EVALUATION OF ANTI-RHEUMATOID ARTHRITIS EFFECT OF *ACROSTICHUM AUREUM* ON COMPLETE FREUND'S ADJUVANT INDUCED RHEUMATOID ARTHRITIS IN RODENTS

Thesis submitted in Partial Fulfilment for the Requirement of

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Submitted By

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CERTIFICATE

This is to certify that, Monihar Pervin has carried out the research on the project entitled "**Exploration of Anti-Rheumatoid Arthritic Potential of *Acrostichum aureum* Linn. on Complete Freund's Adjuvant Induced Rheumatoid Arthritis in Rodents**" under my supervision, in the Division of Pharmacology and Toxicology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700032.

She has incorporated her findings into this thesis of the same title being submitted by her in partial fulfilment of the requirement for the award of Degree of Master of Pharmaceutical Technology, Jadavpur University. I am satisfied that she has carried out her thesis with proper care and confidence to my entire satisfaction.

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DECLARATION

I declare that, "**Exploration of Anti-Rheumatoid Arthritis Potential of *Acrostichum aureum* Linn. on Complete Freund's Adjuvant Induced Rheumatoid Arthritis in Rodents**" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Signature of The Student:

Full Name:

Date:

Dedicated

To

My Family & Guide

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Date: **Monihar Pervin**

PREFACE

The present study entitled "**Exploration of Anti-Rheumatoid Arthritic Potential of *Acrostichum aureum* Linn. on Complete Freund's Adjuvant Induced Rheumatoid Arthritis in Rodents**" covers original research work conducted by the author for the award of Master of Pharmacy in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata

The vast array of diversity seen in plants has drawn the attention of pharmaceutical corporations searching for new drugs and lead compounds. We employ medicinal herbs to treat a wide range of illnesses because of their extensive availability, low toxicity, and little or non-existent side effects. Traditional applications are becoming an important area of research since they require a scientific foundation to be valuable. As a result, in relation to the other research-related parts, the thesis covered the aforementioned topic in a logical and coherent manner.

In conclusion, the detailed study has been put together in a manner that justifies the study's relationship to establishing pharmacological activities, notably anti- rheumatoid arthritis activity.

Monihar Pervin

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CHAPTER 1: INTRODUCTION

1.1 Inflammation and Autoimmunity

Inflammation is a protective response mounted by the body's immune system to eliminate harmful stimuli, such as pathogens, damaged cells, or irritants. It is characterized by the coordinated release of various inflammatory mediators, including cytokines, chemokines, and other signalling molecules, which orchestrate the recruitment and activation of immune cells at the site of injury or infection (Chen et al., 2017). While acute inflammation is a necessary and beneficial process, chronic or uncontrolled inflammation can lead to tissue damage and contribute to the development of various diseases, including autoimmune disorders.

Autoimmunity refers to the aberrant activation of the immune system against the body's own tissues and molecules, resulting in the production of autoantibodies and autoreactive immune cells (Rosenblum et al., 2015). In autoimmune diseases, the immune system fails to recognize self-antigens as harmless and mounts an inappropriate and persistent inflammatory response, leading to tissue damage and dysfunction. The underlying mechanisms that trigger autoimmunity are complex and involve genetic predisposition, environmental factors, and dysregulation of immune tolerance mechanisms (Pollard et al., 2013).

1.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder characterized by persistent inflammation of the synovial joints, leading to progressive joint destruction and disability (Smolen et al., 2016). This debilitating condition affects millions of people worldwide, significantly impacting their quality of life and imposing a substantial economic burden on healthcare systems and societies (Singh et al., 2015). The disease primarily manifests in the synovial joints, causing unbearable pain, fever, chronic proliferative synovitis, symmetrical inflammatory polyarthritis, and synovial tissue and cartilage destruction (Smolen et al., 2016; McInnes & Schett, 2011).

RA is a complex multifactorial disease, with both genetic and environmental factors contributing to its pathogenesis. Epidemiological studies have revealed a global prevalence of RA around 0.46%, with a higher incidence among women compared to men (Smolen et al., 2016; Myasoedova et al., 2010). In India, the prevalence is even higher, estimated at approximately 0.7%, surpassing the global average (Chopra & Abdel-Nasser, 2008). The disease typically manifests in the late twenties to early fifties, with women being five times more susceptible than men (Smolen et al., 2016; Crowson et al., 2011).

This chronic inflammatory state not only affects the joints but can also lead to extra-articular manifestations, such as subcutaneous nodules, pulmonary disease, vasculitis, and neuropathy (Smolen et al., 2016; Cojocaru et al., 2010). Consequently, RA is considered a multi-systemic illness, impacting overall quality of life and increasing the risk of cardiovascular and other comorbidities (Smolen et al., 2016; Nurmohamed & Heslinga, 2007; Kitas & Gabriel, 2011).

1.3 Global Scenario

1.3.1 Global Prevalence Rheumatoid arthritis is a global health concern, affecting individuals across different geographical regions, ethnicities, and socioeconomic backgrounds. According to the World Health Organization (WHO), the global prevalence of RA is estimated to be

around 0.24%, with higher rates observed in developed countries (World Health Organization, Accessed May 15, 2024). However, the prevalence varies significantly among different populations, with reports ranging from 0.3% to 1.2% (Almutairi et al., 2021).

1.3.2 Indian Prevalence In India, the prevalence of RA is estimated to be around 0.7%, which is higher than the global average (Zaman et al., 2022). This highlights the significant burden of the disease in the Indian population. Several factors, including genetic predisposition, environmental exposures, and lifestyle factors, may contribute to the increased prevalence in India (Malaviya et al., 1993).

1.4 Risk Factors

The development of rheumatoid arthritis is influenced by a complex interplay of genetic and environmental factors (Cheng & Tseng, 2022).

Genetic factors: The presence of certain genetic markers, particularly the human leukocyte antigen (HLA) genes, such as HLA-DRB1, has been associated with an increased risk of developing RA (Miller et al., 2022)

1. Environmental factors: Several environmental factors have been implicated in the development of RA, including smoking, infections (e.g., Epstein-Barr virus, Porphyromonas gingivalis), and exposure to certain pollutants or chemicals (Makrygiannakis et al., 2008, (Konig et al., 2022)
2. Hormonal factors: Hormonal changes, particularly related to estrogen levels, may contribute to the higher incidence of RA in women compared to men (Pikwer et al., 2009).
3. Autoantibodies: The presence of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), is a hallmark of RA and is believed to play a crucial role in the disease pathogenesis (Aletaha et al., 2010).

1.5 Signs and Symptoms

The primary symptoms of RA include joint pain, swelling, and stiffness, particularly in the small joints of the hands and feet. These symptoms are typically symmetrical, affecting the same joints on both sides of the body. As the disease progresses, larger joints, such as the knees, hips, and shoulders, may also become involved (Scott et al., 2010).

❖ Common signs and symptoms of RA include:

- **Joint pain and swelling:** Persistent joint pain and swelling, often affecting multiple joints simultaneously, are hallmarks of RA.
- **Morning stiffness:** Patients commonly experience prolonged stiffness in the affected joints, particularly in the morning or after periods of inactivity.
- **Fatigue:** Overwhelming fatigue is a common symptom, often disproportionate to the level of joint involvement.

- **Low-grade fever:** Some patients may experience low-grade fever due to the underlying inflammatory process.
- **Rheumatoid nodules:** Firm, non-tender subcutaneous nodules may develop over bony prominences or in areas subjected to pressure.

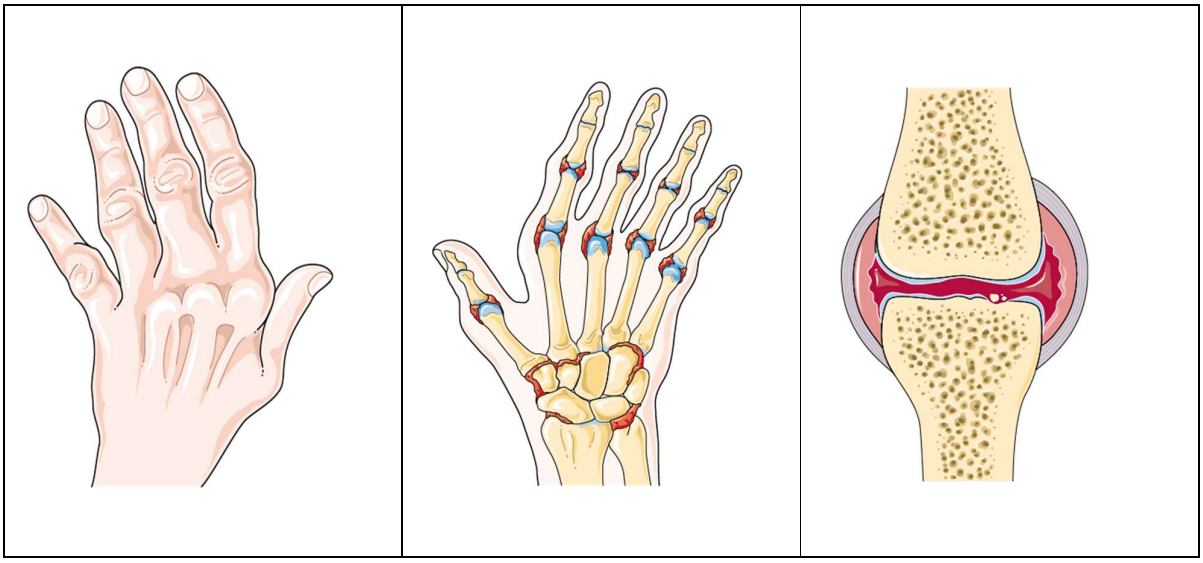

Fig. 1: Sign and Symptoms of Rheumatoid Arthritis

1.6 Types of Effect in Joints

Rheumatoid arthritis can lead to various deformities and joint damage due to the chronic inflammatory process and subsequent destruction of cartilage and bone. Some common joint effects and deformities observed in RA include:

1. Ulnar deviation: A characteristic deformity in which the fingers deviate towards the ulnar (little finger) side of the hand, resulting from the weakening of the tendons and ligaments around the wrist and metacarpophalangeal joints.
2. Swan-neck deformity: A deformity of the fingers in which the proximal interphalangeal (PIP) joint is hyperextended, and the distal interphalangeal (DIP) joint is flexed, resembling the shape of a swan's neck.
3. Boutonnière deformity: A deformity in which the PIP joint is flexed, and the DIP joint is hyperextended, creating a button-like protrusion at the PIP joint.
4. Joint erosions and destruction: The chronic inflammatory process in RA can lead to the erosion of cartilage and underlying bone, resulting in joint deformities, instability, and loss of function.

5. Joint fusion (ankylosis): In severe cases, the continuous inflammation and joint damage can lead to the fusion of joint surfaces, causing complete immobility and loss of joint function.

1.7 Pathophysiology

The pathogenesis of rheumatoid arthritis involves a complex interplay between genetic, environmental, and immunological factors, resulting in a dysregulated immune response and chronic inflammation (McInnes & Schett, 2017). The key events in the pathophysiology of RA include:

1. Autoantibody production: The presence of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), is a hallmark of RA and contributes to the formation of immune complexes and activation of the complement system (McInnes & Schett, 2011)

2. T-cell activation: Autoreactive T cells, particularly CD4+ T helper cells, play a crucial role in the initiation and perpetuation of the inflammatory process by producing pro-inflammatory cytokines and promoting B-cell activation and autoantibody production (McInnes & Schett, 2011)

3. Synovial inflammation: The synovial membrane, which lines the joints, becomes inflamed due to the infiltration of various immune cells, including T cells, B cells, macrophages, and neutrophils. This leads to the production of inflammatory mediators, such as cytokines (e.g., tumor necrosis factor-alpha [TNF-α], interleukin-6 [IL-6], and IL-1β), which further amplify the inflammatory response (Guo et al., 2018)

4. Cartilage and bone destruction: Activated immune cells and inflammatory mediators stimulate the production of matrix metalloproteinases (MMPs) and other enzymes that degrade cartilage and bone. Additionally, the formation of a pannus (abnormal layer of granulation tissue) contributes to the erosion of cartilage and bone (Firestein & McInnes, 2017).

5. Systemic effects: RA is a systemic disease, and the chronic inflammatory state can lead to various extra-articular manifestations and comorbidities, such as cardiovascular disease, osteoporosis, and interstitial lung disease (England et al., 2018).

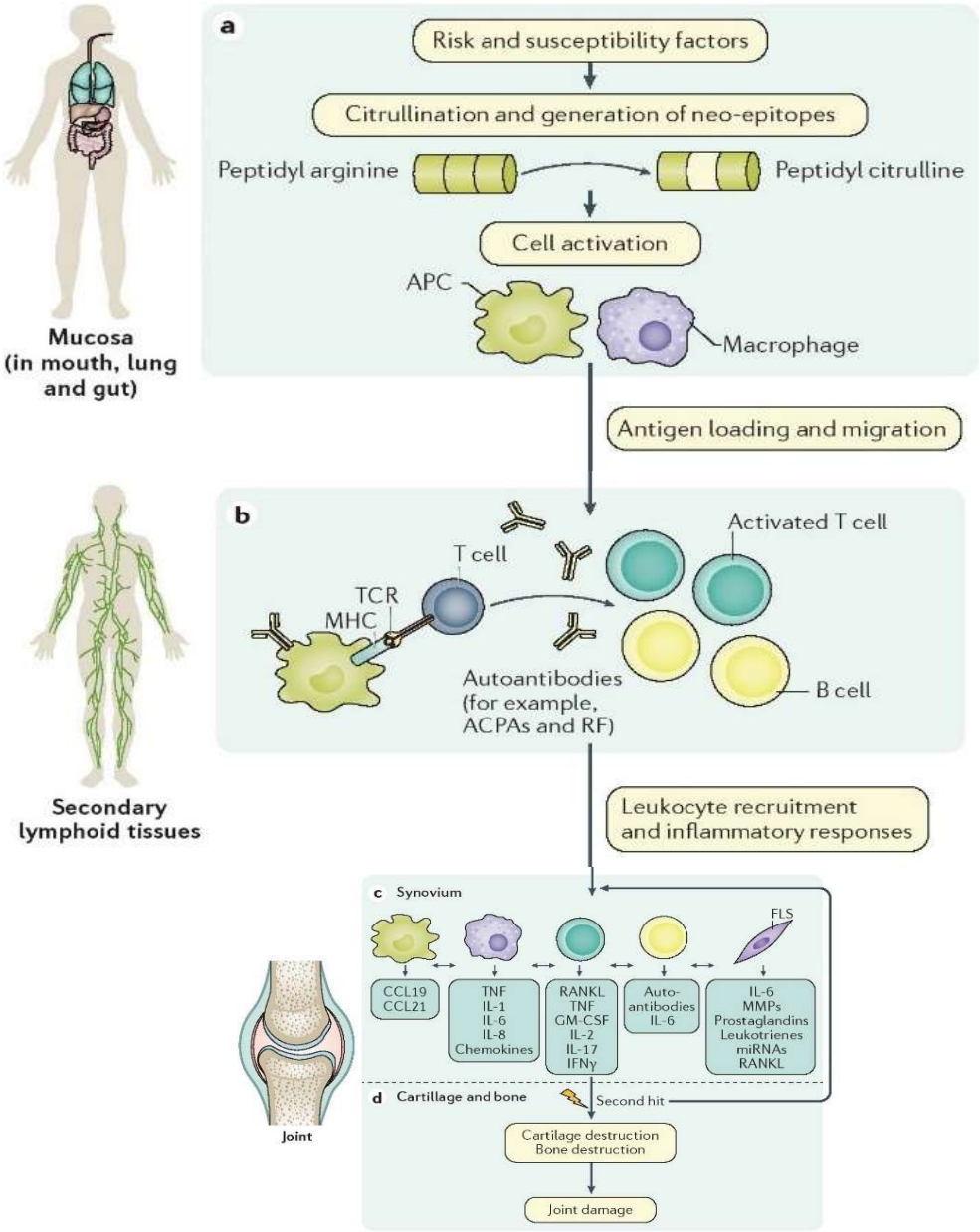

Fig.2: Pathophysiology of Rheumatoid Arthritis

1.8 Etiology

The exact aetiology of rheumatoid arthritis is not fully understood, but it is believed to result from a complex interplay between genetic and environmental factors that trigger an abnormal immune response (Cheng & Tseng, 2022) The main factors contributing to the development of RA include:

1. **Genetic factors:** Certain genetic markers, particularly the HLA-DRB1 alleles, have been consistently associated with an increased risk of developing RA. These genetic

variations may influence the immune system's ability to recognize and respond to self-antigens (Hafkenscheid et al., 2022).

2. **Environmental triggers:**
    - Infections: Certain viral and bacterial infections, such as Epstein-Barr virus and Porphyromonas gingivalis (a bacterium associated with periodontal disease), have been linked to an increased risk of developing RA, potentially through molecular mimicry or triggering of autoimmune responses (Konig et al., 2022, Bingham, 2013)
    - Air pollution: Exposure to air pollutants, such as particulate matter and silica, has been associated with an increased risk of developing RA, possibly due to their ability to induce oxidative stress and inflammation

3. .**Hormonal factors:** The higher prevalence of RA in women, especially during the reproductive years, suggests a potential role of hormonal factors in disease pathogenesis. Estrogen and its metabolites have been shown to modulate immune responses and inflammation (Mohammadnezhad et al.) **Autoantibodies:** The presence of autoantibodies, particularly rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), is a hallmark of RA and is believed to play a crucial role in disease initiation and perpetuation. These autoantibodies can form immune complexes and activate the complement system, leading to inflammation and tissue damage. (Aletaha et al., 2010)

2. **Molecular mimicry:** Certain microbial or viral antigens may share structural similarities with self-antigens, leading to cross-reactivity and activation of autoreactive T and B cells, contributing to the development of autoimmunity. (Ercolini & Miller, 2009)

3. **Dysregulation of Immune tolerance:** Mechanisms that maintain immune tolerance, such as regulatory T cells and anergy, may be impaired in individuals with RA, allowing autoreactive immune cells to escape regulation and perpetuate inflammation. (Mohan et al., 2021)

The interplay of these various factors is thought to initiate and drive the autoimmune response, leading to the chronic inflammation and joint damage characteristic of rheumatoid arthritis.

1.9 Conventional Treatments

The management of rheumatoid arthritis typically involves a multidisciplinary approach, combining pharmacological interventions, non-pharmacological therapies, and lifestyle modifications. The primary goals of treatment are to alleviate symptoms, slow disease progression, preserve joint function, and improve overall quality of life. (Smolen et al., 2016)

Pharmacological Therapy:

1. Non-steroidal anti-inflammatory drugs (NSAIDs): These medications, such as ibuprofen, naproxen, and celecoxib, are used to reduce inflammation and relieve pain

associated with RA. However, they do not modify the underlying disease process. (Singh et al., 2016)

2. Disease-modifying antirheumatic drugs (DMARDs):
    - Conventional synthetic DMARDs: These drugs, including methotrexate, leflunomide, and sulfasalazine, are the mainstay of RA treatment. They can slow disease progression and prevent joint damage by suppressing the underlying inflammatory process. (Smolen et al., 2020)
    - Targeted synthetic DMARDs: These are newer drugs, such as tofacitinib and baricitinib, that inhibit specific signalling pathways involved in the inflammatory process. (Smolen et al., 2020)
3. Biologic agents: These are targeted therapies that inhibit specific inflammatory pathways or immune cells involved in RA pathogenesis. Examples include anti-TNF agents (e.g., etanercept, adalimumab, infliximab), anti-B cell agents (e.g., rituximab), anti-IL-6 agents (e.g., tocilizumab), and inhibitors of other cytokines or cell signalling pathways. (Singh et al., 2016)
4. Corticosteroids: These drugs, such as prednisone and methylprednisolone, are potent anti-inflammatory agents used to rapidly control acute flares of RA. However, long-term use is associated with significant adverse effects. (Strehl et al., 2020)

The choice of pharmacological therapy is guided by various factors, including disease severity, treatment response, comorbidities, and potential adverse effects. In many cases, a combination of different medications may be used to achieve optimal disease control and minimize side effects. (Smolen et al., 2020)

However, long-term use of conventional medications can lead to severe adverse effects, prompting patients and researchers to explore alternative and complementary therapies, particularly those derived from natural sources (Smolen et al., 2016; Efthimiou et al., 2010). Traditional herbal medicine has demonstrated promising results in the management of RA, with recent studies reporting a 60-90% increase in the use of natural product-derived medicines for this condition (Smolen et al., 2016; Efthimiou et al., 2010; Soeken et al., 2003).

Medicinal plants offer a potential avenue for the development of novel therapeutic agents for RA, with numerous plant species currently under continuous evaluation and research for this purpose (Smolen et al., 2016; Mur et al., 1999; Khanna et al., 2007). The use of plant-based remedies for the treatment of RA has a long history, rooted in traditional practices and ethnopharmacological claims across various cultures and regions (Efthimiou et al., 2010; Soeken et al., 2003).

