the demand for the product specifically in the Indian market in terms of usage rate of herbal products among RA patients. Additionally, this analysis will provide insights into the competitive landscape, allowing for a better understanding of the unique value proposition and positioning of the developed product. This analysis can enable us to gather valuable information about the RA drug market at both global and local levels, gain insights into the preferences and adoption of herbal products among RA patients and identify the strengths and weaknesses of existing competitors.

Simultaneously, it is crucial to examine the buying behaviour and attitudes of consumers towards the use of herbal products in treating RA. Conducting a primary survey among RA patients can provide valuable insights into various queries such as:

1. Identifying Target Patients: Determine the specific patient profiles who are most likely to opt for the developed herbal product, considering factors like age, gender, lifestyle, and severity of the condition.
2. Socio-demographic influences: Understand how socio-demographic factors, such as income, education, and geographical location, might influence the buying decisions of the target audience for the herbal product.
3. Preferred Product Form: Investigate the preferred product form (e.g., gels, tablets, oil) among RA patients, enabling the development of the product in a format that aligns with their preferences.
4. Comorbidities: Examine whether patients with co-existing medical conditions are more inclined to opt for herbal products. This knowledge will help determine if the presence of comorbidities influences decision making process.

Studies have indicated that RA patients often chose complementary and alternative medicine (CAM) alongside conventional allopathic drugs to alleviate arthritis pain (Crop et al., 2018; Chatterjee, 2021). Therefore, studying the buying patterns of CAM products among RA patients will provide insights into how our proposed herbal product will be perceived, used and purchased by the target audience. Conducting market research specifically on RA patients who use CAM products will aid in designing an effective marketing plan for our developed herbal product. Through this research we can gain a comprehensive understanding of the consumer buying behaviour and attitudes towards herbal products for RA treatment and enable us to tailor marketing strategies to effectively position and promote the developed herbal product within the target market.

Based on the secondary data obtained from RA drug market analysis and the primary survey on consumer buying patterns, the collected information will assist in formulating appropriate marketing strategies for our developed product. These marketing strategies will encompass the 4Ps: product, place promotion and the price.

Product: The product offering should align with the specific needs and preferences of the target consumers. This involves defining the product’s features, design, quality, branding and packaging. By understanding the requirements of RA patients, we can develop a product that effectively addresses their pain relief and overall treatment needs.

Place: Determining the distribution channels is crucial for ensuring the products availability and accessibility to the target market. Companies must identify the most appropriate channels to reach RA patients, ensuring that the product is conveniently accessible to them. This may involve partnership with pharmacies, healthcare providers, online platforms or other relevant channels.

Promotion: Effective promotion involves developing a messaging strategy that effectively communicates the value of the product to the target market. By understanding the preferences and communication channels of RA patients, we can develop targeted promotional campaigns that resonate with them. This may include utilising social media, online advertising, circulating pamphlets or collaborating with healthcare professionals to raise awareness and generate interest in the product.

Price: Determining the optimal pricing strategy requires considering the perceived value of the product to customers. Companies need to assess the willingness of RA patients to pay for the product and consider factors such as competition, production costs and profit margins. By strategically pricing the product, it can be positioned as both affordable and valuable within the market.

By aligning the marketing strategies with the 4Ps, we can effectively position and promote the developed product to the target audience of RA patients. This approach ensures that the product meets their needs, is conveniently accessible, effectively communicated and appropriately priced.

In this context, this chapter aimed to design marketing strategies for proposed herbal product by conducting secondary studies on RA market along with primary survey on buying behaviour among RA patients. Thus, this chapter covered following 3 sub-objectives in 3 phases namely

- To study the potential of RA market, analyze competitors offerings related to these products.
- To study the demographical, psychological and physiological factors that influence the usage of CAM product among RA patients
- To develop the marketing strategies for launching the developed RA product

5.12.1 Phase 1: Business Environment of RA drugs: Market and Competitor analysis

Conventional Medicines: Conventional medicines are those that are typically prescribed by doctors for the treatment of RA. These include:

1. NSAIDs - These drugs can help to reduce pain and inflammation in RA. Examples include ibuprofen, naproxen, and aspirin.
2. DMARDs - These drugs work to slow down or stop the progression of RA by suppressing the immune system. Examples of DMARDs include methotrexate, sulfasalazine, and hydroxychloroquine.
3. Biologic DMARDs - These drugs are a newer class of DMARDs that target specific parts of the immune system that contribute to inflammation in RA. Examples of biologic DMARDs include adalimumab, etanercept, and rituximab.

4. Corticosteroids - These drugs are powerful anti-inflammatory agents that can help to reduce inflammation and pain in RA. They are typically used in low doses and for short periods of time due to their side effects.

CAM: It encompasses a range of therapies and practices that are used alongside or instead of conventional medical treatments. Some examples of CAM therapies for RA may include:

1. Acupuncture: A traditional Chinese medicine practice that involves the insertion of thin needles into specific points on the body to alleviate pain and promote healing.
2. Massage therapy: A hands-on therapy that can help reduce pain, stiffness, and inflammation in the affected joints.
3. Mind-body therapies: Techniques like meditation, yoga, and tai chi can help reduce stress and improve overall well-being, which can in turn help manage RA symptoms.
4. Herbal supplements: Certain herbs and supplements, such as turmeric and omega-3 fatty acids, may have anti-inflammatory properties that could help manage RA symptoms.

According to a survey conducted by the Arthritis Foundation in the United States in 2019, 60% of people with arthritis reported using some form of complementary therapy in addition to their prescribed medications. These therapies included massage, dietary supplements, and acupuncture, among others. A study published in the Journal of Traditional and Complementary Medicine in 2017 found that about 80% of people with RA in India used complementary and alternative therapies alongside their conventional medications. The most commonly used CAM therapies in this study were Ayurvedic medicine, yoga, and dietary supplements.