Among the plants with ethnobotanical and ethnopharmacological claims in the treatment of RA are mangrove species (Smolen et al., 2016; Bandaranayake, 2002). One such plant, *Acrostichum aureum (A. aureum)* Linn. (Family: Pteridiaceae), also known as golden leather fern, mangrove fern, or tiger fern, has been traditionally used in various parts of Asia for the treatment of snake bites, wounds, and rheumatism (Smolen et al., 2016; Banerjee et al., 2002).

This plant is rich in secondary metabolites, including flavonoids, glycosides, gums, sterols, saponins, alkaloids, tannins, terpenoids, and triterpenoids (Smolen et al., 2016; Dey et al., 2012), which may contribute to its therapeutic potential.

Notably, the aerial parts of *Acrostichum aureum* have demonstrated antioxidant and anti-inflammatory properties in preliminary studies, which could be effective in reducing several inflammatory parameters, such as interleukin-beta (IL-β) and tumour necrosis factor-alpha (TNF-α) levels, thus potentially alleviating the signs and symptoms of RA (Smolen et al., 2016; Dey et al., 2012; Usha et al., 2009). These findings highlight the promising anti-arthritic potential of this plant and warrant further investigation.

Despite the growing interest in natural products for the management of RA, there is a need for rigorous scientific evaluation and validation of the efficacy and safety of these plant-based remedies (Efthimiou et al., 2010; Soeken et al., 2003). The current study aims to contribute to this research area by systematically evaluating the anti-arthritic potential of *Acrostichum aureum* using the well-established Complete Freund's Adjuvant (CFA)-induced arthritis model in rats.

The CFA-induced arthritis model is a widely used experimental model for studying RA, as it closely mimics the pathophysiological features of the human disease, including joint inflammation, cartilage degradation, and bone erosion (Brand et al., 2007; Mia et al., 2017). By inducing arthritis in rats using CFA and subsequently administering the extract of *Acrostichum aureum,* this study aims to assess the plant's ability to alleviate the signs and symptoms of arthritis, as well as its potential mechanisms of action.

The specific objectives of this study are:

- To investigate the Anti-arthritic efficacy of *Acrostichum aureum* hydroalcoholic extract in the CFA-induced arthritis rat model by evaluating various parameters, including paw swelling, arthritis scoring, and joint histology.
- To evaluate the antioxidant potential of *Acrostichum aureum* extract through in vitro antioxidant assays, such as DPPH radical scavenging and superoxide radical scavenging assays.
- To determine the total phenolic and flavonoid content of *Acrostichum aureum* extract, as these phytochemicals are known to contribute to the antioxidant and anti-inflammatory properties of plants (Pandey & Rizvi, 2009; Heim et al., 2002).
- To evaluate the toxicological profile of *Acrostichum aureum* extract through acute oral toxicity studies, ensuring its safety for potential therapeutic applications.
- To explore the potential mechanisms of action underlying the anti-arthritic effects of *Acrostichum aureum,* including its impact on inflammatory mediators, oxidative stress markers, and joint histopathology.

1.10 Diagnosis

The diagnosis of rheumatoid arthritis is based on a combination of clinical evaluation, laboratory tests, and imaging studies. The American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) have developed classification criteria to aid in the diagnosis of RA. (Aletaha et al., 2010)

- **Clinical evaluation:** A detailed medical history and physical examination are essential to assess joint involvement, morning stiffness, fatigue, and other symptoms suggestive of RA.
- **Laboratory tests:**
    - Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA): The presence of these autoantibodies supports the diagnosis of RA, although their absence does not exclude the diagnosis. (Trouw & Mahler, 2012).
    - Acute-phase reactants: Elevated levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) indicate systemic inflammation and can help monitor disease activity. (Firestein & McInnes, 2017)
    - Other laboratory tests: Complete blood count, liver and kidney function tests, and other tests may be performed to assess for potential complications or comorbidities.
- **Imaging studies:**
    - X-rays: Radiographs of affected joints can reveal joint space narrowing, erosions, and other structural changes associated with RA. (Zayat et al., 2019)

Early diagnosis and initiation of appropriate treatment are crucial in managing rheumatoid arthritis, as prompt intervention can help prevent or slow the progression of joint damage and disability.

The outcomes of this research endeavour hold significant implications for the development of novel, plant-derived therapeutic interventions for the management of RA. By elucidating the anti-arthritic potential and mechanisms of action of *Acrostichum aureum,* this study may pave the way for further preclinical and clinical investigations, ultimately leading to the discovery of safe and effective alternative or complementary therapies for this debilitating condition.

Moreover, the findings from this study will contribute to the growing body of knowledge on the therapeutic potential of mangrove-derived natural products, highlighting the importance of conserving and sustainably utilizing these unique ecosystems for drug discovery and development purposes (Bandaranayake, 2002; Kathiresan & Bingham, 2001).

The significance of this research extends beyond the scientific realm, as it addresses a pressing global health issue. RA is a chronic condition that imposes a substantial burden on individuals, healthcare systems, and societies worldwide (Singh et al., 2015; Cross et al., 2014). The development of effective and affordable treatment options, particularly those derived from readily available natural sources, could potentially improve the quality of life for millions of

individuals affected by this debilitating disease, while also reducing the associated healthcare costs and economic burden (Singh et al., 2015; Woolf & Pfleger, 2003).

Furthermore, the potential discovery of novel therapeutic agents from *Acrostichum aureum* aligns with the broader objectives of promoting sustainable and environmentally responsible practices in drug development. By exploring the medicinal properties of this mangrove plant, this study contributes to the valorisation of natural resources and the preservation of biodiversity, while also promoting the incorporation of traditional knowledge and practices into modern scientific research (Bandaranayake, 2002; Kathiresan & Bingham, 2001).

Conclusion:

In summary, the introduction section of this thesis provides a comprehensive overview of RA, its epidemiology, pathophysiology, risk factors, and current treatment approaches, while highlighting the limitations of conventional therapies and the need for alternative and complementary interventions. It establishes the rationale for exploring the therapeutic potential of medicinal plants, specifically *Acrostichum aureum,* based on its traditional use and preliminary scientific evidence. The introduction outlines the specific objectives of the study, emphasizing the systematic evaluation of the anti-arthritic efficacy, antioxidant potential, phytochemical composition, and mechanisms of action of *Acrostichum aureum* extract, as well as its toxicological profile. The significance of this research is highlighted, underscoring its potential contributions to the development of novel therapies, the conservation of natural resources, and the integration of traditional knowledge into modern scientific practices. Overall, this introduction sets the stage for a comprehensive and rigorous investigation into the anti-arthritic properties of *Acrostichum aureum,* with far-reaching implications for the management of RA and the promotion of sustainable drug discovery and development.

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CHAPTER 2: PLAN OF WORK

2.1: RATIONALE

Arthritis and related disorders, including Rheumatoid Arthritis (RA), are common diseases affecting millions of people (Hunter et al., 2017). RA is characterized by articular injuries having an inflammatory propagation of synovial cells, attaining a nearly complete functional defect. It affects about 1% of the general population (Smolen et al., 2016). RA is a kind of chronic inflammatory autoimmune disease. Although a number of drugs used in the treatment of RA, have been developed over the past few decades, there is still a need for more effective drugs with lower side effects (Burmester & Pope, 2017). Conventional medicine, including treatment with steroids, nonsteroidal anti-inflammatory drugs (NSAIDs) and biological agents such as tumour necrosis factor alpha (TNF-) and interleukin-1 beta (IL-1β) antagonists, has shown only limited success against RA. Such therapies are helpful in controlling the symptoms of acute RA, but their effects on chronic, prolonged RA are unsatisfactory.

NSAIDs are conservatively favoured in RA due to their analgesic and anti-inflammatory properties, by inhibiting the production of prostaglandins by targeting Cyclooxygenases 1 and Cyclooxygenases 2. Though it is widely used it has some major side effects such as gastrointestinal, renal, cardiac, and hepatic disorders for COX-1 and myocardial infarction and strokes for COX-2 (Crofford, 2013). Methotrexate “Chemical DMARDs” and corticosteroids “Biologic DMARDs” are also helpful in the management of rheumatoid arthritis. But, methotrexate causes significant hepatic problems, and corticosteroids weaken the immune system, making people more susceptible to infections, as well as causing fragile bone, gut ulcers, bleeding of gastric mucosa, renal and hepatic complications (Thakur et al., 2018). Therefore, more and more patients are experimenting with natural medicinal options in order to cope with this debilitating disease. A recent investigation has estimated that 60–90% of patients with rheumatoid arthritis are very likely to use botanicals (Rao et al., 1999).

Natural goods are more approachable, economically affordable choice than manufactured medicines, with lower toxicity and fewer adverse effects. Medicinal plants have been shown to be strong and effective in the treatment of RA, and numerous medicinal plants are now being researched for the creation of new medicines. Many mangrove plants have been proved to possess a number of biological activities and extensively used to treat various diseases by the local inhabitant.

Acrostichum aureum Linn., a mangrove fern belonging to the family Pteridaceae, mostly grows in mangrove forests and coastal regions of tropical and subtropical areas worldwide, especially in Southeast Asia, Central America, and Africa. Traditionally, *Acrostichum aureum Linn.* is broadly utilized for the treatment of diverse diseases. The rhizomes and leaves of *Acrostichum aureum Linn.* are widely used against **worm infections, wounds, peptic ulcers, boils, and bleeding and the roots and leaves of it are used to treat rheumatism, wounds, and boils**. Modern researches also confirm the anti-inflammatory and antioxidative activities of *Acrostichum aureum Linn.*

Increasing evidence has shown that CFA-Induced Rheumatoid arthritis is closely linked to oxidative stress and inflammation, and the *Acrostichum aureum Linn.* has great antioxidative and anti-inflammatory as well as rheumatism properties. However, the anti-rheumatoid arthritis

activity of *Acrostichum aureum Linn.* still remains unreported. Therefore, in this study, we prepared hydro-alcoholic extract from the aerial parts of *Acrostichum aureum Linn.* (AAHE) and examined its anti-rheumatoid arthritis effect against Complete Freund's Adjuvant (CFA) Induced Rheumatoid Arthritis in Rats by measuring the reduction of inflammation, pain and swelling of the knee joints.

2.2: OBJECTIVE OF THE WORK

- ✓ Antioxidant assay of the selected mangrove plant.
- ✓ Total phenolic content (TPC) and Total flavonoid content (TFC) determination.
- ✓ Acute oral toxicity.
- ✓ Evaluation of the Anti-Rheumatoid Arthritis activity of the selected plants through In-vivo assay.

2.3: PLAN OF WORK

- ✓ Collection and Authentication of *Acrostichum aureum Linn.* plant.
- ✓ Hydro-alcoholic extraction of the selected plant by Soxhlet extraction.
- ✓ Preliminary phytochemical study of the extract.
- ✓ DPPH radical scavenging assay.
- ✓ SOD anion scavenging assay.
- ✓ Total phenolic content (TPC) and Total flavonoid content (TFC) determination.
- ✓ Determination of LD_{50} value (Acute Toxicity).
- ✓ Evaluation of In-vivo Anti-Rheumatoid Arthritis potential.
    - ❖ Physical Parameter Evaluation
        - o Body Weight Measurement.
        - o Paw Diameter Measurement.
        - o Paw Volume Measurement.
        - o Arthritic Score Evaluation.
    - ❖ Sreum Bio-Chemical, Tissue Anti-oxidant Evaluation
        - o Tissue antioxidant parameters.
        - o Tissue free radical parameters.
    - ❖ Enumeration of Total Blood Count.
    - ❖ Imaging Study of Knee Joint.
    - ❖ Histopathology.

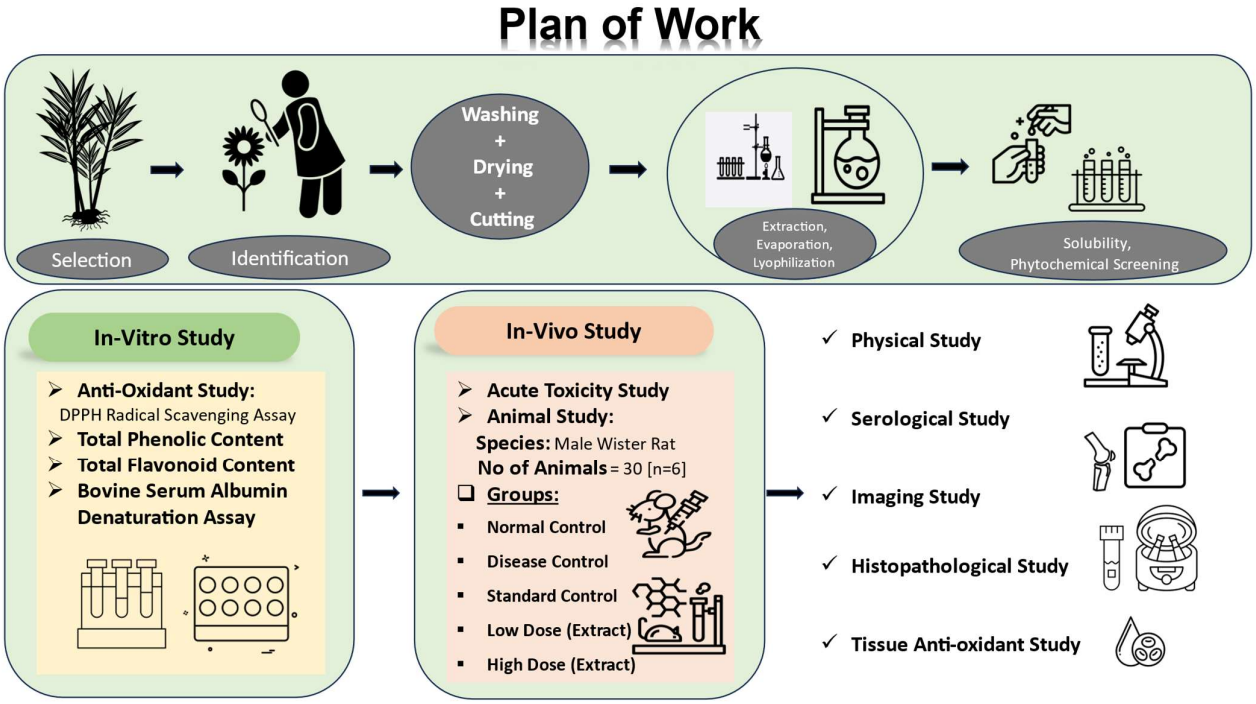

Fig.3: Diagram of Plan of Work

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CHAPTER 3: LITERATURE REVIEW

3.1: PLANT TAXONOMY:

Kingdom: Plantae

Phylum: Tracheophyta

Division: Polypodiophyta

Class: Polypodiopsida

Order: Polypodiales

Family: Pteridaceae

Subfamily: Ceratopteridoideae

Genus: *Acrostichum*

Species: *Acrostichum aureum*

Binomial name: *Acrostichum aureum Linn.*

("WoRMS - World Register of Marine Species - ***Acrostichum aureum L.,***")

Whole plant & leaves of *Acrostichum aureum*

3.2 PLANT DESCRIPTION:

Synonymes : ***Acrostichum longifolium*** *Willd.*

LOCAL NAME:

English: Mangrove Fern
India: Minni
Bangladesh: Hudo
Srilanka: Karen koku
Malaysia: Piuai raya
Chinese: Jin jue
Veitnam: Cary rang la
Philippines: Lagolo
Singapore: Tiger Fern
South Florida: Golden Leather Fern

PARTS USED: Leaves, Roots, Fronds, Rhizome.

3.3: MORPHOLOGY OF PLANT

The shrub grows up to 4m tall and has adventitious roots that are visible at the bottom of the leaves and knots. The stem is distributed horizontally and grows indefinitely. The fern has broad and glossy frond that is oblong and cuneate at the base, while the apex is mucronate. The venation of the leave is reticulate with uniform elongate areoles diverging from the thickened midrib without free vein endings. The stipes is woody and arises from glabrous woody rhizome. The fronds measure approximately 1m long and 50cm wide and are pinnate. There are 8–16 alternate pinnae which are dark green, leathery and widely spaced. At the basal region, the fronds are greenish-yellow in colour and a golden colour at the apex. The central fronds are almost straight, but the outer fronds arch over sideways. Some of the fronds produce sporangia located at the apical region along the veins. The sporangia perform the reproductive function by producing spores and are found in the five to eight distal pinna pairs and terminal pinna. The sporangia are brick red and add a felted appearance to the pinnae. The non-indusiate and densely aggregated sori are formed between June and October. (Duke, 2006)

- **Diagnostic characters:**

Mangrove fern, terrestrial, erect, 1 – 2 m tall. Fronds (leaves of ferns) are pinnate and leathery, sori are found at the extremity of apical pinnae, along with the veins.

- **Botany & morphology:**
  Fronds once-pinnate; leaflet 8-14, alternate, linear-oblong, retusely mucronate at apex, cuneate at base, entire, venation reticulate with uniform elongate areoles diverging from the thickened midrib without free vein endings. Fertile fronds only on upper leaflet, lowest leaflet always distant, long stalked. Sori densely aggregated along the undersurface, non-indusiate. Scales broad, restricted at the base of fronds. Stipes are woody, glabrous, arising from a stout woody rhizome. (Duke, 2006)

- **Regeneration:**
  Vegetative propagation through rhizomatous roots; new individuals also develop from spores via gametophytes, especially in disturbed sites. (Hovenkamp, 1998)

- **Reproductive Biology:**
  Germination of spores seems most successful in fresh water. Sex-organ (antheridia and archegonia) ontogeny occurs in sequence so that, out crossing is promoted. (Global Invasive Species Database, 2021)

- **Ecology:**
  It is a strong weedy and aggressive species occurs in back mangroves and associated tidally influenced estuarine. Light-tolerant or even light-demanding species. It can survive without regular tidal inundation. (Nable & Snow, 1985)

3.4 GEOGRAPHICAL DISTRIBUTION:

The natural habitats of *A. aureum* are swamps and mangrove areas, riverbanks and salt marshes. It tolerates high salinity levels, but fresh water promotes spore germination. It grows on small elevations in the mangrove swampy regions, but it is occasionally found in freshwater locations. Thus, *A. aureum* shows strong adaptation to fluctuating environmental conditions and therefore grows in the intertidal zone, especially those that have been altered by human activities. The plant is native to all over the Southeast Asia, Africa especially in the tropical and sub-tropical regions of many counties including, Brazil, USA, India, Bangladesh, Vietnam, Sri Lanka, China, Costa Rica, Taiwan, Japan, Philippine, Fiji, Trinidad, Senegal, Guinea, Gambia, Nigeria, Zimbabwe, Sierra Leone, Ghana, Mozambique, Kenya, Ivory Coast, Panama, and Jamaica. (Duke et al., 2007)

3.5: ETHNOMEDICINAL EVIDENCES:

Different parts of A. aureum are widely used tradition ally to cure various ailments and diseases in different countries across the world. The following table shows a compact description of validation against the ethnomedicinal claim of the plant.