5.12.1.1 Competitive Environment

The market for RA medications is highly competitive, with many pharmaceutical companies developing and marketing drugs to treat this condition. Some of the major players in the RA medication market include AbbVie, Amgen, Bristol-Myers Squibb, Eli Lilly, Pfizer, Roche, and Sanofi, among others. These companies offer a range of drugs, including NSAIDs, DMARDs, and biologic DMARDs. Competition in the RA market has been increasing in recent years due to the introduction of new biologic drugs, as well as the availability of biosimilars (lower-cost versions of biologic drugs). Biosimilars are expected to become increasingly important in the RA medication market, as they offer a lower-cost alternative to expensive

biologic drugs. In addition to the competition among pharmaceutical companies, there is also competition between different types of RA medications. For example, while biologic DMARDs are effective in treating RA but due to its high cost some patients may prefer to use non-biologic DMARDs or NSAIDs instead. Overall, the competition in the RA medication market is expected to remain intense, as pharmaceutical companies continue to develop new drugs and biosimilars to treat this condition. Competition in the CAM market for RA is also increasing, as more people with RA are seeking out these therapies in addition to conventional medications. Some of the most commonly used CAM therapies for RA include acupuncture, massage therapy, dietary supplements, and herbal remedies. There are many practitioners and companies offering these therapies, and the market for CAM treatments for RA is becoming more competitive. One of the challenges with the CAM market for RA is the lack of regulation. Unlike conventional medications, CAM therapies are not subjected to stringent regulatory requirements, which can make it difficult for patients to know which therapies are safe and effective. Another challenge is the lack of standardized training and certification for CAM practitioners. While some CAM practitioners may have extensive training and experience, others may not have the same level of expertise, which can impact the quality and effectiveness of the therapies they provide. Overall, while the market for CAM therapies for RA is becoming more competitive, it is important for patients to be cautious and do their research before trying any new therapies. It is also important to discuss any CAM therapies with a doctor to ensure that they are safe and do not interfere with conventional medications. According to a survey conducted by the National Center for Complementary and Integrative Health (NCCIH) in the United States in 2012, about one-third of adults with arthritis use some form of CAM therapy, including dietary supplements, massage, and chiropractic care.

In terms of specific CAM therapies used for RA, a systematic review of clinical trials published in 2019 found that acupuncture, herbal medicine, and mindfulness-based stress reduction (MBSR) may be effective in reducing pain and improving physical function in people with RA. However, we noted that more research is needed to fully understand the safety and efficacy of these therapies. Some of the CAM in India are:

1. Rumalaya: Rumalaya contains several herbs that are traditionally used in Ayurvedic medicine to treat joint pain and inflammation, including:
    a. Boswellia (*Boswellia serrata*): A resin extract with anti-inflammatory properties that may help reduce joint pain and inflammation.

   b. Guggulu (*Commiphora mukul*): A resin extract with anti-inflammatory and analgesic properties that may help relieve joint pain.
   c. Indian Winter Green (*Gaultheria fragrantissima*): An essential oil with analgesic properties that may help relieve pain.
   d. Ginger (*Zingiber officinale*): A root with anti-inflammatory and analgesic properties that may help reduce joint pain and inflammation.
2. Rhumayog Gold Emami contains several herbs that are traditionally used in Ayurvedic medicine to treat joint pain and inflammation, including:
   a. Ashwagandha (*Withania somnifera*): An adaptogenic herb that may help reduce inflammation and improve immune function.
   b. Shilajit (*Asphaltum*): A mineral pitch that may help improve joint function and reduce inflammation.
   c. Guggulu (*Commiphora mukul*): A resin extract with anti-inflammatory and analgesic properties that may help relieve joint pain.
   d. Turmeric (*Curcuma longa*): A spice with anti-inflammatory properties that may help reduce joint pain and inflammation.

The results obtained from the secondary data revealed that most of herbal brands were oil or grease based and used "fast action" or "relief in 7 days" as promotion. Most of the herbal marketed drugs contained guggul, curcumin and ashwagandha as main ingredient. None of the marketed products contained any marine source as per the secondary data. Thus, we can conclude that there is a significant competition in the drugs for RA management and there are many people who are affected for the same. The conventional medicines have some side effects and the CAM medicines provide extra relief indicating increase in market size and demand for these medicines.

5.12.2 Phase 2: Consumer Analysis

In order to understand the socio-demographic, physiological and psychological influence of usage of RA product, primary analysis was conducted among RA patients. The profiles of CAM users were identified by surveying 200 RA female patients and thus the data obtained were analysed using logistic regression. The details of the study design, theoretical model, selection of sample size, questionnaire design as well as methodology has been discussed in sections 4.12.2, 4.12.3, 4.12.4 and 4.12.5, respectively

With this objective, this phase of the study aimed to identify the profiles of RA patients that uses CAM alone or along with allopathy drugs. Identifying the factors will help in identifying the right segment of the market where our herbal product could be targeted through right promotion, pricing and distributing strategies.

Results of Consumer analysis

Descriptive analysis indicated that 54% of the patients were of the age group 40-59 years and about 88.66% of the total respondents were literate. 54% used some form of CAM. 79.01% of the RA patients started using CAM to control the excessive pain, 14.81% due to pressure from family/friends, and 67.9% of them started using CAM along with allopathic treatment. About 50.6% of the respondents preferred herbal medicines, while 11.11% of them went for traditional practices like Yoga and 7.4% for acupressure. The percentage of participants using a combination of herbal medicine, yoga, and acupressure was found to be 30.8%. The survey results revealed that the majority of the CAM patients suffering from RA opted for gel formulation (48.48%) compared to oil (31.81%) and tablet formulation (19.69%). However, it was found that 44.45% of the CAM users did not reveal their usage to physicians. To understand the usage of CAM among menopausal women, we studied the behavioural pattern of women aging above 60 and used this information as a proxy to menopause. It was observed that more than half of them (64.7%) use CAM, with 54.5 % uses herbal and 31.3 % uses herbal medicines along with Yoga practices. However, future studies can explicitly ask about the menopause condition directly to RA patients. The results of descriptive analysis are represented in Figure 5.12.2

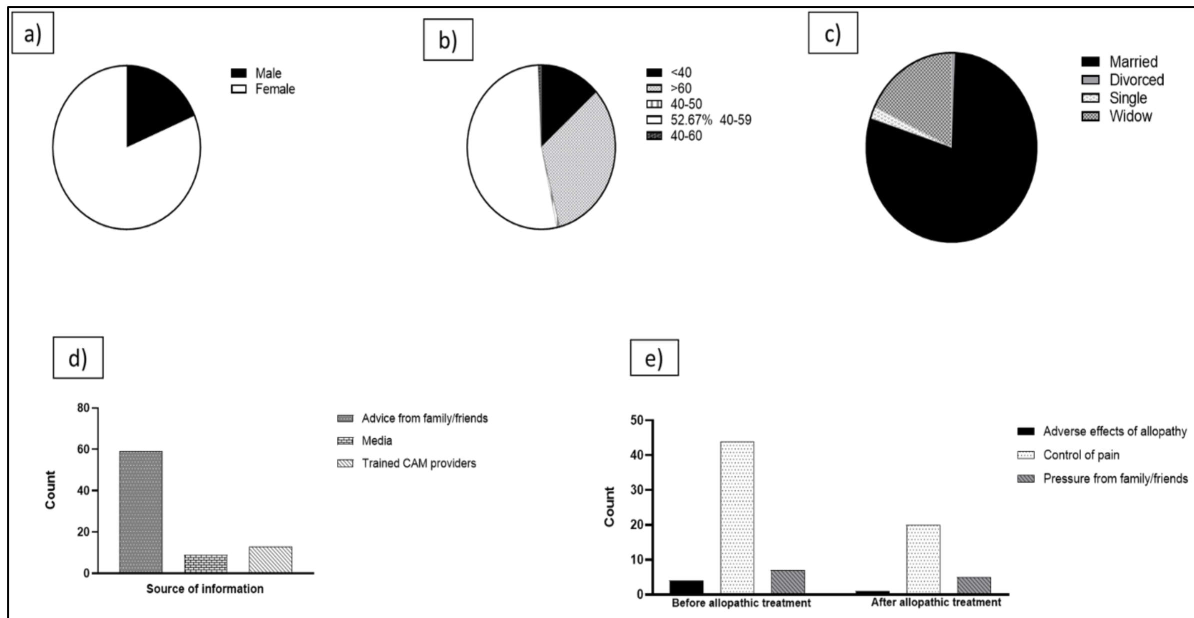


Figure 5.12.2. Results of descriptive analysis (a) Gender; (b) Age; (c) Marital status; (d) Source of information; (e) Reasons to use CAM.

Logistic regression suggested that education had an impact on CAM as its usage was 4.622 times higher among patients with basic education (school/graduation) as compared to the illiterate as shown in Table 5.12.2. A similar trend was observed among married RA patients where CAM usage was 3.141 times higher compared to single/divorced/widowed. RA patients with 1-3 years of sufferings will show lesser odds of using CAM i.e., 0.083 times as compared to the ones with an initial period of sufferings (6-12 months). To put it in simple terms, patients at initial periods of sufferings will use more CAM and usage will decline with an increase in tenure of sufferings. The odds of using CAM by high family income patients (greater than 60000 INR/ month) is lower by 0.165 then lesser income households' patients. Comorbidities like blood pressure, thyroid, and diabetes inhibited patients from using CAM compared to the patients without any comorbidities. Specifically speaking, patients with blood pressure did not use CAM with a probability of 0.062, patients with diabetes used CAM with a lesser probability of 0.034 vis- a vis patient with no comorbidities. Odds of usage of CAM is 0.1 times less in RA patients suffering from thyroid and 0.024 times less in patients suffering from cardiovascular or other complications as compared to patients with no comorbidities.

Further, ordered logistic regression shows that patients suffering from depression used CAM with a probability of 3.43 then the patient showing mild or no depression. Out of the 4 types of pain, patients suffering from moderate sensory pain had a probability of 31.176 to use CAM than the ones with mild/negligible sensory pain. Interestingly, RA patients with severe physiological pain and patients who were unable to perform activities involving the upper

portion of the body (for instance- cutting vegetables/lifting a cup of water/opening taps and door latches) chose to ignore CAM usage with a 0.235 odd ratio as compare to a patient who can perform those activities with little difficulty. Out of 150 observations, 49+62 = 111 predictions were accurate hence placing the model at 74% accuracy making it a reasonably good model.

Table 5.12.2. Variable associated with the use of complementary alternative medicine

Variables		B	S.E.	Wald	Sig.	Exp(B)
Literacy (School/Undergraduation)	Base = Illiterate	1.531	0.806	3.609	**0.037**	4.622
Period of suffering from RA	Base = 6-12 months			10.550	**0.014**	
1-3 years		-2.485	0.924	7.232	**0.007**	0.083
3-5 years		-.427	0.691	0.381	0.537	0.653
>5 years		0.109	0.727	0.022	0.881	1.115
Family income	Base = < 30000 INR			10.607	**0.005**	
30000 INR - 60000 INR		-0.417	0.625	0.444	0.505	0.659
> 60000 INR		-1.802	0.565	10.182	**0.001**	0.165
Comorbidities	Base = None			13.203	**0.010**	
Blood pressure		-2.773	1.100	6.357	**0.012**	0.062
Diabetes/ Diabetes + Blood pressure		-3.385	1.008	11.289	**0.001**	0.034
Thyroid/ Thyroid + Blood pressure		-2.302	1.067	4.655	**0.031**	0.100
Cardiovascular and others		-3.722	1.165	10.198	**0.001**	0.024
Marital status	Base = Single/ Divorced/ Widow	1.145	0.616	3.447	**0.043**	3.141
Pain						
Physiological pain	Base = Mild			3.109	0.211	
Moderate		-0.615	0.883	0.485	0.486	0.541
Severe		-1.125	0.650	2.999	**0.053**	0.325
Sensory pain	Base = Mild			4.874	**0.047**	
Moderate		3.440	1.560	4.863	**0.027**	31.176
Severe		0.559	0.624	0.805	0.370	1.749
Affective pain	Base = Mild			1.904	0.386	
Moderate		-2.264	1.689	1.797	0.180	0.104
Severe		-0.064	0.641	0.010	0.920	0.938
Cognitive pain	Base = Mild			0.938	0.626	
Moderate		-0.646	1.069	0.365	0.546	0.524
Severe		-0.635	0.655	0.938	0.333	0.530
Physical ability to conduct day to day activity *Lower body health index*	Base = Mild			0.950	0.622	
Moderate		0.340	0.859	0.156	0.693	1.405
Severe		0.808	0.906	0.795	0.373	2.242
	Base = Mild			4.599	0.100	
Upper body health index Moderate		-0.907	0.705	1.653	0.199	0.404
Severe		-1.446	0.677	4.557	**0.033**	0.235
Depression	Base = mild/negligible depression	1.235	0.604	4.178	**0.041**	3.438
Constant		2.680	1.442	3.453	**0.043**	14.579
R-square	0.463					
-2 Log Likelihood	143.25					
Chance classification	52%					
Classification table	74%					