Activity	*Part used*	*Extracting solvent*	*Bioassay/ Model*	*Results*	*References*
Antioxidant	T	Methanol	DPPH radical scavenging and Mice brain	DPPH scavenging action with an EC50 of 103.0 µg/ml and an IC50 of 28.99 µg/ml for inhibition of lipid peroxidation	(Vadlapudi et al., 2009)
Antioxidant	L`	Ethanol	DPPH radical scavenging	Significant DPPH scavenging action with an IC50 value of 41.95 µg/ml	(Samydurai & Thangapandian, 2012)
Antioxidant	L`	Methanol	Ferric reducing power	High TPC (524 mg GAE and 51% anti-lipid peroxidation activity	(Lai et al., 2012)
Antioxidant	R	Petroleum ether	DPPH, ABTS, hydroxyl and superoxide	Strong scavenging activity with IC50 values of 31.56, 25.16, 26.12 and 26.18 µg/ml respectively.	(Hari et al., 2014)
		Benzene		Strong scavenging activity with IC50 values of 34.13, 30.18, 22.46 and 24.16 µg/ml respectively	(Hari et al., 2014)
		Ethyl acetate		Strong scavenging activity with IC50 values of 30.36, 31.48, 27.16 and 28.16 µg/ml respectively	(Hari et al., 2014)
		Methanol		Strong scavenging activity with IC50	(Hari et al., 2014)

				values of 36.54, 32.16, 30.11 and 30.96 µg/ml respectively	
		Ethanol		Robust scavenging activity with IC50 values of 32.16, 30.84, 28.36 and 34.84 µg/ml respectively	(Hari et al., 2014)
Antioxidant	L	Ethanol	DPPH radical scavenging	Strong DPPH scavenging action (IC50 = 29.53 ppm) It has high TPC and flavonoid contents of 366.44 mg GAEg−1&28 mg QE g− 1 respectively	(Perumal et al., 2017)
Analgesic	L	Ethanol (250 & 500 mg/kg)	Mice /Acetic acid induced writhing	Dose dependent inhibition of writhing	(Hamid et al., 2014a)
Contraceptive	WP	Ethanol &Acetone	Female rats	100% pregnancy prevention by water soluble fraction of the ethanol extract.	(Asadujjaman et al., 2015)
Cytotoxic/Anti-Cancer	AP	Methanol	Hep-G2, SKLU-1 & MCF-7	Isolated (+)-pinoresinol-4-O-sulfate showed weak cytotoxicity against the cells with IC50 values of 64.73, 65.54 and 73.78 µg/ml	(Minh et al., 2018a)
	WP	Ethyl acetate	HeLa cells	HeLa growth Inhibition (IC50 value of 6.3 µg/mL)	(Chen et al., 2019)
	WP	Methanol	NIH3T3, AGS, HT-29 & MDA-MB- 435S	Low toxicity against NIH3T3, but selective cytotoxicity against the remaining cell lines.	(Islam et al., 2015)

	WP	Methanol	AGS, HT29, MCF-7 MCF-7& MDAMB-231 cells	Isolated patriscabratine exerts moderate cytotoxicity against the cells except NIH3T3. The IC50 values were between 69.8&197.3 µM, while IC50 values for tetracosane ranged from 128.7 to > 250 µM.	(Minh et al., 2019)
	Ar	Methanol	AGS, HT29, MCF-7 & MDA-MB-231cells	IC50 values for cytotoxic activity of isolated (2S,3S) sulfated pterosin C against the cells ranged from 23.9 to 68.8 µM. Lowest value of 23.9 µM was recorded against AGS gastric adenocarcinoma cells.	(Minh et al., 2019)
Antibacterial	L & F	Methanol, Acetone Petroleum ether & Aqueous	*S. marcescens, E. coli, S. aureus, M. luteus* & *P. aeruginosa*	Methanol frond extract inhibited *P. aeruginosa, S. marcescens* & *E. coli*, while acetone extract inhibited all except *M. luteus*	(Biswas et al., 2016)
Antibacterial	L	DMSO (32.25 & 500 mg/ml)	*E. coli, S. paratyphi, S. aureus* & *P. aeruginosa*	*E. coli* and *S. aureus* were more sensitive to the extract & growth inhibition was more profound at 250& 500 mg/ml	(Vats & Tiwari, 2012)
Antibacterial	L	Methanol & Water	Vibrio species	Methanol extract inhibited Vibrio parahaemolyticus.	(Gopal et al., 2019)

Anti- ulcer	Ar	Water (100-400 mg/kg)	Rat/ethanol-induced gastric ulcer	Extract reduced stomach ulcer and oxidative damage by increasing GSH CAT & SOD, while MDA, TNF-α, IL-6,IL-1β, IκBa &p65 were reduced.	(Wu et al., 2018)
Anti-inflammatory	R	Ethanol (400 mg/kg)	Rats/ carrageenan- induced oedema	The extract exerts 65.90% reduction in paw volume similar to indomethacin	(Hamid et al., 2014)
Wound healing	Rh & L	Aqueous	Rabbit/rat excisional wound	Both extracts stimulate collagens production, fibroblasts proliferation and cells epithelization	(Lai et al., 2011) (Herman, 2019)
Anti-diarrheal	R	Ethanol (400 mg/kg)	Mice/ castor oil-induced diarrhea	Decreased diarrhea by 55% similar to loperamide	(Hossain et al., 2013)
Nutritional	Rh			The rhizome is rich in starch, lipid, protein and minerals	(Das et al., 2013)
Antiviral	Ar	Methanol	DENV2, CHIKV and hPiV3	Isolated novel phthalate showed antiviral activity against all the cell lines	(Lien et al., 2020)
Allergenic	Sp &Sm		Allergic rhinitis patients	Positive nasal provocation test	(Kanchan et al., 2008)
	Sp		Human skin	Elicited dermal contact allergy	(Koh et al., 2005)
Anthelmintic	L	Ethanol, Water & Petrolemether	*H. contortus*	Ethanol extract was more effective in causing death and paralysis to H. contortus than other extracts. It exerts 50% reduction in	(Devi et al., 2019)

				faecal egg count of infected sheep.	
Tyrosinase Inhibition	L	Methanol	Dopachrome	Inhibited tyrosinase activity by 33%	(Chai et al., 2011)
Phyto-remediation	WP		Shrimp farmland sediment soil	Root absorbed ciprofloxacin and norfloxacin	(Yu et al., 2021)
	WP		Shrimp farmland effluent	Reduction of pollution parameters including nitrate, BOD & COD	(Huang et al., 2014)
	WP		Plant irrigated with arsenate (0-500 ppm) water	Tolerance and reduction of arsenate toxicity.	(Huang et al., 2014)

R= Roots; L= Leaves; Ar= Arieal Part; WP= Whole Plant; Rh= Rhizome; S=Stem; T=Twig; F= Fronds;

❖ **Biological activity**

In addition to the ethnobotanical uses of *A. aureum,* many pharmacological activities have been reported for different parts and extracts of the plant. The Indians apply the frond as antidote for venomous snakebites, while the fertile fronds and roots are used in treating syphilitic ulcers, pharyngitis and diabetes. The natives of Fiji use different parts of A. aureum to treat chest pains, fever, elephantiasis, asthma, sore throat and constipation. It is also believed to be efficient in increasing the chances of a healthy pregnancy in Fiji. In addition, it is useful in treating respiratory ailments including throat infection and sinusitis. The plant is used as styptic, anthelmintic and also as an astringent in bleeding by the Keralans. Furthermore, the leaves of A. aureum are used to cure women with cloudy urine in Bangladesh and Costa Rica. The Cunas of Panama and Colombia extract fish bones from the throat using the young fiddleheads and as a medicinal bath for infants. In China, the rhizome is used to treat worm infections, inveterate ulcers and blad der ailments. It is a common remedy for treating haemorrhage, myelitis wound, rheumatism and boil in many parts of Asia. Young fronds of A. aureum are sold as vegetables that are eaten fresh and as well-cooked or blanched in Sri Lanka, Malaysia and Indonesia. As depicted in the Fig. 4, the plant has been found to exhibit analgesic, antioxidant, contraceptive, cytotoxic, anti-inflammatory, antibacterial and wound healing activities.

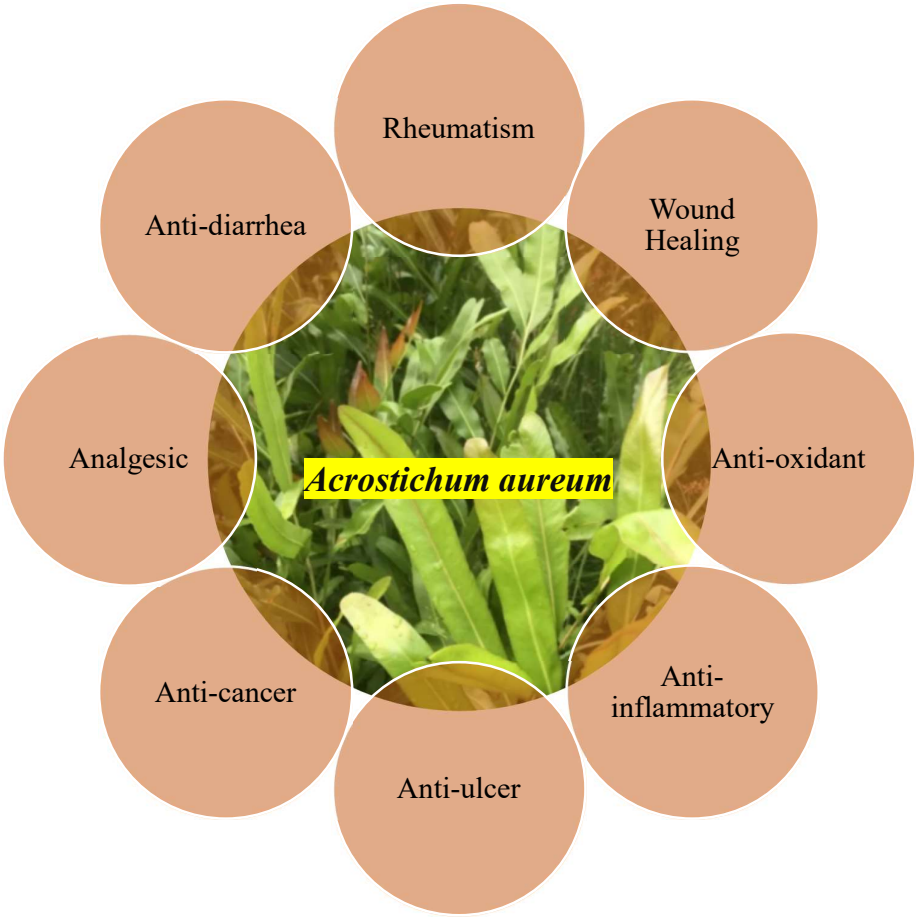

Fig.4: Biological Activity of *Acrostichum aureum* Linn.

- **Antioxidant activity**

  Antioxidants are compounds that prevent oxidation in living and non-living organisms. They can donate hydrogen and thereby reduce reactive oxygen species, reactive nitrogen species or metals in their oxidized forms. Antioxidants also can prevent free radical chain reactions that occur in living organisms. Due to their anti-radical and reducing properties, they play a major role in preparation of pharmaceutical formulations against various diseases. The antioxidant capacities of different extracts of *A. aureum* have been reported by many authors using different antioxidant assays. The methanol twig extract of the plant showed a 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity with an EC50 of 103.0µg/ml, while the IC50 for inhibition of lipid per oxidant was reported to be 28.99µg/ml. The IC50 for the induction of quinone reductase was found to be greater than 20µg/ml. In addition, out of 15 species of ferns that were screened for total phenolic contents and antioxidant activities, *A. aureum* exhibited strong antioxidant capacity and ranked eighth amongst the ferns investigated. The antioxidant activity of *A. aureum* was

attributed to high TPC (524 GAE) and anti-lipid peroxidation activity (51%). It can also be inferred from the study that the phenolics in *A. aureum* are strong metal chelators as the plant had the highest metal chelating potential amongst the investigated ferns. Therefore, the strong antioxidant capacity of *A. aureum* could be attributed to its high phenolic and flavonoid content. The strong antioxidant activity displayed by *A. aureum* suggests that the plant could serve as an important source of antioxidants with varying pharmaceutical and nutraceutical applications. (Minh et al., 2018; Lai et al., 2012).

- **Anti-ulcer and Anti-inflammatory activity**

  The gastroprotective activity of water extract of *A. aureum* in ethanol-induced gastric injury was evaluated where, pretreatment with *A. aureum* dose dependently reduced gastric ulcer and attenuated the pathological damage in the gastric tissue induced by alcohol. The extract also dose-dependently reduced ROS generated by ethanol, but it enhanced the levels of glutathione, catalase and superoxide dismutase in the stomach of rats co-exposed to ethanol and aqueous extract of *A. aureum* in a dose dependent manner. Furthermore, the secretion of pro-inflammatory Cytokines including, Tumour Necrosis Factor-α (TNF-α), Interleukin-1 β (IL-1β) and Interleukin-6 (IL-6) were also decreased by the extract (Wu et al., 2018). It is also experimented that the ethanolic crude extract of *Acrostichum aureum* root possesses anti-inflammatory activity. However, further researches are necessary to find out the active principles responsible for this activity (Hossain et. al., 2011).

- **Analgesic activity**

  The analgesic activity of the ethanol leaf extract of *A. aureum* against acetic-induced writhing was assessed in mice. The results showed that A. aureum at 250 and 500mg/kg body weight respectively showed 28.86% and 46.77% writhing inhibition. The analgesic effect was lower, but comparable to the 69.15% obtained for 25mg/ kg body weight diclofenac sodium that was used as the standard drug. (Hamid et al., 2014)

- **Cytotoxic and anticancer activity**

  Compounds isolated from the methanol extract of *A. aureum* were screened for in-vitro cytotoxicity against Hep-G2, SKLU-1, and MCF-7cells by Minh et al. [14] using sulforhodamine B assay. The ethyl acetate extract of *A. aureum* showed strong cytotoxicity with an IC50 value of 6.3µg/mL among medicinal plants from Hainan in China that were screened for cytotoxic activity against HeLa human cervical cancer cells. The *A. aureum* showed the most potent selective cytotoxicity amongst sixteen Bangladeshi plants that were screened against human colon, gastric, and breast cancer cell lines. The selective cytotoxic effects of some compounds found in *A. aureum* against cancer lines suggest the compounds may have huge potential as anticancer agents. However, this needs to be corroborated in vivo and their detailed mechanisms of actions elucidated. (Minh et al., 2018; Chen et al., 2019; Islam et al., 2015)

- **Contraceptive action:**
  Animal studies have revealed that acetone and ethanol extracts of *A. aureum* have potent anti-implantation activity in rats. Praskash et al. evaluated the antifertility properties of 158 medicinal plant extracts and found that the acetone and ethanol extracts of *A. aureum* exhibited between 60 and 70% anti-implantation activity in rats. Additionally, during administration on day 1–7 postcoital, the water-soluble fraction of the ethanol (95%) extract of *A. aureum* was found to have prevented pregnancy in female rats. The fraction has neither oestrogenic nor anti-oestrogenic activities, which suggest a unique mechanism for the observed antifertility effect. Therefore, the fraction could be a source of novel contraceptive(s) with better efficacy than the existing ones that are limited by side effects including induction of hormonal imbalance. (Praskash et al., 2015; Asadujjaman et al., 2015)

- **Wound healing property:**
  In order to provide the scientific rationale for the use of *Acrostichum* species in many Asian countries, ethanol extract of the plant was evaluated in a rabbit's excision wound model. Topical application of 10% *A. aureum* rhizome resulted in improved wound contraction and epithelization period when compared to the control. The authors attributed the wound healing capacity of *A. aureum* to its antibacterial activity and its phytochemical composition. Similarly, in a thesis submitted to the International Islamic University of Malaysia, Herman demonstrated that aqueous extract of *A. aureum* rhizomes and leaves showed exciting wound healing activities among four different extracts evaluated at doses of 5 and 10% in rabbits infected with back injury. The treated animals displayed enhanced collagen and fibroblasts proliferation in addition to complete epithelized cells taken together, these results may justify the traditional uses of the plant in the treatment of wounds and ulcers. (Herman, 2019)

- **Anti-diarrhoeal activity:**
  Hossain et al investigated the anti-diarrhoea property of ethanol root extract of *A. aureum* in a castor oil model of diarrhoea induction in mice. It was found that the ethanol extract of *A. aureum* root exerted anti-diarrheal effect in treated mice by reducing the rate of defecation and faeces inconsistency. The extract at 400mg/ kg decreased diarrhoea by 55% compared to the standard drug, loperamide that exerted a 66% decrease in diarrhoea. Thus, justifying the ethnobotanical use of the plant in the treatment of diarrhoea. The anti-diarrhoea effect of the root was partly linked to its high tannin content (251.41mg/g of TAE), which probably provoked intestinal mucosa resistance and reduction in secretion. In addition, other secondary metabolites present in the plant such as flavonoids could also reduce intestinal motility and secretion by inhibiting prostaglandins and autacoids release. (Hossain et al., 2013)

- **Anthelmintic activity:**

The potential of *A. aureum* as an anthelmintic agent was investigated with *Haemonchus contortus* in vitro and in sheep by Kalpana Devi et al. The result of in vitro study showed that ethanol extract of *A. aureum* was more effective in causing death and paralysis to *Haemonchus contortus* than water and petroleum ether extracts. Additionally, a 56 % reduction in faecal egg count was also observed in sheep infected with *Haemonchus contortus* before treatment with 100 mg/ml body weight of the ethanol leaf extract. Moreover, treated animals did not show any signs of toxicity. Thus, suggesting that the plant may provide new anthelmintic compounds for effective control of helminthiasis that is devoid of potential to enter food chain, toxicity and therapeutic resistance, which are the limitation of currently used drugs. (Kalpana Devi et al., 2019)

- **Antiviral activity**
  A novel antiviral secondary metabolite isolated from the methanol extract of aerial parts of *A. aureum,* 2″-(methoxycarbonyl)-5″- methyl pentyl 2′-methylhexyl phthalate exhibited only post infection antiviral activity against human parainfluenza virus, dengue virus and chikungunya. The fact that the compound exhibited only post infection activity made the authors to reach a conclusion that the compound was an inhibitor of viral replication rather than viral entry process. (Lien et al., 2020)

Safety of *A. aureum*

Our literature search on the safety of A. aureum returned no information on its in vitro and in vivo toxicity. Therefore, toxicological studies are urgently needed to know the potential toxicity of the plant and to determine the dosage, that can be safely consumed by humans and veterinary animals.

Rational For Selection of Plant:

The wide range of ethnopharmacological claim and traditional uses of *Acrostichum aureum Linn.,* and also the availability of this mangrove plant has given us the valid reasons to select this plant for the study. Moreover, parts of this plant are edible and also uses as an ornamental purpose, provides us sufficient evidence of possessing lesser side effects if used for therapeutic purposes especially in the treatment of Rheumatoid Arthritis.

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CHAPTER 4: COLLECTION, EXTRACTION AND PHYTOCHEMICAL SCREENING

4.1: Collection and Identification:

Acrostichum aureum L is an edible mangrove plant fern that grows mainly in tropical and subtropical regions of the world. The plant especially the aerial part of the plant was collected and purchased from local vendor near Sundarban-Canning region and authenticated from Botanical Survey of India (BSI) Shivpur.

Preparation of plant extract

Crude aerial parts of *Acrostichum aureum* has taken and washed properly in streaming water and then air dried for 25-30 days.

4.2: Extraction:

The dried aerial parts (2kg) were then extracted with cold maceration technique in 70:30 Hydro-Alcoholic mixture of 70% methanol and 30% double distilled water. Then the hydro-alcoholic extract of *Acrostichum aureum* were filtered through vacuum filter and after that the excess moisture of the plant extract was evaporated by using rotary evaporator where temperature was around 50-60°c and the rotation speed was 65-70 r.p.m. After the evaporation the extract was then dried in leaf drier at 55°c and then lyophilized through sublimation procedure for better storage and future use.

The percentage w/w yield of AAHE was 20.14%.

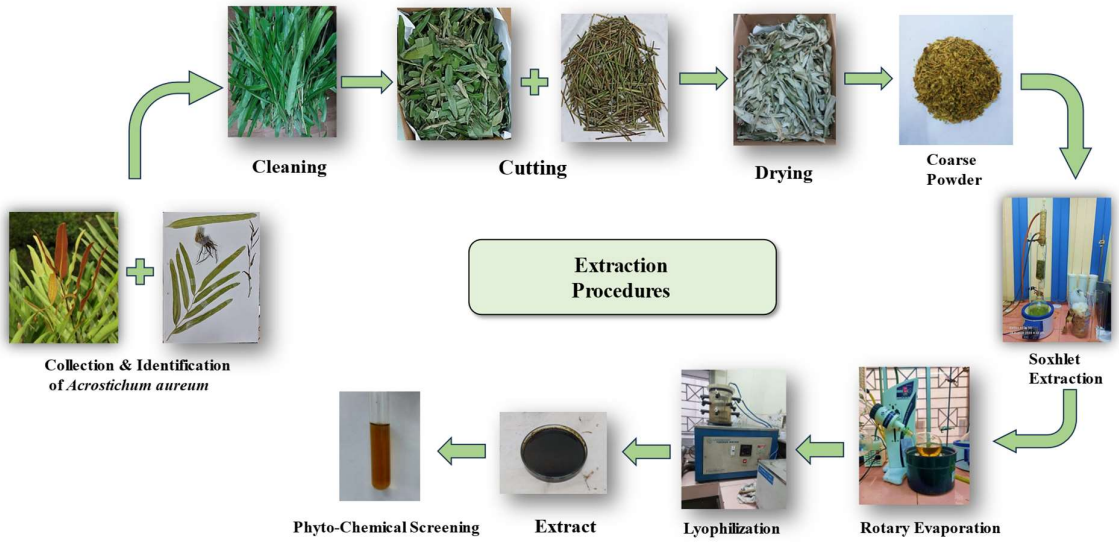

Fig.5: Schematic Diagram of Extraction Procedures

4.3: Preliminary Phytochemical Screening of Hydro-alcoholic Extract of *Acrostichum aureum*:

Preliminary phytochemical tests were carried out to explore the presence of various phytoconstituents or secondary metabolites in the plant. Hydro-alcoholic extract *of Acrostichum aureum* was opted for several procedure to screened the phytoconstituents present in the aerial parts of the plant. As the presence of phytochemical substances, medicinal plants are good for both mending and curing human disorders (Nostro et al., 2000). Phytochemicals are non-nutritive plant components that have disease-preventive or preventative capabilities (Ajuru et al., 2017). As a result, determining the phytoconstituents of a plant material or extract is critical. The extract was then submitted to qualitative phytochemical tests in order to detect unique phytoconstituents.