Notes: SE-Standard errors; B-standard coefficient; Exp(B)-odd ratios

The results obtained from the questionnaire-based survey of patients suffering from arthritis are in line with previously published studies. The results revealed that more than 54% of the respondents used some form of CAM as a measure to cope with arthritis. This certitude in CAM can be attributed to several factors like failure of conventional therapies to address their pain, common belief of CAM being safer compared to allopathic medicines, and frustration with long term regimen for management of arthritis. Several studies have reported similar findings underlying the disappointment with conventional therapy (Álvarez-Hernández et al., 2006; Alaaeddine et al., 2012; Almuhareb et al., 2019). Another result found in the study was 45% of the patients were reluctant to reveal the use of CAM to their physicians. Several studies have reported similar findings where the patients either concealed CAM usage or were hesitant to share with their physicians (Geisler and Cheung, 2015; Yang et al., 2017; Zhao et al., 2017). The probable reasons cited in this study were either they were not asked by the physicians or they forgot to inform. Another probable reason behind it could be fear of disapproval by the physician. A study by Alaaeddine et al (2012) showed that 75% of the physicians disapproved of the usage of CAM which highlights the reason for poor disclosure of CAM to their physicians. Contrary to this, another study by Wallen and Brooks (2012) revealed that shared decision-making practice with the physician eased the patients to reveal the usage of CAM which is in line with the study conducted by Sleath et al, (2005). The education level of the patients showed to have a direct association with the usage of CAM. The results unfolded that patient with basic education (school/undergraduate) used CAM with a probability of 4.622 compared to an illiterate. Education opens the horizon of knowledge and instils an ability to reason any given thing. It also unlocks the ocean of information available online with a single click in today's digital era. The obtained results are contrary to the results obtained by Han and his co-workers (2015) who found higher education levels decrease the usage of CAM in a study conducted in South Korea.

The usage of CAM in the married group of RA patients was found to be significant. This can be attributed to the privilege of economic freedom compared to the single and widowed group of patients. The results are congruent with the findings of Han (2015). Further, our result indicated that patients at initial periods of suffering will use more of CAM and the usage will decline with an increase in the period of suffering.

Another interesting result that came upon during the study was that the patients experiencing moderate sensory pain had a probability of 31.176 to use CAM than the ones with mild/negligible sensory pain. CAM usually involves touch therapies and acupressure, hence

people with a moderate level of sensory pain were observed to use CAM more than the ones who had mild/negligible sensory pain. The results are in line with the studies conducted by Zaman et al, (2007). The failure of conventional therapies and desperation for pain relief could be the encouragement for the patients to adopt the usage of CAM so significant that about 8.3% of them suffering from pain start using alternative medicine. Also, the chronic nature of the disease provides a wide window for the adoption of any promising therapy for pain relief.

It is also a standard observation by many studies that patients suffering from mild physiological pain tend to use CAM, however, once the intensity of pain becomes severe, they start relying on allopathy for a quick remedy (Tan et al., 2007).

The results were in line with Lee et al, (2008) who found similar results in a study conducted on the usage of CAM by RA patients in Korea. Further, our result indicated that patients rely less on CAM when they experience severe physiological pain as compared to a mild one. As hypothesized, there is no significant relationship between the usage of CAM and cognitive as well as affective pain (Lee et al., 2008). The relationship between CAM usage and depression when explored in the present study revealed patients suffering from depression used CAM with a probability of 3.43 compared to patients with no depression. This direct relation between depression and CAM usage among RA patients is well established and has been highlighted in several studies so far strengthening the results obtained in our study (Kojima et al., 2014; Han et al., 2015; Berner, 2018; Chancay et al., 2019; Peterson et al., 2019; Larice et al., 2020). Zairko et al, (2019) entrenched in his study that pain experienced in RA was responsible for the anxiety and depression built up in patients receiving conventional therapies.

The study brings out comorbidities like hypertension, diabetes, thyroid, etc., discouraged RA patients to use CAM. The probable factors could be fear of possible drug interaction with multiple drug regimen simultaneously, economic constraints, physician disapproval, lack of knowledge and information, etc. A detailed review done by Bishop and Lewith, (2010) failed to establish any significant association between comorbidities and CAM usage. Also, the severity of pain often leads RA patients to go for allopathy for fast relief. This fact was evident from the results of the survey where patients experiencing difficulty in carrying out basic activities like eating, drinking, cutting vegetables/ lifting a glass of water/ opening door latches, etc. used lesser CAM which is in line with the findings of Tan G et al, (2007).

Primary study conducted among RA patient indicated that patient who are at an initial period of suffering (1-3 years) with no comorbidities shows high CAM usage. CAM has been sought by most of the RA patients experiencing mild physiological or moderate sensory pain mostly to ease the difficulties in carrying out day to day activity. Depression and higher education were found to significantly promote higher CAM usage. The study showed most of the patients were reluctant to consult their physicians for CAM usage in fear of disapproval. The physicians and other healthcare providers must acknowledge the use of CAM and discuss treatment options with their patients to avoid possible interaction with other therapies. Further, study indicated that patient suffering from severe pains opt for allopathy for their treatment.

This consumer study helped in selecting the primary target market for our proposed product. In other words, helped in answering the basic question, "Who are we really trying to serve?". Thus, our developed herbal product should be targeted among women who are at initial period of suffering i.e., 1 to 3 years with moderate sensory pain, having no commodities. Women with basic education, and showing certain sign of depression should be targeted with our herbal product for greater market coverage.

Once the target market segment of our product has been identified, marketing strategies will be specifically tailored to cater to this particular patient group. The next phase of the study will delve into the details of this strategies.

5.12.3 Phase 3: Marketing Strategies

A well-designed marketing strategy incorporates the 4Ps of the marketing mix, which include product, place, promotion and price. It can provide a roadmap for creating and executing marketing activities that will deliver a return on investment. The following section explains the marketing mix for our proposed product.

Product: The Product refers to the specific good or service that a business offers to its customers. In the case of our proposed product development careful consideration will be given to ensure it aligns with the needs and preferences of our target market. The product will be formulated to effectively address the symptoms and provide fast relief from joint pains. Given that the product is herbal-based all the excipients used will be used from natural sources. The use of complete natural ingredients will appeal to individuals who prefer alternative treatment and are concerned about potential side effects. It will be ensured that the product is available in various forms such as gels, tablets or oils. This will provide flexibility to cater to the diverse preferences and usage habits of RA patients, allowing them to choose the form that best suits

their needs. Attention will be given to packaging and branding to create a positive and appealing customer experience. The packaging will be designed to be user-friendly and informative, conveying the product's benefits and usage instructions clearly. The branding will reflect the product's benefits and usage instructions clearly. The branding will reflect the product's quality, reliability and alignment with the values and aspirations of the target market. By developing a product that effectively addresses the needs of RA patients, utilizing natural ingredients, providing it through appealing packaging and branding, we can position our product as a desirable solution within the target market.

Price: The price of a product or service is the amount customers are willing to pay for it. When determining the price, companies must consider various factors, including production costs, competition and customer demand. There are three types of pricing which can be done namely competitive based pricing, value-based pricing and cost-based pricing. In this case, we will opt for competitive-based pricing by conducting a competitor analysis of similar products in the market (Table 5.12.3). Based on the pricing information gathered, we recommend setting the price of our product in the range of Rs 3 to 20 per tablet. This range aligns with the prices offered by competitors such as Baidyanath, Dabur, Himalaya, Zandu, Patanjali etc. its important to note that specific price within this range will depend on further analysis and considerations. Factors such as the unique features, formulation, quality and branding of our product will be taken into account to determine the optimal price point within the suggested range. Additionally, ongoing market research, customer feedback and profitability analysis may further refine the pricing strategy to ensure it remains competitive and profitable.

Place: The distribution channel chosen for a product are crucial in making it available to the customers. In the case of our product, we can utilise two channels: physician and direct-to-consumer through over-the-counter (OTC) availability. The physician channel involves targeting healthcare professionals like rheumatologists, orthopaedic specialist, physiotherapists and neurologists. By partnering with these healthcare providers, we can have our product recommended and prescribed to patients. In addition to the physician channel, our product can also be made available directly to consumers as an OTC drug. This means that the consumers can purchase without a prescription from retail outlets and pharmacies. Potential retail outlets and pharmacy chains like Hetero and Apollo Pharmacy can be targeted to ensure widespread availability and accessibility to consumers. Thus, by utilizing both these channels we can ensure a broader reach for our product. Also, it is important to establish strong partnerships

with health professionals and ensure that our product is prominently displayed and well-stocked in retail outlets and pharmacies. This multichannel distribution strategy will enhance the accessibility of our product to the target market, ultimately increasing its chances of commercial success.

Promotion: Promotion involves the marketing activities used to communicate the value of a product or service to customers. In the case of our product, there are two key decision-makers involved: physicians and patients. Since physicians are mostly reluctant to prescribe a new drug the sales force or medical representatives can detail our product, providing comprehensive information. Free samples can also be provided to encourage trial and familiarity. In order to increase the knowledge of the developed herbal product, various conference presentations and peer reviewed articles which will report on the clinical testing of the drug will be encouraged. Physiotherapists and dermatologists who frequently encounter patients with conditions involving sensory and muscular pain can be targeted as they can recommend it to relevant patients. A persuasive advertising campaign highlighting benefits and effectiveness in reducing RA symptoms and creating a sense of urgency or early intervention should be adopted. At the same time, the advertisement should display women approximately 25–49-year age, who is finding moderate difficulty in doing day today activities. Online platforms and medical apps like Pharmeasy, Truemeds, Practo, Netmeds etc. will be opted to reach to RA patients.

The promotion strategy should include a 6-month detailing campaign aimed at raising awarness among both the physicians and patients. This can be followed by direct to consumer advertising campaigns to encourage patients to request the product from pharmacist and reinforce its availability as an OTC drug.

Table 5.12.3. Marketing strategies (4Ps) of the available CAM products for RA

S.No	Competitor	Product	Ingredients	Place	Promotion	Price
1	Dabur	Rheumatil oil	Gandhapura oil, Guggulu, Pudina, Neelgiri oil	CAM	ayurvedic oil for joint pain relief	Rs.228/100ml
2	Emami ltd (Zandu)	Zandu ortho vedic oil	more than 100 Ayurvedic ingredients and enriched with the goodness of classical oils like Mahanarayan Taila, Mahamasha Taila and Vishgrabha Taila,	CAM	"Visible improvement in mobility within 7 days of usage"*.	Rs 218/100ml
3.	Himalaya	Rhumalaya forte	Boswellias (shallaki), Indian bdellium(guggul)	Prescription	none	Rs180/30tablet
		Reosto	Arjuna (Terminalia arjuna), Indian Bdellium (Guggulu)	Prescription	none	Rs180/30tablet
		Shallaki	Shallaki		Reduces pain and inflammation	Rs200/60tablet
		Rhumalaya gel	Boswellias (shallaki), Indian Winter Green (Gandhapura taila) oil	CAM	Fast action Quick relief	Rs 110/30g
		Rhumalaya	Drumstick (Shigru), Indian Tinospora (Guduchi)	Prescription	None	Rs150/60tablet
		Rhumalaya liniment	Camphor (Karpoora), Indian Winter Green (Gandhapura taila) oil	CAM	RAF- Rapid action formula	Rs 130/60ml
		Rhumalaya acive spray	Gandhapura (Gaultheria fragrantissima), Maricha, Sarala (Pinus roxburghii)	CAM	Starts relieving pain in 2 minutes	Rs 165/60g
4	Hamdard (Unani)	Majun surajan	Not known	CAM	none	Rs50/100g
		Habb-E-Surajan	Not known	CAM	none	Rs 132/300 capsule
		Majun Chobchini	Not known	CAM	none	Rs109/100g
		Aujai	Not known	CAM	none	Rs 105/60 capsule
		Roghan surajan	Not known	CAM	none	Rs 25/80ml
5.	Baidyanath	Rhuma oil	Aswagandha, haldi, Shatavari, Vatsnabh, Kuchla	CAM	none	Rs220/100ml
		Rheumartho gold	Multiple ayurvedic ingredients	CAM	Enriched with pure gold	Rs 486/30 capsules
		Mahanarayan oil	Multiple ayurvedic ingredients	CAM	The Renowned oil for Joints and Muscular pains	Rs 410/100ml
6.	Charak	Go 365 nutra tablet	Glucosamine, chondritin, turmeric and guggul	CAM	Faster, more effective	Rs 323/30 tablet
7.	Kerela Ayurdeva limited	GT capsules	Multiple ayurvedic ingredients	CAM	none	Rs680/100tablet