4.4: Phyto-Chemical Screening Test:

4.4.1: Test for Steroids

Liebermann- Burchard Test:

- 10 mg of extract was dissolved in 1 ml of chloroform. After adding 2 mL of concentrated sulphuric acid, 1 mL of acetic anhydride was added. The emergence of a reddish violet tint indicated the existence of steroids. (Zhou and Yu, 2004)

Salkowski Test:

- One ml of concentrated sulphuric acid was added to 10 mg of extract diluted in 1 ml of chloroform. The presence of steroid was shown by the formation of reddish blue color of the chloroform layer and the green fluorescence of the acid layer. (Bosila and El-Sharabasy, 2009)

4.4.2: Test for Flavonoids

- Alkaline Reagent Test:
  To 2 mL of extract, a few drops of 20% sodium hydroxide solution were added. Flavonoids are detected by the production of a bright yellow color that fades to colorless when mild hydrochloric acid is introduced. (Solomon et al., 2013)
- Shinoda Test:
  In alcohol, a small amount of extract was dissolved. Two to three pieces of magnesium were added, then powerful hydrochloric acid was added, and the mixture was boated. Appearance The appearance of magenta indicates the presence of flavonoids. (Pethappachetty et al., 2012)

4.4.3: Test for Saponins

- 1ml of the extract was diluted up to 20 ml with distilled water and agitated for 15 minutes in a graduated cylinder. The production of stable foam revealed the presence of saponins.

- One mL of extract was treated with a 1% lead acetate solution. The production of white precipitate confirmed the presence of saponins. (Verma et al., 2021)

4.4.4: Test for Tannins

- To 1 mL of 5% ferric chloride solution 5 mL of extract solution was added. The greenish black color suggested the presence of tannins.
- To 5 mL of extract, 1 mL of 10% aqueous potassium dichromate solution was added. The production of a yellowish-brown precipitate revealed the presence of tannins.
- To 5 mL of extract, 1 mL of a 10% lead acetate solution in water was added. The emergence of yellow precipitate indicated the presence of tannins. (Segelman et al., 1969)

4.4.5: Test for Glycoside

- Legal's Test:
  The extract was dissolved in pyridine and a solution of sodium nitroprusside was added to make it alkaline. The production of pink red to crimson color indicates the presence of glycosides.
- Bontrager's Test:
  To 1 ml of extract solution few ml of mild sulphuric acid was mixed. Then boiled, filtered and extracted with chloroform. The chloroform layer was treated with 1 ml of ammonia. The formation of a red color indicates the presence of anthraquinone glycosides. (Salwaan et al., 2012)

4.4.6: Test for Carbohydrate

Benedict's Test:

The test solution was mixed with a few drops of Benedict's reagent (an alkaline solution containing cupric citrate complex) and heated in a water bath to observe the formation of reddish-brown precipitate, which confirmed the presence of carbohydrate. (Bhandary et al., 2012)

Molish Test:

To 2 mL of extract, 1 mL of a-naphthol solution and 1 mL of concentrated Sulphuric acid were added via the test tube sides. Carbohydrates are detected by the presence of a purple or reddish violet tint at the junction of the two liquids. (Salwaan et al., 2012)

4.4.7: Test for Alkaloids

- Mayer's Test:
  In a test tube 1.2 mL of the extract was taken. When 0.2 mL of dilute hydrochloric acid and 0.1 mL of Mayer's reagent are mixed together, a yellowish buff precipitate forms, indicating a positive test for alkaloids.
- Dragendroff's Test:

In a test tube 2 ml of the extract was taken. Then 0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendroff's reagent were added. The presence of alkaloids was confirmed by the production of orange brown precipitate.

- Wagner's Test:
  To 2 ml of extract solution, 0.1 ml of Wagner's reagent and 2 ml of dilute hydrochloric acid were added. A reddish-brown coloration indicated a positive response to alkaloids.
- Hager's Test:
  In 2 ml of extract solution, 0.2 ml of dilute hydrochloric acid and 0.1 ml of Hager's reagent were added. Formation of yellowish precipitate indicated the presence of alkaloids (Bruck de Souza et al., 2020)

4.4.8: Test for Phenols

To the extract solution, 3-4 drops of $FeCl_3$ was mixed. The formation of a bluish black color indicates the presence of phenol. (Saxena et al., 2015)

4.4.9: Test for Triterpenoid

- Salkowski Test:
  The test extract was mixed with a few drops of concentrated H_2SO_4. The emergence of a yellow color in the lower layer suggested the presence of triterpenoids. (Nayak et al., 2010)

4.4.10: Fixed oils and fats

- After the organic solvent was extracting out, a small amount of extract was pressed between two filter papers. The presence of oil streaks on the paper confirmed the presence of fixed oil. (Saxena et al., 2015)
- After the organic solvent was extracting out, a few drops of 0.5 (N) alcoholic potassium hydroxide solutions and a drop of phenolphthalein were mixed with a small quantity of extract. The reaction mixture is heated on a hot plate for 1 hour. The presence of fixed oils and fats is indicated by soap formation or partial alkali neutralization.

4.4.11: Gum

- To 10 ml of extract solution, 20 ml of absolute alcohol added along with continuous stirring. The precipitate is filtered and dried in air. The precipitate is examined for its swelling properties and for the existence of gum

4.4.12: Mucilage

- To the extract solution ruthenium red solution was added, pink color is obtained. The test extract is treated with thionine solution, after 15 min the mixture was washed with alcohol. The formation of violet-red color indicated the presence of mucilage.

4.4.13: Proteins and amino acids

- Biuret's Test:

To 2 mL of test solution, 1 mL of 40% NaOH solution is added. Then 1 or 2 drops of 1% $CuSO_4$ solution is added. The formation of violet colour confirmed the presence of peptide linkage in the molecule. As a generic protein test, it has been applied. (Saxena et al., 2015)

- Millon's reagent:
  In 9 mL of fuming nitric acid, 1 gm of mercury was dissolved, and the fluid was kept cool throughout the reaction. When the procedure is completed, an equal volume of distilled water is added. Protein becomes crimson when heated. (Saxena et al., 2015)
- Ninhydrin Test:
  A 0.1% solution of ninhydrin in n-butanol is prepared. It is warmed with the test extract. Purple color is produced when amino acids are present. This is the amino acid test in general. (Saxena et al., 2015)

4.4.14: Volatile (essential) oils

• Sudan red Ill solution is given to a specific thin section of plant material; the red color acquired by globules shows the presence of volatile oil.

• The presence of volatile oil was suggested by the adding of a few drops of tincture alkana on a thin section of plant material.

• About 50 gm of powdered material is exposed to hydro-distillation in a volatile oil estimation apparatus. The distillate is collected in the graduated tube of the assembly, where the aqueous component is automatically separated from any volatile oil present. (Saxena et al., 2015)

4.5. Results:

Table 1: Preliminary Phytochemical Screening of *Acrostichum aureum L.*, Hydro-Alcoholic Extract

SL NO.	Phytoconstituents	Hydro alcoholic extract of *Acrostichum aureum l.*
1	Flavonoids	+
2	Phenolics	+
3	Triterpenoid	+
4	Steroids	+
5	Carbohydrates	+
6	Cardiac Glycosides	+
7	Tannins	+
8	Proteins	+
9	Saponin	+

10	Alkaloids	-
11	Volatile oil	+
12	Mucilage	-
13	Gum	+
14	Fixed oils & Fats	+

['+' represents 'Presence', '-' represents 'Absence']

4.6. Conclusion:

The presence of flavonoid, alkaloid, phenolic, steroids, carbohydrates, glycosides, tannins, saponin, gum and proteins were found during preliminary phytochemical investigation. Polyphenols, which include flavonoids and phenolic acid, are a diverse collection of phytochemicals. Flavonoids act as antioxidants, modulating oxidative stress in the body by neutralizing the action of nitrogen and oxygen species, avoiding illness and improving the sign and symptoms of rheumatoid arthritis by showing anti-oxidant and anti-inflammatory activities. It is demonstrated that flavonoids can modulate various players in synovial inflammation, regulate immune cell function, decrease synoviocytes proliferation and balance the apoptotic process, decrease angiogenesis, and stop/prevent bone and cartilage degradation, which are all dominant features of RA. Due to the chemical structure of phenolic groups of phenolic compounds may accept an electron or proton, forming relatively stable phenolic radicals preventing chain oxidation reactions in cell compartments. Phenolic compounds act as antioxidants protecting human tissues against oxidative stress and conditions associated with RA. As the plant extract (AAHE) itself rich in flavonoid and poly-phenolic compounds can show a promising assistance in the treatment of Rheumatoid Arthritis. Although further investigation is necessary to determine the effectiveness of flavonoids in humans, the available data from in vitro and in vivo models suggest their potential as new disease-modifying anti-rheumatic drugs. As a result, AAHE is high in polyphenols. More research is needed to acquire a better understanding of the mechanism of action against rheumatoid arthritis.

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CHAPTER 5: EVALUATION DPPH RADICAL SCAVENGING ASSAY, TPC & TFC DETERMINATION

5.1: Introduction

Oxidative stress is a pivotal player in the aggravation of chronic inflammatory joint disease. Both experimental models and assessments in patients showed, in addition to elevated ROS and lipid peroxidation formation, a decrease in antioxidant defences. The involvement of oxidative stress in the pathogenesis of inflammatory diseases, such as RA, has been demonstrated in several studies. (Mittal et al., 2014). ROS and RNS are important categories of molecules generated in living systems for cellular metabolism. However, when such reactive species reach concentrations above the upper limit of normal range, they damage cellular components. (Pizzino et al., 2017). In this way, therapies that decrease oxidants and/or increase antioxidants are promising in the treatment of various oxidative stress-related inflammatory diseases. The antioxidant potential of AAHE was determined using the DPPH radical scavenging assay and Super oxide dismutase (SOD) Assay in this study. Terpenoids, phenolic metabolites, and alkaloids are the three principal types of plant compounds. Among these three classes, phenolic chemicals are the most essential for nutritional applications and have received the greatest attention. Phenolic acids (hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolysable and condensed tannins), and flavonoids are examples of phenolic chemicals. These chemicals protect plants from oxidative damage and have been employed by humans as antioxidants. (Kumar and Pandey, 2013) It is of tremendous interest to discover new and safe antioxidants from natural sources for use in natural antioxidants, functional foods, and nutraceuticals.

5.2: Drugs & Chemicals

Ascorbic acid (Hi-media, Mumbai, Maharashtra, India), Gallic Acid (Sigma-Aldrich Co., USA.), Quercetin (Hi-media, Mumbai, Maharashtra, India) and all other Chemicals of analytical grade were obtained commercially.

5.3: Evaluation of the *in-vitro* antioxidant activity

DPPH radical scavenging assay:

Free radicals are molecules, usually of oxygen, which have lost an electron and are continuously generated during human body metabolism. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate the radical scavenging activity. In DPPH radical scavenging assay, antioxidants react with deep violet colour DPPH (1, 1-diphenyl-2-picryl-hydrazyl) and convert it to yellow coloured 1, 1-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the radical-scavenging potential of the antioxidant. (Brand-Williams et al., 1995)

The antioxidant properties of Hydro-Alcoholic Extract *Acrostichum aureum* were assessed using a 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay established in our lab. In this test, 15.625 – 500 µg/µl of both the sample and the DPPH solution (0.2 mg/ml) followed by serial dilution in a 96-well microplate and left to stand in the dark for 10 minutes

at room temperature. A spectrophotometer was used to quantify the reduction in absorbance of the sample solution at 517 nm. Ascorbic acid was utilised as a Reference Control. The IC50 value (g/ml) was used to express the free radical scavenging activity. The Percentage Inhibition was calculate using the following formula(Brand-Williams et al., 1995). (Brand-Williams et al., 1995)

Percentage Inhibition= $[(A_0-A_1)/A_0 \times 100]$

Where, A_0 = Absorbance of the control

A_1 = Absorbance of AAHE or Reference Control (Ascorbic Acid).

5.4: Assessment of the total phenolic and flavonoid content

The total phenolic and flavonoid content of aerial parts of *Acrostichum aureum* hydro-alcoholic extract was determined using a previously disclosed method from our lab. (Gupta et al., n.d.)

5.4.1. Total Flavonoid Content of The Plant Extract:

The estimation of Total Flavonoid Content (TFC) was done on the plant extract AAHE and the standard Quercetin. To perform this assay, we have used different concentrations of AAHE and Plant extract in methanol and the ranges are 31.25-500 µg/ml concentrations and followed serial dilution method. We used 96 well plate in which firstly we have added 90 µl distilled water and then 10 µl of NaNo2 (50g/l) and after that we have added 25 of Standard (Quercetin) or Plant extract solution (AAHE). After 5 min, 15 µl of AlCl3 solution and 50 µl of 1M NaOH solution was added. Then the plate was shaken about 20 seconds inside the plate reader. The Optical Density or absorbance was detected at 415 nm thricely. (Alam et al. 2013)

The Total Flavonoid Content (TFC) of the plant was analyzed as Quercetin Equivalent/ gm.

The Calculation was depicted as mean±SD (n=3).

5.4.2. Total Phenolic Content of The Plant Extract:

To analyze Total Phenolic Content of the Plant extract (AAHE), spectrophotometric method was utilized where, Gallic Acid was used as Reference Control. The ranges of concentration reference and test sample (AAHE) were used 31.25-500 µg/ml. 75 µl of Distilled water, then 25 µl of sample/standard, and 25 µl of Folin- Ciocalteu's reagent were mixed then, the reaction mixture was stand for 6 minutes, Then, 100 µl of Na_2CO_3 was added to each well and the plate was covered and left for 90 minutes in the dark. Repeat the procedure as same for the blank. Then plate was placed into the analyzing chamber for 60 seconds of shaking and then the absorbance was measured at 725 nm.

The estimation of TPC was expressed as mg of gallic acid equivalent/g (GAE/g) of the sample.

5.5: Statistical analysis

Results from statistical analysis are presented as Mean±SEM (n=3). Following One-way ANOVA, the statistical significance between the groups was examined using Dunnet's multiple comparison test. Statistics were judged significant at $P<0.05$.

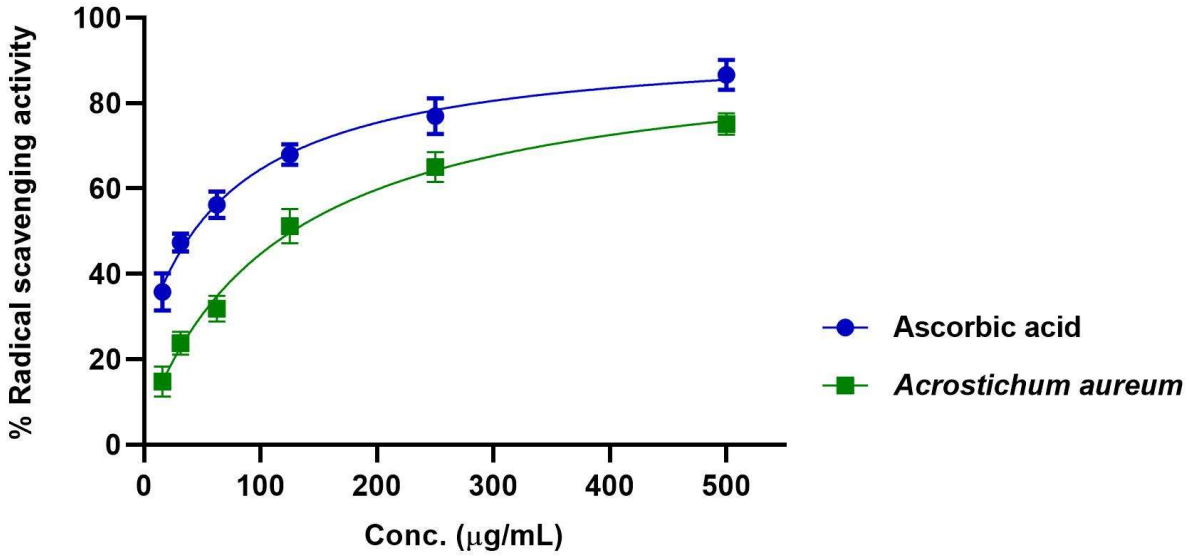
- **DPPH- Radical Scavenging Activity of The Plant Extract:**
  The assay showed the inhibitory ability of extract and standard ascorbic acid on DPPH in a dose dependent manner (Fig. 1). The IC50 value for DPPH scavenging of AAHE and standard ascorbic acid were found to be 94.03± 0.67 µg/ml and 9.37±0.90 µg/ml (Fig. 9), which indicate the efficient DPPH scavenging activity.

Table 2: IC_{50} values of antioxidant assay of AAHE

TYPE OF EXTRACT	DPPH IC_{50} (µg/ml)
AAHE	77.28 ±0.29
Ascorbic Acid	119.9 ±4.92

Each value expressed as Mean±SEM (n=3)

Fig.6: DPPH- Radical Scavenging Activity of *Acrostichum aureum* Hydro-alcoholic Extract



Percentage Inhibition of DPPH shown by Different Concentrations of AAHE and Ascorbic Acid

❖ **Estimation of TPC and TFC:**

Gallic acid equivalents (GAE) per gram of dry extract were used to assess the number of total phenols present in the dry extracts. The Total Phenolic Content (TPC) was found to be 4.06±0.010 mg GAE/gm of dry extract. The equation for the gallic acid standard curve was Y= 0.002783*x+ 0.01357, and the correlation coefficient was R2 = 0.9917. Quercetin equivalents (QE) per gram of dry extracts were used to assess the number of total flavonoids present in the samples. Total flavonoids content of AAHE was 0.77±0.0277 mg QE/g of dry extract. The standard curve equation of quercetin was Y = 0.001512*X + 0.4767 with a correlation coefficient of R2 = 0.9713.

5.6: Discussion

The aim of this study was to evaluate the in-vitro-antioxidant activity of a hydroalcoholic extract of *Acrostichum aureum* aerial part. The presence of phenolic and flavonoid content ensured this plant possess good antioxidant activity, which was confirmed by an invitro DPPH radical scavenging assay using ascorbic acid as a reference. (Ghosh et al., 2023; Cai et al., 2004)

5.7: Conclusion

The current investigation confirmed that the hydro alcoholic extract of *Acrostichum aureum* aerial parts portion had substantial in-vitro antioxidant activity.

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CHAPTER 6: IN-VITRO ANTI-RHEUMATOID ARTHRITIS ACTIVITY OF HYDRO-ALCOHOLIC EXTRACT OF *ACROSTICHUM AUREUM* AERIAL PART

6.1 In- Vitro Anti-Inflammatory Study:

6.1.1 Introduction:

The serum albumin protein obtained from cows is known as bovine serum albumin (BSA) or "Fraction V". In laboratory research, it is frequently used as a reference for protein concentration.

Albumin is referred to as "Fraction V" because it was the fifth fraction in the original Edwin Cohn purification process, which used the varied solubility properties of plasma proteins. Cohn was able to extract different "fractions" of blood plasma by varying the temperature, pH, salt concentrations, and solvent concentrations. Human albumin was used in the method' initial commercialization for medical purposes before BSA was produced using it.

It is suggested to use the in-vitro anti-denaturation effects of natural products and non-steroidal compounds in heat-treated (Immunogenic) bovine serum albumin as a screening assay for the early stages of drug discovery to identify anti-inflammatory compounds without using animals.

The annual mangrove fern *Acrostichum aureum* has long been used as a remedy for rheumatism. This fern is well recognized for its anti-inflammatory properties. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) that inhibits the activity of the enzymes cyclo-oxygenase-1 (COX1) and cyclo-oxygenase-2 (COX2), which in turn inhibits the synthesis of inflammatory mediators such as prostacyclin, thromboxane, and prostaglandin E2 (PGE2). Diclofenac also has analgesic antipyretic activity. Furthermore, it has the ability to lower the elevated substance P levels in rheumatoid arthritis patients' synovial fluid. Additionally, it has been demonstrated that diclofenac has better intrinsic anti-inflammatory efficacy than the other NSAIDs. (Scholer & Associates, 1986) Diclofenac was thus employed as the reference control in this investigation.

The aim of the present study was to evaluate the anti-inflammatory effect of the hydro-alcoholic extract of *Acrostichum aureum* by using Bovine Serum Albumin (BSA) assay, with diclofenac sodium as reference.

- **Chemicals & Reagents:**

Bovine Serum Albumin (BSA) and diclofenac sodium (DICF-S) were purchased from Sigma Aldrich.

6.1.2 Bovine serum albumin denaturation Assay Procedure:

Protein denaturation is one of the most key factors in the analysis of rheumatoid arthritis or any inflammatory disease.

To evaluate albumin denaturation 0.5 ml reaction mixture was prepared. To prepare the reaction mixture 0.45 ml of Bovine Serum Albumin (BSA) (5% aqueous solution) and 0.05 ml of different concentrations (31.25-500 µg/µl) of Plant Extract (AAHE) or Diclofenac Sodium as a reference was used. The reaction mixture was then incubated at 37°c for 20 minutes and after that heated at 57°c for 3 min and then cooled to room temperature. 2.5 ml of phosphate buffer saline (pH 6.3) was prepared. After cooling the

reaction mixture 2.5 ml of phosphate buffer was added into the mixture. For control, only 0.05 ml distilled water was used. The outcome was analyzed by using spectrophotometer at 660 nm. (Sur et al., 2023), (Dhivya et al., 2015)

Percentage Inhibition was calculated by using following method:

Percentage inhibition of denaturation = Abs(control) − Abs(sample)/Abs (ref) X100.

The procedure was repeated thrice and the result was calculated by using the statistical method of ANOVA.