Conclusion

- The proposed herbal product to be developed should be targeted to patient who are at initial phase of the treatment (1-3) years, experiencing depression, moderate sensory pain, married, educated with no commodities.
- Our product could adopt two channels namely "physician" as well as reach directly to consumer as OTC drugs.
- Price of the product should be in the range of Rs. 3 to 20 per tablet.
- Physician should be detailed about the product through sales force, free samples should be provided for effective promotion.
- Extensive 6 months awareness program should be conducted and physiotherapist should be made aware of the product so as to recommend patient within initial phase of treatment
- Various media such as TV, radio, catalogue should be used apart from direct salesforce.
- Advertisement in social media, especially testimonials should be uploaded, along with placing the advertisement of the product in various medical apps such as Pharmeasy, Practo etc.
- Message in advertisement should focus on functional and beneficial aspects of the product, showing women of middle age finding mild to moderate difficulty in doing upper body activities like cutting vegetables, lifting a glass of water, etc.

Chapter 6

Summary and Conclusion

- RA is a systemic chronic autoimmune disease characterised by inflammation of the synovium, synovial hyperplasia, angiogenesis, and cartilage damage. Pharmacologic treatment of RA involves combinations of NSAIDs, GCs, and DMARDs. The serious side effects associated with the long-term use of these drugs, the high cost of DMARDs, and the high economic burden of RA treatment add a limitation to their use. In view of the above facts, an attempt was made to explore and develop cost-effective and safe oral herbal compositions for the treatment of RA.

- *H. pinifolia,* a marine seagrass was selected as the major ingredient of the compositions based on the pro-inflammatory cytokine inhibition property exhibited by it in our pilot study. Three HP-based compositions: composition 1 containing HP+ licorice (LQ); composition 2 containing HP+ glycyrrhizin (Gly), and composition 3 containing HP+ mono ammonium glycyrrhizinate (MAG) were designed and subjected to in-vitro assays.

- Composition 1 was prepared by mixing HP and LQ, respectively in different ratios 1:1, 1:0.5, 1:0.25 and 1:0.125. Composition 2 was prepared by mixing HP and Gly, respectively in different ratios 1:0.5, 1:0.25, 1:0.125 and 1:0.0625. Composition 3 was prepared by mixing HP and MAG, respectively in different ratios 1:0.5, 1:0.25, 1:0.125 and 1:0.0625.

- The individual ingredients and the compositions were screened for their pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and NO inhibition effect under in-vitro (ELISA and Griess method) in LPS-stimulated RAW 264.7 cell lines. The results revealed significant pro-inflammatory cytokines and NO inhibition effect by all the tested samples with the compositions showing synergistic effect (CI score <1) and based on which 1:0.5 was fixed as the ratio of mixing of HP and LQ/Gly/MAG for the in-vivo experiments and the compositions were respectively coded as C1, C2 and C3.

- The samples HP and LQ used for the study were standardized by HPLC method using their anti-inflammatory constituents as marker compounds (rosemarinic acid, vanillic acid, ethyl protocatechuate and glycyrrhizin). The standardised extracts were used for preparing the compositions for in-vivo study.

- The heavy metal test on HP and LQ extracts using ICP-MS analysis showed the presence of As, Cd, Hg and Pb within the regulatory threshold values. Further, the acute toxicity study performed on Balb/c mice showed no adverse action or health-related acute toxicity due to the oral administration of HP (2000 mg/kg body weight). Thus, HP, a pro-inflammatory cytokine and NO inhibitory extract was proved to be safe for oral administration.

- An oral suspension of 100 mg HP combined with 50 mg/kg of LQ/Gly/MAG (C1/C2/C3) was prepared to test the efficacy of compositions and their individual components under LPS-induced mouse endotoxaemia model and carrageenan-induced mouse paw oedema model. All compositions showed excellent inhibition effects under LPS-induced endotexaemia model, with C3 and C2 showing 65.05% and 62.56% inhibition of IL-1β, respectively. C2 showed the highest inhibition of TNF-α levels (60.40%) among the tested samples. C1 showed a similar inhibition effect over all the tested cytokines and it was found to be more than 50%.

- Under the carrageenan-induced oedema model, a significant reduction in paw oedema was shown by C1 and C2. The tested compositions significantly ($P<0.001$) inhibited the mRNA expression of TNF-α, IL-1β and IL-6 and the protein expression of NF-κB along with NO production, MPO activity. The results of acute inflammation models demonstrated the potential anti-inflammatory activity of HP and the HP-based oral compositions and hence were taken forward for evaluating their anti-arthritic properties.

- In the first set of experiments, samples HP, LQ and C1 were tested in Freund's complete adjuvant induced arthritis (FCA) model. The results revealed that the treatment with HP, LQ and C1 for 14 days reduced paw swelling in arthritis-induced rats. Treatment with HP significantly restored the platelets, ESR and WBC levels suggesting the protective effect of HP against adjuvant arthritis-related inflammation. The results of ELISA and RT-PCR assay revealed that the treatment with HP, LQ and C1 significantly ($P<0.0001$) reduced the cytokine levels (IL-1β, IL-6, TNF-α) and osteoclast markers (RANK, TRAP and Cathepsin K) in paw tissue homogenate. HP showed better results among all. Histopathology and radiological scanning demonstrated lesser joint deterioration in sample-treated rats, as evident phenotypically. The downregulation of

CD51 and MMP-3 corroborated the anti-arthritic effect of HP, LQ and C1. The results of the FCA- induced arthritis model proved the strong anti-arthritic effect of HP, LQ and C1. Further, it was observed that sample C1 showed a better effect in downregulating mRNA expression of IL-6 and TRAP compared to the individual administration of HP and LQ, which widened the scope for further exploration.

- A lipid nano-emulsion of composition 1 (C1-N) containing HP and LQ in the same ratio of 1:0.5 was prepared. C1-N was characterized by testing various properties in the aspect of formulation development like particle size, polydispersity index (PDI), zeta potential and SEM analysis to assess the uniformity of size, shape, and physical stability features of aggregation or irregularity. The size of C1-N ranged from 292 to 400 nm, and the PDI was 0.3 with more than 90% of particles being in the nanometer range and having a characteristic spherical form, indicating a homogenous nano-formulation which was reproducible. The total phenolic content of C1-N revealed that there was no incompatibility in the formulation. Further, C1-N was found to have better physical properties in terms of size, PDI and morphology when compared to the conventionally prepared suspension C1.

- Both C1 and C1-N were tested under collagen-induced arthritis model. C1-N was more effective in reducing the paw swelling. The spleen and liver index and the haematological levels of red blood cells, white blood cells, haemoglobin and platelets of both C1 and C1-N groups were significantly reduced compared to the arthritic control group. C1-N showed remarkable inhibition of TRAP (62.21%) compared to C1 (36.63%) highlighting the better effect of the nano-formulation. C1-N showed a similar trend in downregulating the mRNA expression of cathepsin K (6-fold) and TNF-α (9-fold) and downregulating both CD51 and MMP-3 expression. establishing the improved efficacy of the formulation. The significantly low level of ESR in the C1, C1-N and standard drug-treated animals, highlighted their anti-inflammatory potential. The considerable increase in the concentration of RF in the arthritic rats was significantly reduced by C1 and C1-N treatment in this study. Therefore, both C1 and C1-N were found to be potentially active under collagen-induced arthritis model. The results proved that nanonisation (292 to 400 nm) and formulation of C1 as lipid nano-emulsion (C1-N) improved the stability as well the activity.

- Furthermore, the evaluation of anti-arthritic activity (FCA model) of C2 and C3 was continued along with another novel combination DEX-H, containing 100 mg of HP + 0.05 mg of dexamethasone. The study included a DEX group administered with 0.1 mg/kg dose. The dose of dexamethasone was reduced to half in DEX-H to test the steroid-sparing effect of the novel composition. Treatment with DEX-H (0.05 mg of DEX + 100 mg of extract) and C2 (50 mg of Gly+ 100mg 0f extract) suppressed the arthritic progression comparable to DEX (0.1 mg/kg body weight) alone treated group and showed mark pro-inflammatory cytokine inhibition and downregulation of osteoclast markers. Paw swelling was significantly ($P<0.0001$) inhibited with DEX-H and C2 group. The decrease in plasma concentration of IL-6 and TRAP by DEX-H, C2 and C3 was similar to that of DEX and downregulation of mRNA expression of IL-1β by C2 (5.8-fold), cathepsin K by DEX-H (6.3-fold) and MAG (4.2-fold) were higher than DEX alone treated group (IL-1β: 3.7-fold; Cathepsin K: 3.6-fold), which highlighted the steroid-sparing effect of the novel compositions. These results were supported by the radiographic and histological study of the ankle joint in arthritis. The expressions of CD51 and MMP-3 in western blot analysis were found to be downregulated by all the treatment groups with DEX-H and C2 showing a better effect. The results reflected the potential of the new combinations as an alternative therapy for rheumatoid arthritis. Despite the reduced amount of dexamethasone present in DEX-H i.e. contained half that of DEX, a similar effect was observed between them, which proved the steroid-sparing effect of DEX-H.

- Furthermore, a thorough market analysis, competitor analysis, consumer analysis and marketing strategies were employed to understand the journey of our product, proposed to have anti-arthritic activity from laboratory to market. The results obtained from the thorough market analysis revealed that most herbal brands were oil or grease based and used “fast action” or “relief in 7 days” as promotion. Most of the herbal marketed drugs contained guggul, curcumin, and ashwagandha as main ingredients. None of the marketed products contained any marine source as per the secondary data. CAM is often opted by RA patients when the employed first-line therapies fail to offer satisfactory efficacy. Therefore, the buying behaviour and attitudes of consumers towards the use of herbal products in treating RA were analysed by conducting a primary survey among RA patients. The survey revealed that more than 54% of the

respondents used some form of CAM and 45% of the patients were reluctant to reveal the use of CAM to their allopathic clinicians. The results showed that patients with basic education (school/undergraduate) used CAM with a probability of 4.622 compared to an illiterate. It was found that patients experiencing moderate sensory pain had a probability of 31.176 to use CAM than the ones with no sensory pain. A significant relationship between depression and CAM usage was found which revealed CAM usage with a probability of 3.43 compared to patients with no depression. Comorbidities like hypertension, diabetes, thyroid, etc., discouraged CAM usage. It was concluded that CAM is preferred at the initial stage of pain among RA patients but as the pain reaches higher levels, patients opt for allopathy or allopathy along with CAM for faster relief. The in-depth analysis of marketing strategy and marketing mix, which included product, place, promotion and price helped to frame and provide a roadmap for creating and executing marketing activities that will deliver a return on the proposed product investment.

- Thus, the research explored the anti-arthritic effect of HP for the first time and yielded five novel oral compositions C1, C1-N, C2, C3 and DEX-H possessing significant anti-inflammatory and anti-rheumatoid arthritis activity. Additionally, the preliminary analysis of the challenges associated with the positioning and pre-launching of herbal products for rheumatoid arthritis provided a roadmap to ensure a return on the proposed product investment.