6.2 Results:

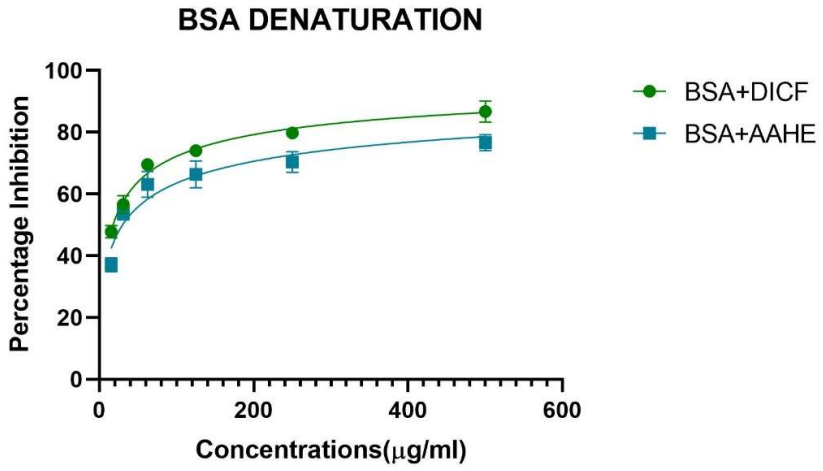
6.2.1 Statistical Analysis:

Results from statistical analysis are presented as Mean±SEM (n=3), following the one-way ANOVA. Statistics were judged significant at $P<0.05$.

Table 4: Effect of Hydro-alcoholic Extract of Acrostichum aureum Aerial Parts on Bovine Serum Albumin Denaturation

Concentration (µg/ml)	***Inhibition (%) (Mean±SEM)***		***IC50 (µg/ml)***	
	BSA+DICF	***BSA+AAHE***	***BSA+DICF***	***BSA+AAHE***
15.625	47.818±1.188	37.003±1.325	***17.88***	***30.23***
31.250	56.585±1.656	53.676±1.260		
62.500	69.569±1.073	63.112±2.396		
125.000	74.000±0.937	66.348±2.499		
250.000	79.769±1.116	70.341±1.943		
500.000	86.746±2.006	76.658±1.520		

Fig.8: Bovine Serum Albumin Denaturation Curve



Percentage Inhibition of Bovine Serum Albumin Denaturation Shown by AAHE and DICF

6.3 Conclusion:

This study proves that at a maximum tested concentration of 500 µg of hydro-alcoholic extracts of *Acrostichum aureum* showed 76.658% inhibition which is almost equivalent to that of reference Diclofenac sodium (DICF). It is concluded that, hydro-alcoholic extract of *Acrostichum aureum* have a potential anti-inflammatory effect in concentration dependent manner by in-vitro method.

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CHAPTER 7: ACUTE TOXICITY STUDY OF HYDRO-ALCOHOLIC EXTRACT OF *ACROSTICHUM AUREUM* AERIAL PART

7.1: Acute Toxicity Study

7.1.1 INTRODUCTION:

The number of pharmacological substances and chemicals being used in the human community today, have increased at almost an innumerable amount (Sperling F,1979). These may be presented today in the form or as constituents of food substances, medicines, beverages, other industrial and household products. However, these chemicals or pharmacological substances result in chronic toxicity in the living system when used over a long period of time or acute toxicity may also occur when used in large quantities capable of eliciting immediate toxic effect. These effects may be mild or severe, depending on the nature of substance.

The term toxicology derived from the word ""toxion" means poison and 'logos' means science. Toxicology is the science which deals with the harmful effects of chemicals and drugs on living systems. It helps us to determine the quality and quantity of chemical which will turn it into poison. The potential uses of toxicity testing data include:

•Establishing the therapeutic dose.

•Acquiring information about the harmful effects on specific organs.

•Establishment of the mode of toxic action.

•Establishment of the toxic substance as a future reference. (Sperling F, 1979).

OECD Guidelines 425 (Up and Down Procedure) were followed for the oral acute toxicity study. Healthy Wister Albino rats were purchased from…, aged 6 to 7 weeks, and weighed 200±20 gr. The animals were then kept for seven days in an animal house with ideal condition like temperature, humidity, day-night cycle along with sufficient food, and water ad libitum. Water was employed as the vehicle in a limit test of a hydro-alcoholic extract of *Acrostichum aureum* at 2000 mg/kg per-oral single dose (Patrick-Iwuanyanwu et al., 2012). Rats were fasted for 3–4 hours before starting the treatment, with continuous accessibility to water. Food was offered following a two-hour dosing of period. First, a single Rat received a dose based on its body weight (Khazdair et al., 2015). It was then thoroughly monitored for the first 30 minutes, and then for another 4 hours. After the first animal survived, 4 others were taken and given the same dose under the same conditions. The five animals were then thoroughly monitored over the following six hours for any toxic signs, and also for the following 14 days at regular intervals, body weight and behavioural patterns were observed. The same procedure was applied for the control group, where five animals were taken with sole exception that water was used as the treatment vehicle rather than any extract.

7.2 Evaluation:

Table 5: Effects of the extracts on the body weight of Rats in acute toxicity study

Groups	1st day Body weight (gm.)	7th day Body weight (gm.)	14th day Body weight (gm.)
Vehicle Control	165±15	182±7.5	191±5.0
AAHE	167±15	177±15	191±7.5

AAHE: *A. aureum* extract; values are presented as mean ± SEM; (n=3)

No mortality was observed after administering the maximum dose of 2000 mg/kg AAHE. The test animals were closely monitored for the first 30 minutes and then for the following 4 hours. The animals were monitored at regular intervals for the duration of the 16-day study period. Over the course of the trial, both the treatment group and the control group the changes in body weight and other behavioural patterns are tabulated grew in Table 2.

In the first 30 minutes, the extract-treated group's respiration rate was normal. There was evidence of sleepiness in both groups. No convulsions and tremors were seen for the first four hours in the extract-treated group. Within the first 30 minutes, both groups experienced shivering and itching.

Table 6: Behavioural patterns of rats in extract treated (2000 mg/kg p.o) and vehicle treated groups

Parameters	30 minutes		4 h		24 h		48 h		7 days		14 days	
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
Fur & skin	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Respiration	N	N	N	N	N	N	N	N	N	N	N	N
Urination	N	N	N	N	N	N	N	N	N	N	N	N
Faeces consistency	N	N	N	N	N	N	N	N	N	N	N	N
Somatomotor activity & behaviour pattern	N		N		N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N	N	N

Itching	P	P	N. F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F
Convulsions & tremors	N. F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F	N.F

Key: CG= Vehicle Control group, TG= AAHE group, N= Normal, P=Present, I = increased, N.F= not found

7.3 CONCLUSIONS:

During the 14-day at the dose of 2000 mg/kg acute toxicity study, no morality was observed, with normal food and water consumption along with minimal weight fluctuations were also noted.

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CHAPTER 8: ANTI-RHEUMATOID ARTHRITIS ACTIVITY OF HYDRO-ALCOHOLIC EXTRACT OF *ACROSTICHUM AUREUM* AGAINST COMPLETE FREUND'S ADJUVANT INDUCED RHEUMATOID ARTHRITIS IN RATS

8.1 INTRODUCTION:

Rheumatoid Arthritis (RA) is an inflammatory and autoimmune disease characterized by inflammation in the affected areas brought on by the immune system attacking healthy cells. Rheumatoid arthritis is a chronic inflammatory illness that affects more than only joints. Skin, eyes, lungs, heart, and blood vessels are just a few of the systems in the body that might sustain harm from the illness in certain individuals. Rheumatoid arthritis affects the lining of the joints, generating a painful swelling that can lead to bone erosion and joint deformity, in contrast to the wear-and-tear deterioration of osteoarthritis. (Mayo Clinic, 2021)

Multiple inflammatory cells infiltrate the pannus and joint fluid, causing tissue death as a result. This is the hallmark of RA. Along with other inflammatory mediators, chemokines are significant in the pathophysiology of RA. The inflammatory responses seen in RA patients are largely orchestrated by the coordinated production of proinflammatory cytokines and chemokines. (Szekanecz & Koch, 2007)

Imbalance between pro- inflammatory and anti-inflammatory cytokine activities favours the induction of autoimmunity, chronic inflammation, and thereby joint damage. Monocytes that are attracted to the RA joint differentiate into macrophages and become activated. These macrophages play a pivotal role in RA because they are numerous in the inflamed synovial membrane and at the cartilage-pannus junction. They activate MHC Class-II (Major Histocompatibility Complex Class-II) molecules and secrete proinflammatory or regulatory cytokines and growth factors like IL-1, IL-2, IL-6, IL-10, IL-13, TNF-Alpha (Tumour Necrosis Factor), GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), chemokines and chemoattractant e.g. IL-8, MIP1 [Macrophage Inflammatory Protein-1] and MCP1 [Monocyte Chemoattractant Protein]), metalloproteinases, and neopterin. (Kinne et al., 2000; Szekanecz et al., 2008)

TNF regulates IL-1β expression, which is important for the induction of prostanoid and MMP (Matrix Metalloproteinases) production by synovial fibroblasts and chondrocytes. Cellular interactions mediated by TNF and IL-1, cytokines that are mainly produced by activated macrophages are prominent factors leading to cartilage damage in RA. TNF increases the expression of adhesion molecules on endothelial cells, which recruit more cells to the joint. (Szekanecz et al., 2008)

MCP1 and IL-8 are also secreted by macrophages and attract more cells into the joint. IL-1 and TNF induce synovial fibroblasts to express IL-6, chemokines (IL-8), GM-CSF, and MMPs, which contribute to cartilage and bone destruction. In addition, IL-1 mediates cartilage degradation directly by inducing the expression of MMPs by chondrocytes. (Szekanecz et al., 2008)

These innate immune system cells, however, have extensive proinflammatory, damaging, and remodeling properties, and they significantly contribute to inflammation and joint degradation during both the acute and chronic stages of RA. Furthermore, RA synovial stromal cells release these chemokines, which promote monocyte movement. (Szekanecz et al., 2008)

It is known that autoreactive B cells play a crucial role in activating CD4+ T cells, which in turn can cause autoreactive B cells to create IgG autoantibodies that may be directly linked to joint injury. It makes sense to think about ways to lessen or avoid B cell-mediated effects in RA patients because these cells seem to be crucial to the disease's progression. Their survival and proliferation under constant stimulation in RA patients suggests that clones of autoreactive B lymphocytes. Beyond just

producing autoantibodies, B cells can also effectively communicate antigens to T lymphocytes in the synovial milieu and secrete numerous other significant cytokines. (Marston et al., 2010)

The aetiology of RA also involves abnormal presentation of self-antigen(s) by APCs (antigen-presenting cells) and activation of autoreactive T-cells. T lymphocytes play a central role in the disease process. The rheumatoid synovial membrane is rich in MHC Class-II, APCs, and CD4+ T-cells. However, it is not clear whether T-cell activation occurs before entry to the tissue, during trans endothelial migration, or in the synovium. APCs require signals from activated T-cells for their differentiation and maturation; this subsequently enables APCs to activate newly arrived T-cells in a specific or unspecific manner in the local inflammation. Activated T-cells promote the disease progression by inducing the secretion of pro-inflammatory cytokines (in particular, TNF-Alpha) from macrophages and synovial cells in a contact-dependent manner. (Kinne et al., 2000)

Traditional herbal medicine has been proof enough in the betterment of rheumatoid arthritis and recent studies has shown 60-90% increased use of natural product derived medicines in the management of RA. Thus, medicinal plants could be a possible way out for the management of RA and there are several medicinal plants which are in continuous evaluation and research to bring out a novel drug. Mangrove plants also reported to have ethnobotanical, ethnopharmacological claim and has been used traditionally in the treatment of rheumatoid arthritis. (Bandaranayake, 2002)

The mangrove plant *Acrostichum aureum (A. aureum)* Linn. has ethnopharmacological claim in the treatment of snake bites, wound, rheumatism and used traditionally in wide parts of Asia, the plant is enriched in secondary metabolites like flavonoids, glycosides, gum, sterols, saponins, alkaloids, gums, tannins terpenoids and triterpenoids. The aerial parts of *Acrostichum aureum* shows antioxidant and anti-inflammatory property which can be effective in the reduction of several inflammatory parameters like IL-β, TNF-α, thus, could be effective against the sign and symptoms of RA. (Bandaranayake, 2002) Therefore, the current study was designed to evaluate the anti-arthritic potential of *Acrostichum aureum* on Complete Freund's Adjuvant-induced arthritis (CFA) rheumatoid arthritis in rats. (Bendele, 2001)

8.2. Materials and methods

8.2.1: Reagents and apparatus

Complete Freund's Adjuvant (CFA), Bovine Serum Albumin (BSA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), DTNB, reduced glutathione (GSH), NADH were purchased from Sigma (Sigma Chemical Co., USA), Diclofenac sodium, Plethysmometer, Vernier Calliper, and other reagents used for in-vitro, in-vivo study were of the highest grade available in the Laboratory and Hi-Media Laboratories Pvt. Ltd. provided all other chemicals. (Mumbai, Maharashtra, India).

Fig.9: Digital Calliper & Plethysmometer

8.2.2: Animals

Twenty-Five Male Albino Wister rats (160-180 grams), aged between 6-7 weeks were purchased from The State Centre for Laboratory Animal Breeding (SCLAB), Buddha Park, B 14, Block B, Kalyani, West Bengal 741235 (FSSAI REG NO. 10012031000104). Animals were kept in Departmental animal house with a suitable condition including temperature of 20-26 °C, relative humidity of 44-56%, 12-hour cycles of light and dark, along with proper bedding and fooding. Prior to the primary animal experiment, the Institutional Animal Ethical Committee (IAEC), Jadavpur University has approved for the experimental work (Approval Number- JU/IAEC-24/54)

8.3: Induction of Rheumatoid Arthritis:

There is a critical need for better and well-defined animal models for rheumatoid arthritis (RA) that display the specific aspects of the human disease and can serve as platforms for research on the underlying pathology, as well as for drug discovery and validation. Currently, adjuvant arthritis (AA) in rats is the most widely used arthritis models in academia and industry. AA, which is induced by a mixture of paraffin oils, mannide monooleate, and heat-killed mycobacteria (Mb), known as **Complete Freund's Adjuvant (CFA)**, is an acute model that tends to have an aggressive disease course.

8.3.1 Complete Freund's Adjuvant Induced Rheumatoid Arthritis:

In 1956, Pearson et al. reported that rats immunized with Complete Freund's Adjuvant (CFA) containing *Mycobacterium tuberculosis (M. tuberculosis*: MT) developed a form of arthritis termed Adjuvant-Induced Arthritis (AIA). Since this discovery, AIA has been widely used as a model for rheumatoid arthritis (RA). AIA has also been induced using other strains of bacteria, including *M. butyricum* and *Staphylococcus epidermidis*. Peptidoglycans (PGs), a key component of the bacterial cell membrane, as a common arthritogenic factor among these bacterial cell walls, and muramyl dipeptide (MDP) as the minimum arthritogenic structure of PG's derivatives (Kohashi et al.).

CFA containing 1 mg/ml MT is seldom effective for inducing AIA. For effective induction, CFA containing 10 mg/ml MT is recommended, although CFA containing 5 mg/ml of MT is also capable of inducing AIA. (Bendele et al., 2001)

8.3.2 Administration of CFA in Laboratory Rats:

There are several sites for the administration of CFA in rats for example, either the footpad or the base of the tail, depending on the purpose of the study. Here I have chosen the footpad of the hind limb of the Rats. (Smolen et al., 2016)

Subcutaneously inject 0.1 ml of CFA containing 10 mg/ml of heat-killed MT into the footpad of a rear paw. The needle should be inserted just under the skin of the footpad pointing toward the ankle: this maximizes delivery of the adjuvant to the draining popliteal lymph nodes. All the Rats excluding the normal control rats were induced with 0.1 ml of CFA by injecting into the sub-plantar region of the left posterior paw. Severe and acute inflammation is observed within 30 minutes of injection, reaches its' peaks within 4-14 days, and often persists for 20 to 25 days. The injected foot should not be included in an arthritis severity scoring protocol because swelling always occurs in the injected foot even when arthritis is absent in other limbs. (Smolen et al., 2016)

8.4*:* Experimental Design:

The experimental protocol was designed for 28 days. The rats were divided into Five groups, with five animals in each group (n=5).

❖ **Group I:** Normal Control Group: The Rats were received normal saline (0.5 ml/kg, p.o) for 28 days.

❖ **Group II:** Rheumatoid Arthritis (CFA) Control Group: The rheumatoid Arthritis Control rats treated with CFA (0.1 ml; s.c)

❖ **Group III:** Extract-Treated (AAHE 200 mg/kg) Group: The rheumatoid arthritis rats were treated with AAHE (200mg/kg B.W, p.o) from the 7th day to 28th day.

❖ **Group IV:** Extract-Treated (AAHE 400 mg/kg) Group: The rheumatoid arthritis rats were treated with AAHE (400mg/kg B.W, p.o) from the 7th day to 28th day.

❖ **Group V:** Reference (DICF-S) Control Group: The rheumatoid arthritis rats were treated with 5mg/kg of Reference Drug Diclofenac sodium (DICF-S); per-oral route (p.o) from the 7th day to 28th day.

After 24 hours of the last dose and after 18 hours of fasting condition blood was collected from five rats of each group, by cardiac puncture for the estimation of haematological parameters associated with Rheumatoid Arthritis and then sacrificed by cervical dislocation for the study of liver, kidney and bone antioxidant parameters. Histopathological studies of the liver, kidney and bone were also carried out.

8.5: Evaluation of Rheumatoid Arthritis:

8.5.1: Evaluation of Physical Parameter of Rheumatoid Arthritis:

Macroscopic evaluation of each group of rats were analyzed by determining the measurement of paw diameter, paw volume, arthritic score which is the principle arthritic index of the rats and also body weight of the rats were also evaluated.

8.5.1.1: Measurement of Paw Diameter:

The changes in joint diameter of left hind paws of all experimental rats were evaluated by using a digital vernier caliper on the respective days of 0, 4th, 7th, 14th, 21st, 28th day during the study period. The percentage in changes of paw diameter was calculated by using the formula:

$$Paw\ Diameter\ (\%) = \frac{Hind\ paw\ diameter\ on\ day\ X - Hind\ paw\ diameter\ on\ day\ 0}{Hind\ paw\ diameter\ on\ day\ 0} \times 100$$

Where, **X** = Mean of paw diameter at day 4th/7th/14th/21st/28th

0= Mean of paw diameter at day 0 (Before induction of CFA)

The values were compared with the negative control group statistically. (Ekambaram et al., 2010)

8.5.1.2: Measurement of Paw Volume:

Paw volume of left hind paw of all experimental rats were observed by evaluating the paw edema formation using the Plethysmometer manually before and after induction of CFA on the 0, 4th, 7th, 14th, 21st, 28th day of the experiment period.

The percentage in changes of paw volume was calculated by using the formula:

$$\text{Paw Volume (\%)} = \frac{\text{Hind paw volume on day X} - \text{Hind paw volume on day 0}}{\text{Hind paw volume on day 0 X 100}}$$

Where, **X** = Mean of paw volume at day 4ᵗʰ/7ᵗʰ/14ᵗʰ/21ˢᵗ/28ᵗʰ

0= Mean of paw volume at day 0 (Before induction of CFA)

The values of the experimental rats were statistically compared with CFA-control group. (Ekambaram et al., 2010)

8.5.1.3: Determination of Arthritis score:

A macroscopical investigation was conducted on all of the experimental rats to evaluate any significant changes including degree of swelling and redness, edema or nodule formation over the paw region including joints and particular tissues.

The experimental animals were subjected to macroscopical examination to measure the arthritic score by observing the degree of swelling and redness of joints, edema of periarticular tissues in the injected and non-injected paw

There was no significant change in paw volume or nodule formation in rats before the Adjuvant administration. After the CFA induced RA initiation the left hind paw joints physical changes were monitored, recorded and analyzed. There was criterion by depending upon that the paw score were determined and recorded. The criterions were:

0 = If there is no significant alteration in the paw area and joints

1= Formation of little amount of erythema in the rat's joints

2 = moderate or serious erythema in the rat's joints

3 = Severe erythema and significant swelling or inflammation in the rat's joints

4 = Severe erythema and swelling exceeding interphalangeal joints in rats. (Ekambaram et al., 2010)

8.5.1.4: Measurement of Body Weight:

Body weight of all the experimental rats was monitored before and after induction of CFA from the experiment from the Day 0, 4, 7, 21, 28. The difference in the data was correlated with both the normal control and CFA control groups statistically by using One-way Anova. (Ekambaram et al., 2010)

8.5.2: Estimation of Haematological and Serum Biochemical Parameters:

After completion of the 28 days of study, on day 29th all the experimental rats were sacrificed by using cervical decapitation procedure and through cardiac puncture method the blood was collected to analyse the haematological parameter analyzation including Red Blood Cell (RBC), White Blood Cell (WBC), haemoglobin (Hb), Erythrocyte Sedimentation Rate (ESR). The biochemical parameter including aspartate aminotransferase (AST) or Serum glutamic oxaloacetic transaminase (SGOT), alanine amino transferase (ALT) or Serum glutamic-pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin (TB), total cholesterol (TC), total protein (TP), and urea was checked and analysed by using commercially available assay kits (Arkray Healthcare Pvt. Ltd., Mumbai, Maharashtra, India).

8.5.2.1 Estimation of Serum SGOT AND SGPT

SGOT and SGPT are the enzymes found mainly in heart muscle, skeletal muscle, liver cells, Kidneys. Injury to these tissues causes releases of these enzymes in blood. Elevated levels are found in myocardial infraction, cardiac operations, hepatitis, acute renal diseases and lower level found in pregnancy, diabetic keto acidosis. All the reagents are used in this assay was already prepared in kit which was ready to be used. The reagents are named as enzyme reagent and starter reagent. Clean and dry test tubes with proper label were used for this assay. Enzyme reagent (0.8 ml) mixed with 0.2 ml samples (serum collected from each animal groups free from haemolysis), and serum was incubated at room temperature for 1 minute After that initiator reagent (0.2 ml) was added to the mixture. All the reaction mixture was mix properly and read the initial absorbance was taken at 340 nm Ao and repeated measure of the absorbance was taken in every 1, 2& 3 minutes. Mean was calculated for absorbance change per minute, value was estimated by following calculations

SGOT activity in U/L 250C/ 30C = Amin x 952

8.5.2.2 Estimation of ALP

Serum ALP is an important determinant of inflammatory condition of body. Pathological conditions like liver disease, parathyroidism, increased blood sugar level causes increase level of ALP in serum. In this study serum ALP is estimated by commercially available kit, Buffer and Substrate liquid reagents were supplied in kit, ready-to-use. Working Reagent was prepared in the ratio of 5 parts Buffer (R1) to 1 part Substrate (R2) (i.e., 25 mL Buffer and 5 ml Substrate). Serum was separated from each animal group, where, n=10 and haemolysis free serum was used for this experiment. For each sample and control, 1.0 ml Working Reagent was added to cuvette or test tube and warm to 37°C for 3 minutes. In sample tube 20 µL (0.020 mL) of serum was added and mixed gently. After that absorbance was taken at 405nm against distil water. At first absorbance was recorded for 1 minute and incubated at 37°C and repeated absorbance was measured up to 3 minutes. Reaction rate remain constant.

Calculation:

Values are derived based on the "absorptivity micromolar extinction coefficient" of 4-nitrophenol at 405 nm (0.01845). Units per litre (U/L) of Alkaline Phosphatase activity is that amount of enzyme which products one mmol/L of 4-nitrophenol per minute.

U/ L= AA/ Min/ Absorptivity x Total Volume/Sample volume

U/L = A/ Min / 0.01845 x 1.020/0.020

U/L = A/Min x 2764

8.5.2.3 Estimation of Total Protein

Proteins are the constitute of muscle, enzymes, hormones and other structural and functional entities of the body. Main plasma proteins like albumin and globulin fractions vary widely depend upon various disease condition. In this study, plasma protein level was estimated by using kit. Biuret reagent, which was provided in kit was used for the detection of serum protein. Serum was collected from each group, where n= 6. Three test tubes were labelled as blank, test and standard, where blank contains only biuret reagent and distil water, standard tube contains biuret reagent and protein standard and sample tube contains serum sample and biuret reagent. Samples of each tube were mixed properly and followed by incubation at 37C for 10min. Absorbance was measured for test and standard tubes at 550 nm against blank within 60 min

Calculations:

Total proteins (g/dl) = Abs Test/Abs. Sample X 8

8.5.2.4. Estimation Of Serum Lipid Profile

Total cholesterol, triglycerides, high density lipoproteins are the main lipids found in serum. Certain pathophysiological conditions, such as hypercholesterolaemia, hyperlipidaemia, hypothyroidism, uncontrolled diabetes, nephrotic syndrome and cirrhosis result increased levels of serum lipid. Malabsorption, malnutrition, hyperthyroidism, anaemias and liver diseases are the causes of lower level of serum lipid. Cholesterol kit uses CHOD / PAP method to determine cholesterol activity in serum or plasma. All the reagents were ready to use.

All the contents were mixed properly and incubated at 37C for 5 min, followed by measuring the absorbance at 505nm against blank.

Calculations:

Cholesterol in mg/dl = Abs. Of test/ Abs. Of standard x 200

8.5.3: Estimation of In-vivo antioxidant activity:

In-vivo antioxidant activity of bone, liver and kidney of experimental rats was evaluated. Firstly, the bones were crushed and then the crushed liver, kidney and bone were homogenized distinctively in 25 ml of phosphate buffer (20 mM, pH: 7.4). After complete homogenization the mixture was then centrifuged to obtain the clear supernatant at 12000 rpm for 30 min at 4°c using a Remi microcentrifuge (RM-12C). After obtaining the supernatant the ascertaining of the level of Lipid

Peroxidation (LPO), Reduced Glutathione (GSH), Superoxide Dismutase (SOD), Catalase (CAT) was also evaluated. The research of antioxidants, such as Superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT), as well as studies of free radicals, such as nitric oxide (NO), lipid peroxidation (LPO) were then conducted using these homogenized tissues. On the basis of the production of NADH-phenazine methosulphate-nitro blue tetrazolium formazan, the evaluation of SOD was developed. Based on GSH's potential to diminish DTNB within 5 minutes, an assay of GSH was conducted. The ability of catalase to oxidise hydrogen peroxide was used to estimate catalase activity. Griess reagent, where nitrates and nitrites are generated, was used to evaluate the measurement of NO. Malondialdehyde (MDA) emitted at the time of lipid peroxidation serves as the basis for the evaluation of LPO (Jana et al., 2023).

8.5.4: X-ray and Histopathological examination

On the 29th day after sacrificing the animal's liver, kidney and ankle joints were separated and preserved in 10 % formalin and later on assigned to a diagnostic lab for X-ray and histopathological study. For the assessment of the samples, the tissues were stained with haematoxylin-eosin dye for photo microscopic observations with resolution of 10x of microscope. Degree of pannus formation, synovial hyperplasia, and erosion of cartilage and bone were estimated. To facilitate the study photomicrographs were also taken. (Ekambaram et al., 2010)

8.6: Statistical Analysis:

All the data were presented as the mean ± Standard error of mean (SEM). The results were analysed for statistical significance by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test for significance. The p values less than 0.05 ($p < 0.05$) were considered as statistically significant.

8.7: Results:

8.7.1 Estimation of Hydro-alcoholic Extraction *Acrostichum aureum* on Paw Diameter:

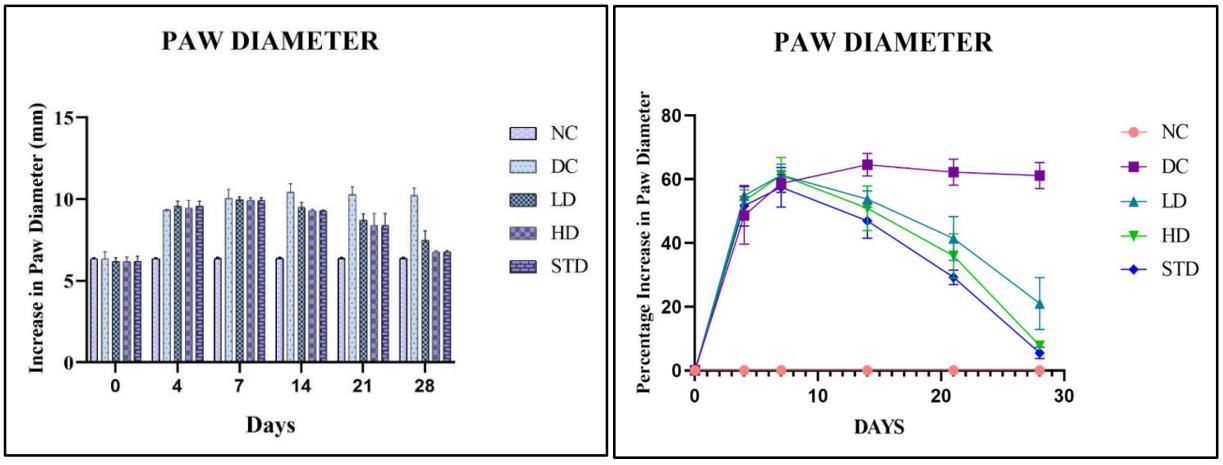
All the experimental rats were exhibited the increase in paw diameter on day 4 and reach maximum on day 14 in contrast to normal control rats. CFA induced rats showed significant redness, swelling, erythema, and edema at their joints. Treatment with Reference Diclofenac had significantly ($P<0.001$) reduced the paw swelling in contrast to the CFA induced group. Administration of formulations from day 15th to 28th day had significantly decreased the paw diameter in the order of STD, HD, LD. However, normal healthy control rats did not show any change in paw diameter. The results are depicted below (Table. 7); (Fig. 10).

Table 7: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Paw Diameter***

Group	DAY 0	DAY 4	DAY 7	DAY 14	DAY 21	DAY 28
Normal Control	6.35±0.032	6.35±0.032	6.363±0.032	6.363±0.032	6.363±0.032	6.363±0.032
Disease Control	6.343±0.255a*	9.316±0.076a*	10.06±0.307a*	10.43±0.299a*	10.28±0.274a*	10.21±0.0268a*
Low Dose (200mg/kg AAHE)	6.176±0.137b*	9.566±0.184b*	9.956±0.109b*	9.496±0.187b*	8.72±0.222b*	7.476±0.331b*
High Dose (400mg/kg AAHE)	6.166±0.165b*	9.450±0.269b*	9.936±0.093b*	9.293±0.048b*	8.393±0.418b*	6.776±0.037b*
Reference Control (DICF-S 5mg/kg)	6.190±0.185b*	9.56±0.0913b*	9.936±0.131b*	9.276±0.090b*	8.293±0.236b*	6.521±0.170b*

Each value is expressed as Mean±SEM (n=5), a* Disease Control (Rheumatoid Arthritis Control group) versus Normal Control group (P<0.0001), b* Treatment Control groups (200 mg/kg AAHE & 400 mg/kg AAHE) versus Disease Control group on corresponding day (P<0.0001), *Values significantly differ from each other where (P<0.0001). SEM: Standard Error Mean, AAHE: *Acrostichum aureum* Hydro-alcoholic Extract; DICF-S: Diclofenac sodium.

Fig.10: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Paw Diameter***



Each value is expressed as Mean±SEM where n=5; a* p<0.05 when compared to Normal Control and b* p<0.05 when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

8.7.2 Estimation of Hydro-alcoholic Extraction *Acrostichum aureum* on Paw Volume:

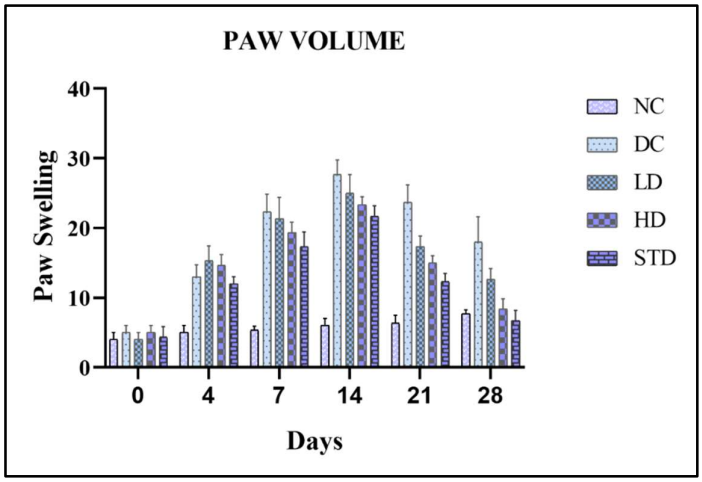
The activity of Hydro-alcoholic extract of *Acrostichum aureum* at doses 200 and 400mg/kg body weight and Diclofenac sodium to inhibit inflammatory paw edema or paw volume was examined against CFA-control group and found to be significant at $p < 0.001$ and $p < 0.05$ respectively. Highest percent of inhibition was expressed by AAHE extract at a dose of 400mg/kg, especially at chronic phase at which is relatively similar to DICF-S. The results are depicted below (Table. 8); (Fig. 11).

Table 8: ***Estimation of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Paw Volume***

Group	DAY 0	DAY 4	DAY 7	DAY 14	DAY 21	DAY 28
Normal Control	4±0.577	5±1.00	5.333±01.152	6±1.201	6.333±1.452	7.666±1.154
Disease Control	5±0.881a*	13±0.577a*	22.333±1.201 a*	27.666±0.881 a*	23.666±0.666 a*	18±0.881a*
Low Dose (200mg/kg AAHE)	4±0.577b*	15.333±1.201 b*	21.333±1.763 b*	25±1.527b*	17.333±0.882 b*	12.666±0.881 b*
High Dose (400mg/kg AAHE)	5±0.577b*	14.666±0.881 b*	19.333±0.882 b*	23.333±0.666 b*	15±0.577b*	8.333±0.666b *
Reference Control (DICF-S 5mg/kg)	4.333±0.881b *	12±0.269b*	17.333±0.093 b*	21.666±0.048 b*	12.333±0.418 b*	6.666±0.037b *

Each value is expressed as Mean±SEM (n=5), a*Disease Control (Rheumatoid Arthritis Control group) versus Normal Control group (P<0.05), b* Treatment Control groups (200 mg/kg AAHE & 400 mg/kg AAHE) versus Disease Control group on corresponding day (P<0.05), *Values significantly differ from each other where (P<0.05). SEM: Reference Error Mean, AAHE: *Acrostichum aureum* Hydro-alcoholic Extract; DICF-S: Diclofenac sodium.

Fig.11: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Paw Volume***



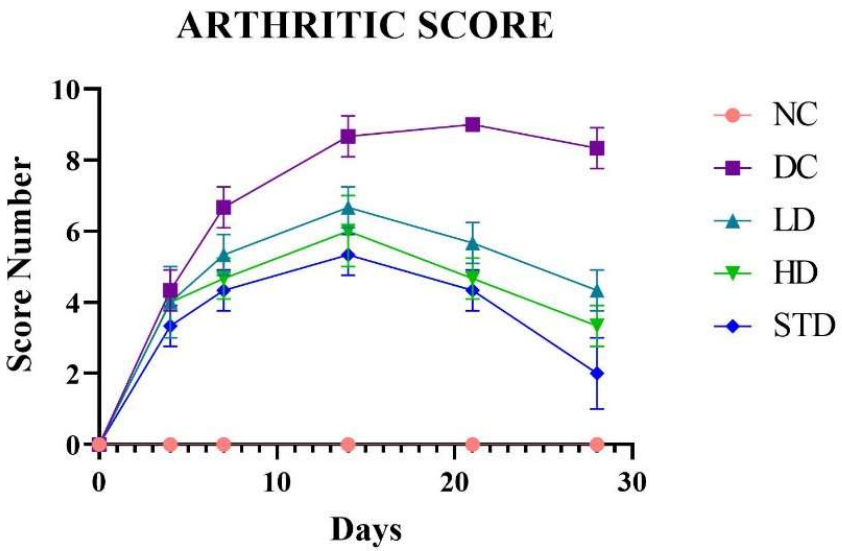
Each value is expressed as Mean±SEM where n=5; a* p<0.05 when compared to Normal Control and b* p<0.05 when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

8.7.3 Estimation of Hydro-alcoholic Extraction *Acrostichum aureum* on the Arthritic Score

The morphological examination of hind paws of experimental rats was given in Fig. 1c. The arthritic score was found to be maximum (9.5 ± 0.28) on 28th day in vehicle-treated CFA immunized rats in contrast to healthy rats. The animal treated with diclofenac sodium remarkably (P<0.001) declined the arthritic score on the 28th day. However, supplementation of extract from 15th to 28th day was noticed with a significant reduction in arthritic score in the order of HD and then LD. The normal healthy control rats did not show any sign of erythema on the hind paw. The macroscopic investigation of hind paws of the experimental rat is given in Fig.

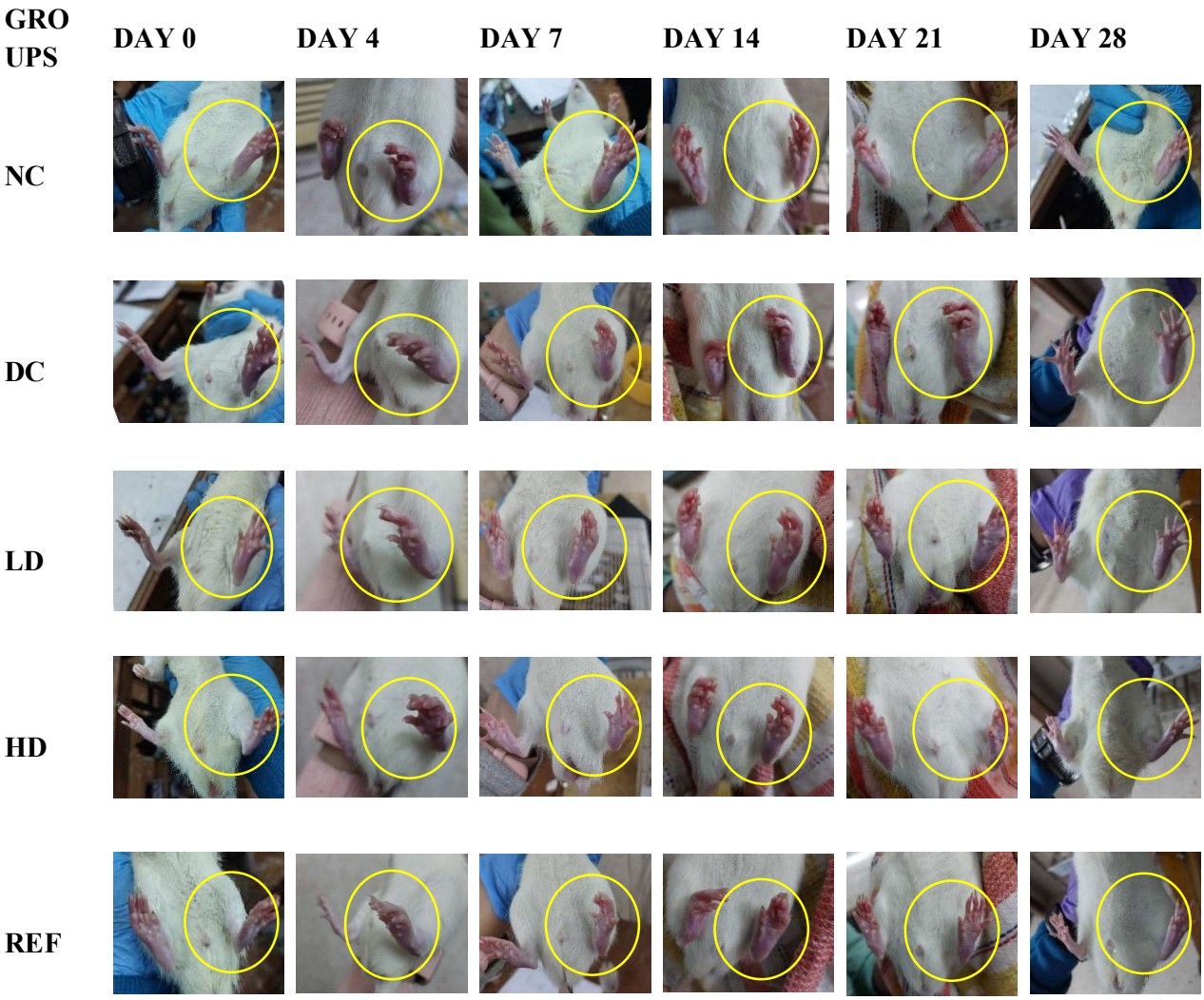
Fig.12: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Arthritic Score***



Values significantly differ from each other where (P<0.05). Each value is expressed as Mean±SEM where n=5; a p<0.05 when compared to Normal Control and b* p<0.05 when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

Fig.13: Macroscopic Evaluation of Arthritis Index



"NC= Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= Extract Treated Group (200mg/kg AAHE); HD= Extract Treated Group (400mg/kg AAHE); REF= Reference Control Group (DICF-S 5mg/kg)"

8.7.4 Estimation of Hydro-alcoholic Extraction *Acrostichum aureum* on Body Weight:

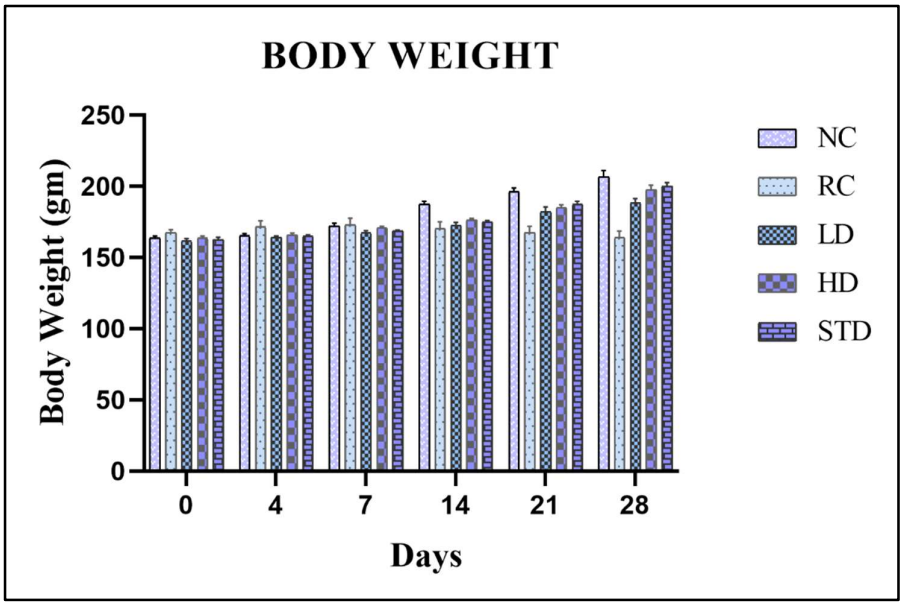
The vehicle-treated CFA rats were marked with no such significant (P<0.001) reduction in body weight in between 14th to 28th days in contrast to Rheumatoid Arthritis rats. The normal control rats were noticed with progressive improvement in body weight with respect to time. A continual increase in body weight was also marked in rats treated with Diclofenac sodium. The rats treated with AAHE showed a gradual increase in body weight up to 28th day. Table. 9 and Fig. 14.