Chapter 7
Future Perspectives

The use of herbal drugs has gained increasing attention in recent years with their systematic scientific validation and assurance of quality. Also, there is a growing interest in their potential as a source of new medicines. Plants have a vast array of chemical compounds, and many of these compounds are yet to be fully explored. As new technologies and research methods emerge, there is potential to discover new and innovative therapies derived from plants. These could potentially treat a range of conditions such as inflammation, cancer and neurodegenerative diseases. As research continues to uncover the therapeutic potential of plant-based remedies, future developments in this area are promising. In view of this, a detailed investigation was carried out on *H. pinifolia* along with *G. glabra* following in-vitro and in-vivo models of inflammation and arthritis. The study yielded five novel compositions inhibiting the key pro-inflammatory cytokines in the in-vitro models and potentially acting on key inflammatory and osteoclast targets in in-vivo models of inflammation and arthritis. The future plans of this project are given below.

- Pharmacokinetic studies on the developed compositions could be performed. Also, the effective therapeutic dose of the compositions could be determined and thereby suitable formulation can be prepared leading to a phytopharmaceutical product.
- The compositions can be tested under other chronic inflammatory disease models like inflammatory bowel syndrome, asthma, fatty liver disease, cancer etc. to prove their therapeutic effect.
- A detailed mechanistic study could be performed to evaluate the pathway involved.
- The chemical investigation work on the secondary metabolites of *H. pinifolia* can be continued to explore novel compounds possessing anti-inflammatory and anti-arthritic properties.
- The study design for marketing strategies can be extrapolated to rheumatologists, pharmacists and other health care professionals.

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- **Samrun Nessa,** Kirti Hira, Pragya Paramita Pal, Sajeli Begum A. In vitro and in vivo exploratory studies evidencing the anti-inflammatory effect of new oral compositions containing *Halodule pinifolia* and natural sweeteners by downregulating pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), NO and MPO through NF-κB pathway. ***Molecular and Cellular Biochemistry (Under review)***.

- **Samrun Nessa,** Rajesh K. Routray, Kirti Hira, Lakshmi P. Soukya, Suraj Gupta, Venkatesh Erram, Lavanya S, Onkar P. Kulkarni, Sajeli Begum A. New Natural Agents Showing Steroid (Dexamethasone)-Sparing Effect and Anti-Arthritic Effect in Complete Freund's Adjuvant-Induced Arthritic Rats. ***International Immunopharmacology (Under Review)***.

OTHER PUBLICATIONS

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PAPER PRESENTED AT NATIONAL AND INTERNATIONAL CONFERENCES

- Oral presentation on "Pro-inflammatory cytokine inhibition effect of *Halodule pinifolia"* at 5th IUPHAR WCP-NP and 51st Annual conference of Indian Pharmacological Society (IPS), Hyderabad, India. 4th-7th December, 2019

- Oral presentation on "GPS-Aided study of Larval Indices of Aedes aegypti and analysis of Risk of Dengue Outbreak*"* at ICHTR conference organized by Manipal University, Manipal, India. 29th – 31st October, 2018.

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Samrun Nessa completed her B. Pharm degree from Manipal College of Pharmaceutical Sciences in the year 2014 and her M. Pharm degree from Birla Institute of Technology, Mesra in the year 2016. After her masters she had joined as an Assistant Professor in Global College of Pharmacy, Hyderabad in 2016 and worked for a year. In October 2017, she joined as Senior Research Fellow in ICMR-ICSSR sponsored project under the guidance of Dr. A. Sajeli Begum and Dr. Swati Alok. She got registered for PhD program in Department of Pharmacy, BITS-Pilani, Hyderabad campus in January 2018. As a part of her research achievements, she has contributed to 5 publications. She was also awarded with ICMR- SRF fellowship in 2019 for a period of 3 years.

Biography of Prof. A. Sajeli Begum

Prof. Ahil Sajeli Begum is the Professor and Head of the Department of Pharmacy at BITS Pilani-Hyderabad Campus. She received her undergraduate degree from Dr. M.G.R Medical University, Chennai and Master's degree in Pharmaceutical Chemistry from IIT-BHU. In 2005, she was awarded a Ph.D. in Medicinal Chemistry from the Institute of Medical Sciences, Banaras Hindu University, Varanasi for research conducted at Eberhard Karls University of Tuebingen, Germany (DAAD Fellow) and IMS-BHU (CSIR-SRF). After completing her PhD in 2005, she joined as an Assistant Professor in the Department of Pharmaceutics, IIT-BHU and worked for four and half years. In 2010 she moved to BITS-Pilani Hyderabad Campus. She has around 18 years of teaching and research experience. She was appointed as the Head of the Department of Pharmacy in 2019, which is being continued to date. Under her supervision and guidance, six students successfully defended their PhD thesis and currently, five students are doing their PhD research under her. Five government-funded projects from agencies UGC, CSIR, DST, ICMR-ICSSR and TSCOST have been completed by her during her tenure in BITS-Pilani HyderabadCampus. She has published more than 50 research papers and has filed 03 patents. She is also an author of 2 book chapters published by Springer Wien New York. Prof Sajeli Begum is a lifetime member of various scientific forums like the Association of Pharmaceutical Teachers of India (APTI), Indian Pharmacy Graduates Association (IPGA), Indian Chemical Society, the Society for Ethno Pharmacology, Member of Indian Phytopathological Society and Member of International Cytokine and Interferon Society.

Biography of Prof. Swati Alok

Prof. Swati Alok is currently an Associate Professor in Department of Economics and Finance, BITS Pilani-Hyderabad Campus. She was awarded a PhD in Management from BITS Pilani. She has five years of industry experience as a human resource professional and around 12 years of teaching and research experience. Her research accomplishments include 39 articles in reputed national and international journals and has 7 book chapters. Additionally, she has presented her works at 20 international conferences. Also, she has completed several external projects on themes of flexi-working, public health, women's empowerment, and women's career persistence funded by august institutions such as the Indian Council of Medical Research and the Indian Council of Social Science Research. She is currently involved in three new, externally funded projects on themes of health management, public health, women's entrepreneurship, and women's labour force participation. These are funded by Indian Council of Medical Research, Indian Council of Social Science Research, National Human Rights Commission, and Telangana State Council of Science & Technology.