Table 9: *Estimation of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Body Weight*

Group	DAY 0	DAY 4	DAY 7	DAY 14	DAY 21	DAY 28
Normal Control	163.66±0.881	165.33±0.882	172±1.154	187.33±1.201	196.33±1.452	206.66±2.603
Disease Control	167.33±1.201a*	171.66±2.403 a*	173±2.645 a*	170.33±2.728 a*	167.33±2.728 a*	164±2.645 a*
Low Dose (200mg/kg AAHE)	161.66±0.881 b*	164±0.577 b*	167.33±0.881 b*	172.66±1.201 b*	182±2.081 b*	188.33±1.763 b*
High Dose (400mg/kg AAHE)	163.66±0.881 b*	165.66±0.881 b*	171±0.577 b*	176.33±0.666 b*	185±1.154 b*	197.66±1.763 b*
Reference Control (DICF-S 5mg/kg)	162.66±0.881 b*	165±0.577 b*	168.66±0.333 b*	175±0.577 b*	187.33±1.732 b*	200±1.201 b*

Each value is expressed as Mean±SEM (n=5), a*Disease Control (Rheumatoid Arthritis Control group) versus Normal Control group (P<0.05), b* Treatment Control groups (200 mg/kg AAHE & 400 mg/kg AAHE) versus Disease Control group on corresponding day (P<0.05), *Values significantly differ from each other where (P<0.05). SEM: Reference Error Mean, AAHE: *Acrostichum aureum* Hydro-alcoholic Extract; DICF-S: Diclofenac sodium.

Fig.14: *Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Body Weight*



Each value is expressed as Mean±SEM where n=5; a* p<0.05 when compared to Normal Control and b* p<0.05 when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; RC= Rheumatoid Arthritis Control; LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

8.7.5 Estimation of Hydro-alcoholic Extraction *Acrostichum aureum* on Haematological parameters

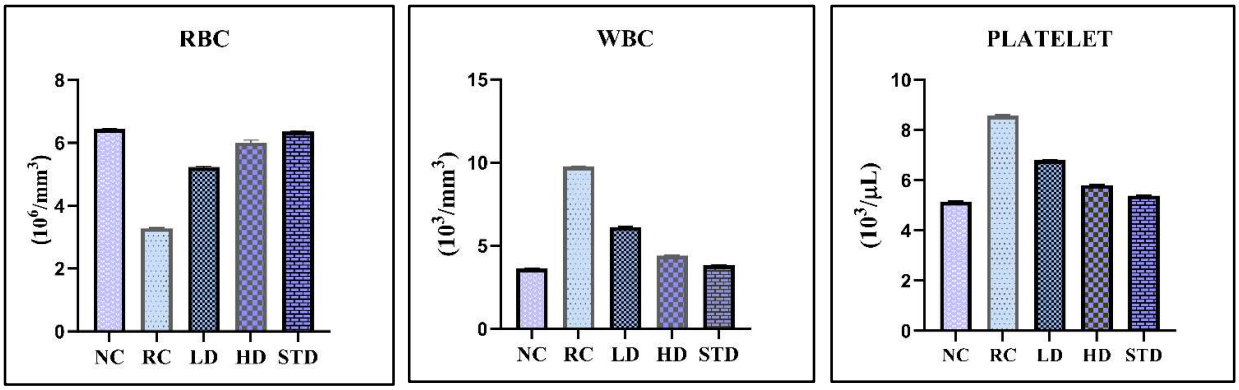
The experimental rats induced with CFA were distinguished with a significant decrease in RBC count, haemoglobin, packed cell volume and increase in WBC count, ESR and platelet count in contrast to the normal healthy rats. The group treated with indomethacin significantly (P<0.001) retrieved the altered haematological parameter to the normal state. These haematological parameters were also remarkably restored to normal value with the administration of AAHE. The data are given in Table 10 and Fig. 15

Table 10: *Estimation of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Haematological Parameters:*

Groups	RBC (10^6/mm^3)	WBC (10^3/mm^3)	PLATELET (10^3/µL)	HAEMOGLOBIN (g/dl)
Normal Control	6.45±0.15	3.62±0.04	5.29±0.03	11.54±0.11
Disease Control	3.35±0.08a*	9.62±0.08a*	9.17±0.09a*	5.62±0.12a*
Low Dose (200mg/kg AAHE)	5.59±0.09b*	5.82±0.05b*	6.75±0.05b*	8.18±0.010b*
High Dose (400mg/kg AAHE)	6.12±0.07b*	4.31±0.06b*	5.82±0.02b*	10.22±0.05b*
Reference Control (DICF-S 5mg/kg)	6.35±0.05b*	3.85±0.03b*	5.43±0.01b*	11.23±0.11b*

"Effect of AAHE on Haematological Parameters: Each value is expressed as Mean±SEM where n=5. a*p<0.05 when Disease Control (Rheumatoid Arthritis Control group) compared to Normal Control and b* p<0.05 when Treatment Control groups (200 mg/kg AAHE & 400 mg/kg AAHE) compared to Disease Control**."**

Fig.15: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Haematological Parameters***



"Effect of AAHE on RBC, WBC and Platelet. Each value is expressed as Mean±SEM where n=5; a* $p<0.05$ when compared to Normal Control and b* $p<0.05$ when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; RC= Rheumatoid Arthritis Control; LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

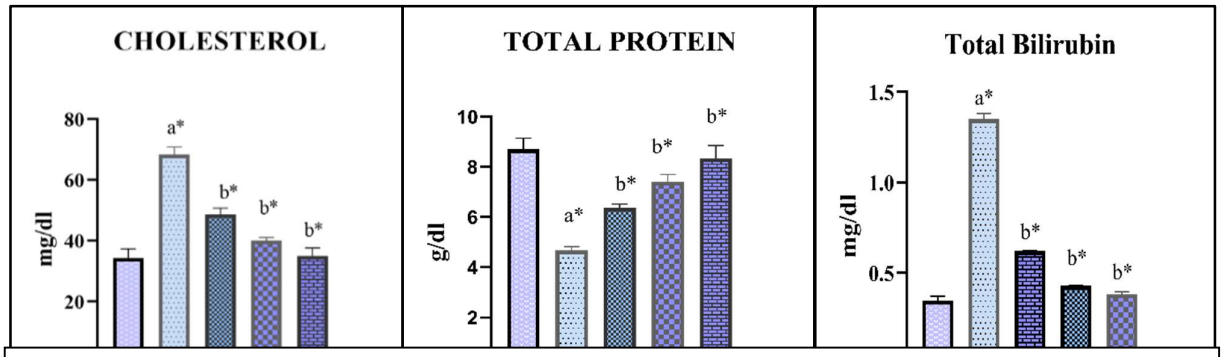
8.7.6 Estimation of Hydro-alcoholic Extraction of *Acrostichum aureum* on the Sreum Biochemical Parameters:

The significant elevation in the level of Cholesterol, SGPT, SGOT, ALP, Total Bilirubin, Total Protein, Urea was noticed in CFA induced groups in comparison to normal control rats. There was also a remarkable increase Cholesterol, SGPT, SGOT, ALP, Total Bilirubin, Urea and, a decline of total protein and albumin in case of CFA treated rats in comparison to Normal rats. Treatment with DICF-S (5mg/kg) and AAHE (200mg/kg) and 400 mg/kg noticeably decreased these levels of SGPT, SGOT, ALP, Cholesterol, Total Bilirubin, and significantly increased the Total Protein level as compared to CFA treated rats Table 11 and Fig. 16.

Table 11: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Serum Biochemical Parameters***

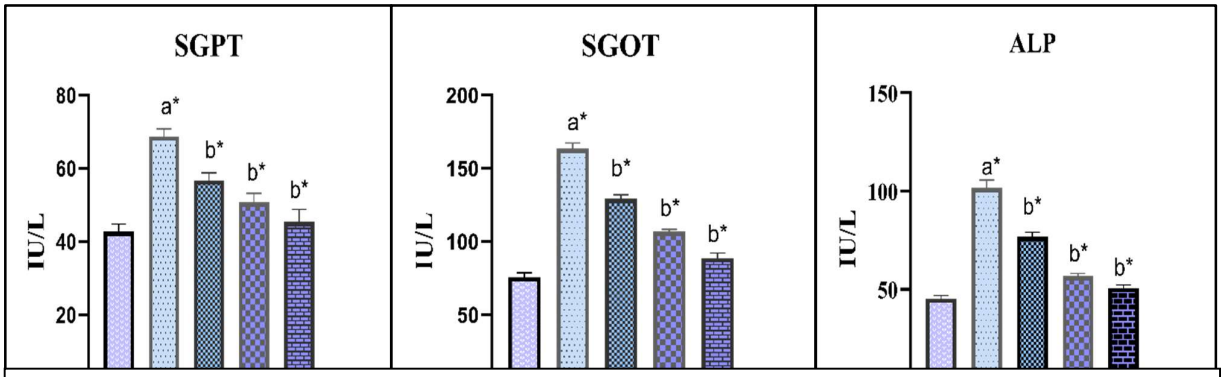
Groups	Cholesterol (mg/dl)	Total Protein (g/dl)	Total Bilirubin (mg/dl)	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	Urea (mg/dl)
Normal Control	34.333±1.763	7.133±0.145	0.346±0.014	42.667±1.201	75.666±1.763	45.333±0.881	16.20±0.608
Disease Control	63.666±1.201a*	5±0.10a*	1.29±0.015a*	68.666±1.201 a*	163.33±2.333 a*	101.65±2.963 a*	26.866±0.688 a*
Low Dose (200mg/kg AAHE)	43.666±0.881b*	6.233±0.145 b*	0.593±0.005 b*	56.666±1.154 b*	129.33±1.452 b*	76.833±2.059 b*	17.50±0.503 b*
High Dose (400mg/kg AAHE)	38.666±0.881 b*	7.1±0.185 b*	0.208±0.003 b*	50.666±1.452 b*	109±1.527 b*	56.666±1.452 b*	17.333±0.346 b*
Reference Control (DICF-S 5mg/kg)	36±2.081 b*	7.533±0.173 b*	0.349±0.005 b*	45.333±1.154 b*	90.333±2.728 b*	50.667±1.763 b*	16.3±0.611 b*

Fig.16: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Serum Biochemical Parameters***



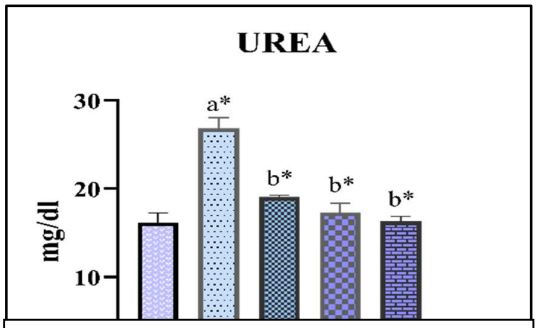
"Effect of AAHE on Cholesterol, Total Protein and Bilirubin: Each value is expressed as Mean±SEM where n=5; a* p<0.05 when compared to Normal Control and b* p<0.05 when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"



"Effect of AAHE on SGPT, SGOT, ALP: Each value is expressed as Mean±SEM where n=5; a* p<0.05 when compared to Normal Control and b* p<0.05 when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"



"Effect of AAHE on UREA: Each value is expressed as Mean±SEM where n=5; a* p<0.05 when compared to Normal Control and b* p<0.05 when compared with Rheumatoid Arthritis Control**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

8.7.7 Evaluation of in-vivo antioxidant activity

The level of LPO, enzymatic SOD and CAT, and nonenzymatic GSH activity were estimated in the tissues of joints, liver, and kidney of experimental animals. The increase in LPO, decrease in SOD, CAT and GSH activity were observed in vehicle-treated CFA groups in comparison to the normal control group. The LPO was measured by determining the amount of thio-barbituric acid reactive substances (TBARS) in tissues of experimental rats (Fig. 17.1). The elevated level of LPO was perceived in the Liver followed by kidneys, Joint of arthritic animals as compared to normal control rats. This altered level of LPO was remarkably brought to the normal by the treatment with AAHE

treatments. The group treated with Diclofenac sodium was also noticed with a significant reduction in the LPO level. The SOD activity was assessed to determine the endogenous defences against superoxide radicals. Joint tissues exhibited the highest SOD activity and lowest in the kidney of CFA vehicle-treated rats. The negative control group has showed with a decrease in catalase activity by in the Joint tissues followed by Liver, and kidney (Fig. 17.2). The GSH level was calculated as acid soluble sulfhydryl group (-SH) in tissues of normal and CFA induced rats. Fig.17.3 showed a significant decline of GSH quantity in disease control rats in contrast to normal control rats. The onset of CFA caused a remarkable depletion in the levels of GSH in the joints, liver, and kidney respectively. These altered enzymatic and non-enzymatic anti-oxidant levels were significantly maintained by the Diclofenac sodium and AAHE treated rats as compared to the disease control (CFA) rats. Fig 17.4 showed a marked decrease in the level of CAT in case of CFA treated groups whereas increase in the Treatment control groups including Reference control group as well as Extract treated group (AAHE 200 mg/kg and 400 mg/kg).

Table 12.1: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on the Level of Lipid Peroxidant:***

Groups	LPO (µm/100gm Tissue homogenate) [LIVER]	LPO (µm/100gm Tissue homogenate) [KIDNEY]	LPO (µm/100gm Tissue homogenate) [JOINT]
Normal Control	1.366±0.012	1.193±0.008	1.253±0.008
Disease Control	3.296±0.017a*	3.193±0.018 a*	3.173±0.018 a*
Low Dose (200mg/kg AAHE)	2.880±0.013b*	2.566±0.041b*	2.716±0.015b*
High Dose (400mg/kg AAHE)	1.970±0.017b*	1.870±0.043b*	1.870±0.023b*
Reference Control (DICF-S 5mg/kg)	1.550±0.023b*	1.366±0.014b*	1.466±0.014b*

"Values are represented as Mean±SEM, where n=6. a*p<0.05 when compared Disease control compared with Normal Control, b* when Treatment Control (200 mg/kg AAHE and 400 mg/kg AAHE) compared to Disease Control."

Table 12.1: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on the Level of Superoxide Dismutase:***

Groups	SOD (U/mg) [LIVER]	SOD (U/mg) [KIDNEY]	SOD (U/mg) [JOINT]
Normal Control	3.403±0.029	3.680±0.017	3.790±0.011
Disease Control	0.813±0.176a*	1.033±0.023a*	1.176±0.023a*
Low Dose (200mg/kg AAHE)	1.956±0.024b*	2.113±0.017b*	2.303±0.018b*
High Dose (400mg/kg AAHE)	2.793±0.017b*	2.813±0.041b*	2.980±0.017b*
Reference Control (DICF-S 5mg/kg)	3.203±0.012b*	3.326±0.023b*	3.536±0.031b*

"Values are represented as Mean±SEM, where n=6. A*p<0.05 when compared Disease control compared with Normal Control, b* when Treatment Control (200 mg/kg AAHE and 400 mg/kg AAHE) compared to Disease Control."

Table 12.1: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on the Level of Glutathione Reductase:***

Groups	GSH (µg/mg Tissue) [LIVER]	GSH (µg/mg Tissue) [KIDNEY]	GSH (µg/mg Tissue) [JOINT]
Normal Control	3.443±0.012	3.643±0.089	3.786±0.008
Disease Control	1.620±0.005a*	1.863±0.008a*	1.720±0.017a*
Low Dose (200mg/kg AAHE)	2.501±0.017b*	2.710±0.020b*	2.811±0.020b*
High Dose (400mg/kg AAHE)	3.190±0.011b*	3.246±0.023b*	3.486±0.027b*
Reference Control (DICF-S 5mg/kg)	3.366±0.027b*	3.592±0.015b*	3.753±0.020b*

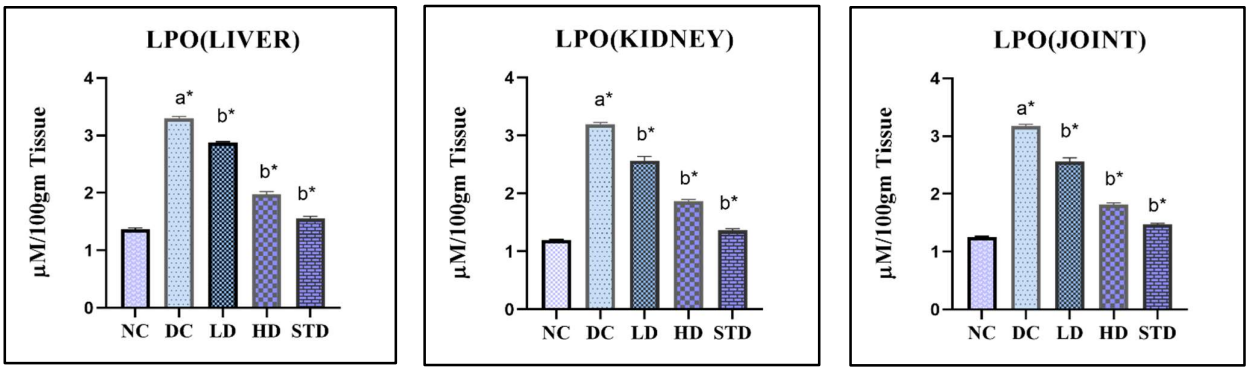
"Values are represented as Mean±SEM, where n=6. a*p<0.05 when compared Disease control compared with Normal Control, b* when Treatment Control (200 mg/kg AAHE and 400 mg/kg AAHE) compared to Disease Control."

Table 12.1: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on the Level of Catalase:***

Groups	CATALASE (KU/min/mg Protein) [LIVER]	CATALASE (KU/min/mg Protein) [KIDNEY]	CATALASE (KU/min/mg Protein) [JOINT]
Normal Control	2.596±0.017	2.363±0.017	2.253±0.008
Disease Control	0.946±0.064a*	0.633±0.023a*	0.281±0.020a*
Low Dose (200mg/kg AAHE)	1.986±0.021b*	1.790±0.050b*	1.426±0.018b*
High Dose (400mg/kg AAHE)	2.326±0.039b*	2.060±0.037b*	1.801±0.049b*
Reference Control (DICF-S 5mg/kg)	2.406±0.021b*	2.179±0.017b*	1.976±0.047b*

"Values are represented as Mean±SEM, where n=6. a*p<0.05 when compared Disease control compared with Normal Control, b* when Treatment Control (200 mg/kg AAHE and 400 mg/kg AAHE) compared to Disease Control."

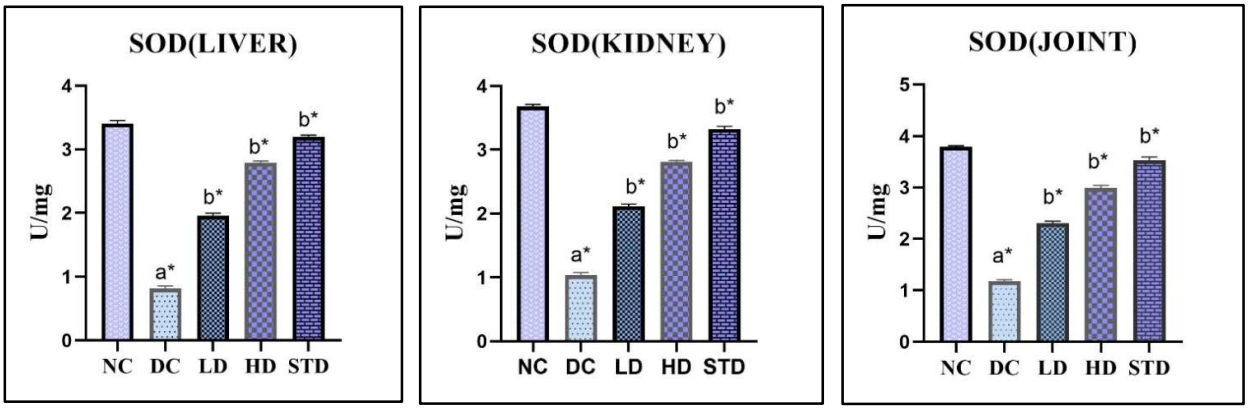
Fig.17.1: Estimation of LPO Level in Liver, Kidney and Joint



"Effects of Different Concentrations of AAHE on Lipid Peroxidation: The values are represented as Mean±SEM; a* p<0.05 when Disease Control compared with Normal Control Liver, Kidney and Joint; b* p<0.05 when Treated Group compared with Disease Control Liver, Kidney and Joint.**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

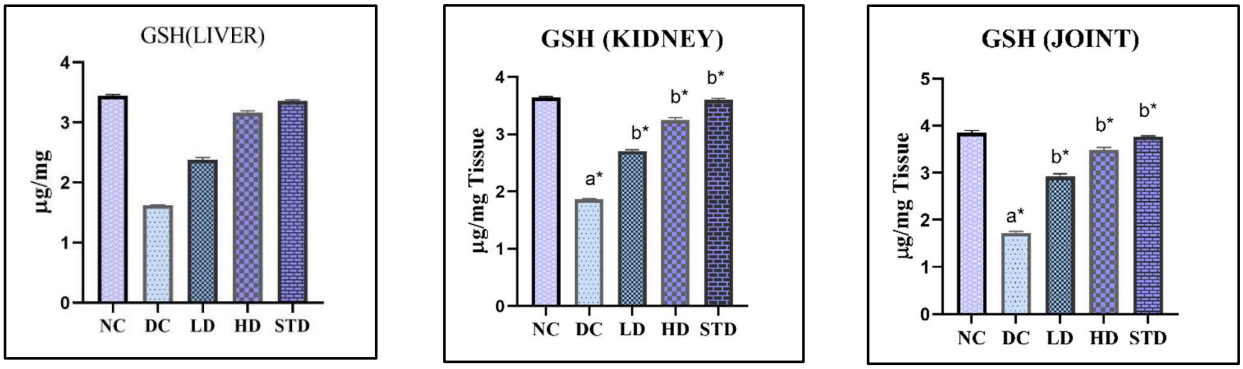
Fig.17.2: Estimation of SOD Level in Liver, Kidney and Joint



"Effects of Different Concentrations of AAHE on Super Oxide Dismutase: The values are represented as Mean±SEM; a* p<0.05 when Disease Control compared with Normal Control Liver, Kidney and Joint; b* p<0.05 when Treated Group compared with Disease Control Liver, Kidney and Joint.**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

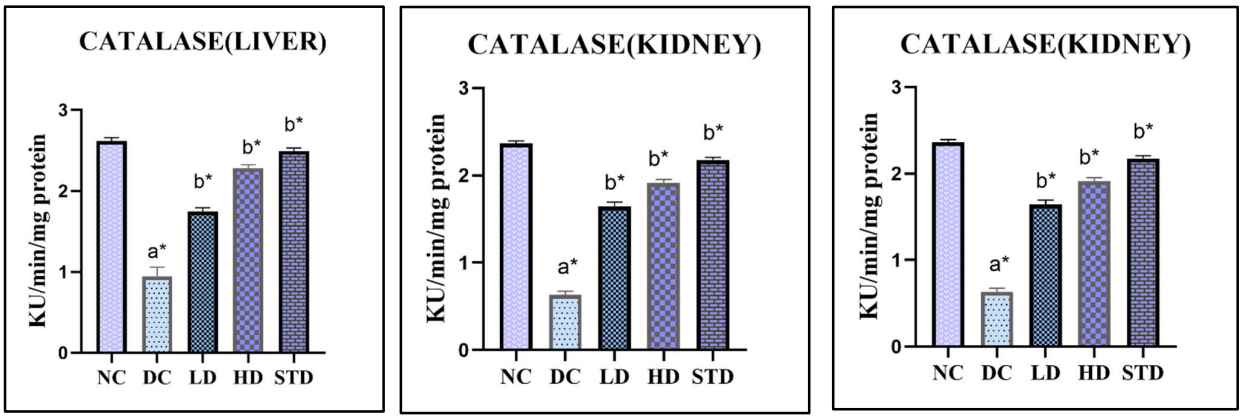
Fig.17.3: Estimation of GSH Level in Liver, Kidney and Joint



"Effects of Different Concentrations of AAHE on Glutathione Reductase: The values are represented as Mean±SEM; a* p<0.05 when Disease Control compared with Normal Control Liver, Kidney and Joint; b* p<0.05 when Treated Group compared with Disease Control Liver, Kidney and Joint.**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

Fig.17.4: Estimation of CAT Level in Liver, Kidney and Joint



"Effects of Different Concentrations of AAHE on Glutathione Reductase: The values are represented as, Mean±SEM; a* $p<0.05$ when Disease Control compared with Normal Control Liver, Kidney and Joint; b* $p<0.05$ when Treated Group compared with Disease Control Liver, Kidney and Joint.**"**

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

8.7.8 Histopathology of Liver, Kidney and Joint:

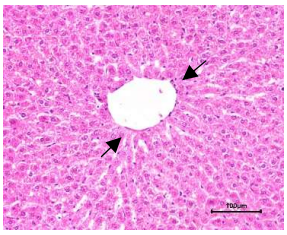
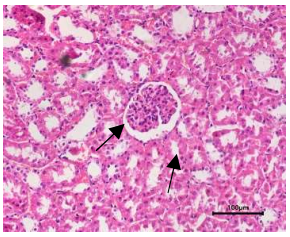
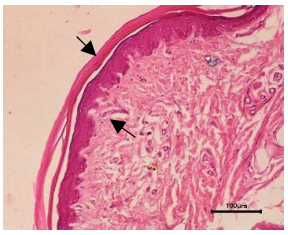
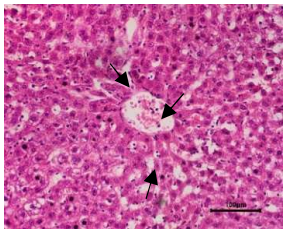
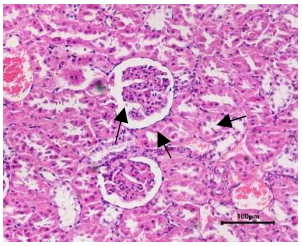
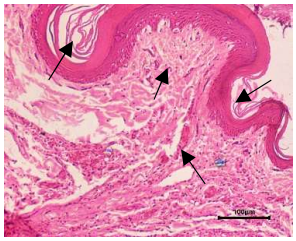
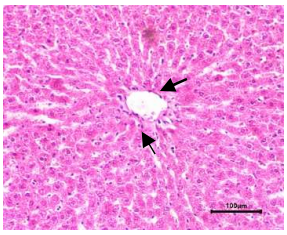
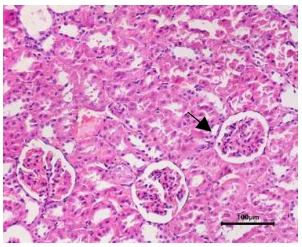
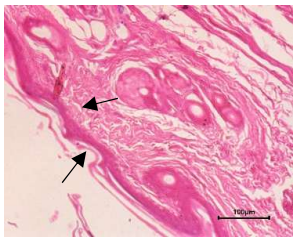
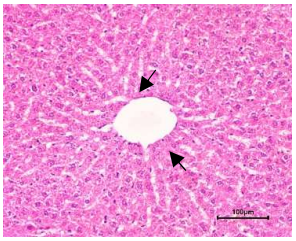
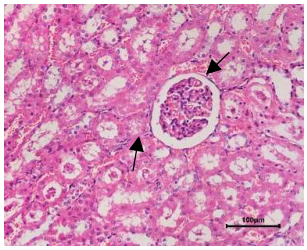
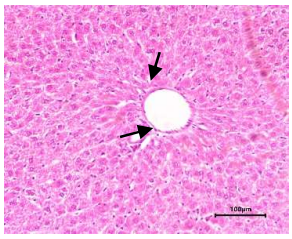
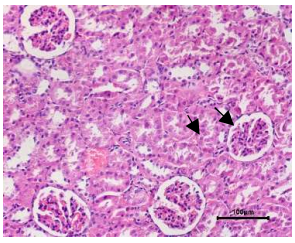
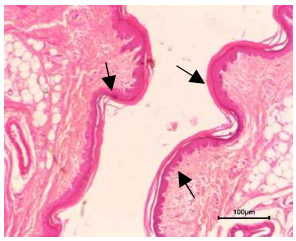
The histological study of haematoxylin and eosin-stained ankle joints of normal and CFA treated rats were depicted in Fig.18.

The livers of CFA treated rats were noticed with destruction in cells surrounding portal vein in compare to normal control groups but there is a recovering area in dose dependent manner Low dose (200 mg/kg) and High Dose (400 mg/kg) and also in case of Reference Control (DICF-S 5 mg/kg) with respect to Disease control group rats.

The kidneys of CFA treated rats were noticed with thickening of glomerular membrane in compare to normal control groups but there is decrease in thickness of glomerular membrane in dose dependent manner Low dose (200 mg/kg) and High Dose (400 mg/kg) and also in case of Reference Control (DICF-S 5 mg/kg) with respect to Disease control group rats.

The joints of CFA vehicle-treated rats were noticed with distinct bone and cartilage erosion indicating the successful induction of arthritis by CFA followed by the development of synovial hyperplasia and pannus formation. The ankle joints of healthy rats revealed normal histology of joints. The histological section of joints of Diclofenac treated and AAHE treated rats observed increased in smooth articular surface and articular cartilage layer, normal joint space, and reduction in synovial hyperplasia and pannus formation. The therapeutic administration of AAHE from 15th to 28th day had remarkably reduced the histological changes in experimental rats.

Fig.18: ***Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on Histopathology:***

Group	Liver	Kidney	Joint
Normal Control			
Disease Control			
Low Dose (200mg/kg AAHE)			
High Dose (400mg/kg AAHE)			
Reference Control (DICF-S 5mg/kg)			

8.7.9 Effect of Hydro-alcoholic Extraction of *Acrostichum aureum* on X-ray analysis

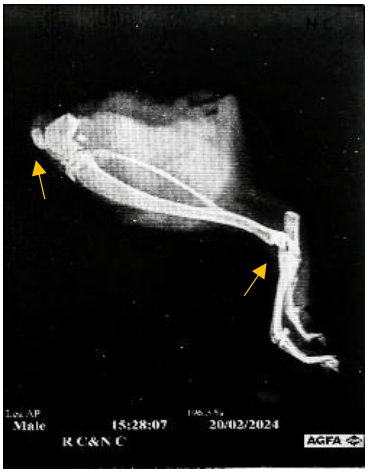
Histopathological Changes in Liver, kidney and Joint Tissues: Light microscopy images of haematoxylin and eosin-stained liver (day 29), kidney (day 29) and joint (day 29) were taken at a magnification of x20. Tissue samples were collected at day 29. Scale bar 100 µm.

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

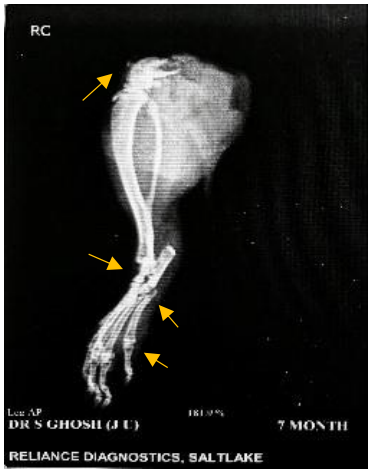
The radiographic examinations of ankle joints of normal and treated animals were shown in Fig.19. The CFA induced rats showed significant swelling of soft tissue, narrowing of joint space and resorption of bone matrix, whereas no such evidence was noticed in treatment control groups. These radiological changes were significantly protected by AAHE treated and reference drug treated rats in comparison to CFA treated rats.

Fig.19: Effect of Hydro-Alcoholic Extract of Acrostichum aureum Aerial Part on X-ray analysis:

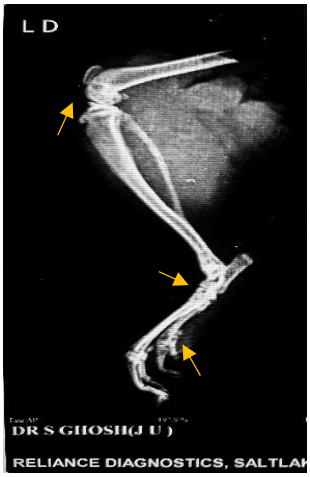
NC



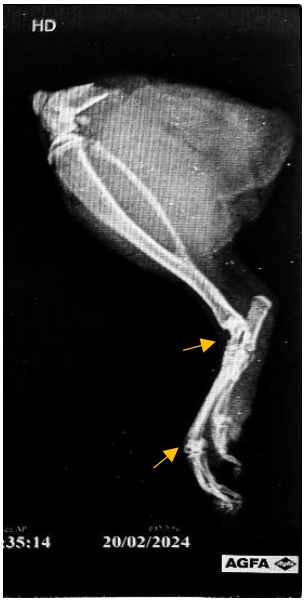
DC



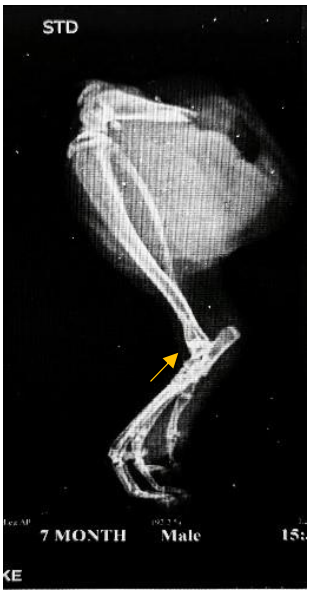
LD



HD



STD



Bone and cartilage erosion and (E) Radiological study: soft tissue swelling (white arrow), degenerative changes (yellow arrow) of proximal interphalangeal joints and knee joints (stained with hematoxylin and eosin) of experimental rats.

"NC=Normal Control; DC= Disease Control (CFA induced Rheumatoid arthritis); LD= CFA+AAHE 200 mg/kg; HD= CFA+AAHE 400 mg/kg; STD= CFA+ DICF-S 5 mg/kg"

8.8. Discussions

The result of the present study revealed that hydro-alcoholic extract of *Acrostichum aureum* aerial part has successively supresses the disease progression in RA by inhibiting the production of inflammatory mediators and oxidative stress. The rats induced with CFA were noticed with remarkable inflammation on hind paws. In the acute phase of inflammation, there is the production of immune cells that are drifted to the injected area and causes the vascular exudative phenomenon. In the chronic phase, there is the liberation of pro-inflammatory mediators (IL-6, IL-1β etc.), which causes synovial hyperplasia, pannus formation, and bone and cartilage destruction. In the present investigation, the significant reduction in the paw oedema, paw diameter arthritic score, arthritic index, and histological study results could be due to the interaction of active principles of *Acrostichum aureum* with these immune cells and thereby inhibiting the release of inflammatory cytokines. From the present investigation it is found that the dosage progression supressed in dose dependent manner.

Rheumatoid cachexia, recognized by a decrease in body weight due to the poor absorption of nutrients through intestine during inflammation, which was noticed in CFA induced rats. The data of the present study revealed the significant protection against loss of body weight in AAHE treated rats. It is reported that the anti-inflammatory agents can retrieve the impended absorption potentiality of the intestine. Thus, the anti-inflammatory property of the compounds present in *Acrostichum aureum* provides significant protection against weight loss.

It has been seen that a patient with RA was associated with several haematological and biochemical alterations, which are considered as crucial markers in an arthritic diseased condition indicating a change in the immune system. The variation in haematological parameters such as rise in WBC count, platelet, and Hb. Values are expressed as mean ± SEM, n= 6 and was estimated by using one-way ANOVA.

The significant increase in serum enzyme level; AST, ALT, and ALP indicate functional impairment of liver and kidney of the body. It is reported that ROS plays a significant role in the pathogenesis of RA. In arthritic conditions, the inflamed cells release free radicals and cause tissue oxidative stress, which was overcame, in this study, by the AAHE via decreasing the inflammation as well as oxidative stress of arthritic rats. In the present investigation the elevated malondialdehyde (MDA) content was noticed in the tissue of liver, kidney, and joint of CFA induced arthritic rats in contrast to normal rats indicates increased in LPO. This might be due to the compromised intracellular antioxidant defence system with insufficiently scavenge free radicals resulting in increased oxidative stress in RA.

Decrease in SOD and CAT activity was observed in CFA induced rats in comparison to the normal healthy group. This may be due to the excessive production of superoxide radicals and H2O2 that obstruct the enzymatic activity. It was noticed in the study that excessive lipid peroxidation caused an increase in GSH consumption and thereby depletion of GSH in CFA induced rats as compared to normal rats. The oral administration of AAHE treated extract in CFA induced rats has improved the SOD and CAT activity followed by GHS and the decreased level of MDA suggesting the antioxidant activity of the AAHE (200 mg/kg and 400 mg/kg). This may be due to the presence of bioactive principles such as Flavonoids, Tri-terpenoids and alkaloids in *Acrostichum aureum,* which have been proven to scavenge the free radicals in the ethanol-induced gastric acid in animal model. *Acrostichum aureum* is an important traditional medicinal plant, which is rich in poly-phenolic compounds. Thus, the presence of active Phyto-constituents present in AAHE could potentiate the antioxidant efficacy.

The experimental rats, subjected to histological study, were marked with degenerative and inflammatory changes in the ankle joint. The decreased histopathological score has indicated a significant protective role of the AAHE against arthritic conditions. The reduction in tissue injuries and inflammatory changes in ankle joint might be due to the antioxidant activity of formulation by scavenging the free radicals. Thus, the immunomodulatory effects of AAHE against arthritic rats indicate its potential as an anti-arthritic agent. In RA, the destruction of bone and cartilage causes a decrease in joint space which leads to bone fusion. X-ray analyses of experimental rats were marked with the loss of joint space owing to the fusion of ankle joint. Radiographs of the ankle joints of CFA induced rats were noticed with a significant reduction in fusion and bone destruction after treatment with the Hydro-alcoholic extract of *Acrostichum aureum* aerial parts. (Mahdi et al., 2018)

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CHAPTER 9: SUMMARY AND CONCLUSION

9.1 Summary and Conclusion:

Rheumatoid arthritis (RA) is a systemic autoimmune and inflammatory joint disease characterized by persistent inflammation and synovium hyperplasia, leading to cartilage erosion and bone destruction. It can rapidly progress into a multi-system inflammation with irreversible joint destruction, thus increasing the risk of mortality (McInnes and Schett, 2011). Recent studies have revealed that pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) produced by T helper type-1 (Th1) cells, play a key role in the pathogenesis of RA (Brennan and McInnes, 2008).

While RA is not strictly a genetic disorder, some individuals possess genes that make them vulnerable to the disease. These genetic predispositions may be activated by environmental triggers such as infections or other factors (Karlson and Deane, 2012). The inappropriate reactions by the body's immune system to these stimuli culminate in the production of substances that attack the joint instead of protecting it, resulting in the development of rheumatoid arthritis.

Oxidative stress, due to the accumulation of reactive oxygen and nitrogen species (ROS and RNS), plays a significant role in the development and progression of RA (Hitchon and El-Gabalawy, 2004). Free radicals generated from macrophages, lymphocytes, neutrophils, and endothelial cells contribute to the pathophysiology of synovial inflammation. The imbalance between pro-oxidants and anti-oxidants in RA has been suggested to be due to acceleration of certain cellular reactions or perturbed antioxidant defence mechanisms.

Medicinal plants have shown potential in the management of RA, providing an alternative approach to conventional treatments. Several plants such as *Zingiber officinale*, *Aloe barbadense*, and *Withania somnifera* Linn have demonstrated anti-arthritic properties (Mahajan et al., 2020). Among these, *Acrostichum aureum* Linn., a mangrove fern, has shown diverse pharmacological effects (Behera et al., 2021).

In this study, we investigated the prophylactic action of *Acrostichum aureum* hydro-alcoholic extract (AAHE) against Complete Freund's adjuvant (CFA)-induced rheumatoid arthritis in rats. The CFA model has been widely used in preclinical trials for the exploration of novel drugs for RA treatment (Guo et al., 2019). Our results demonstrated that treatment with AAHE significantly alleviated RA signs and symptoms by reducing inflammation, pain, and oxidative stress.

The study revealed that AAHE provided significant protection against weight loss in arthritic rats, possibly due to the anti-inflammatory properties of its phytochemicals. AAHE treatment also markedly reduced rat paw swelling, paw diameter, and arthritis scores, suggesting immunological protection and prevention of joint destruction.

Haematological and biochemical changes, important indicators of arthritic disease conditions, were also observed. These included increases in platelet count, white blood cell count, and erythrocyte sedimentation rate, along with decreases in packed cell volume, haemoglobin, and red blood cell count (Möller et al., 2018).

Our study also found increased lipid peroxidation and decreased antioxidant enzyme activities (SOD and CAT) in CFA-induced arthritic rats, indicating heightened oxidative stress. AAHE treatment showed protective effects against these changes, likely due to its antioxidant properties (Quiñonez-Flores et al., 2016).

Histological examination of ankle joints and X-ray analysis further confirmed the protective effects of AAHE against arthritic conditions. The presence of various phytochemicals in AAHE, including flavonoids, phenols, alkaloids, steroids, and terpenoids, might have contributed to its potent antioxidant and anti-arthritic potential.

In conclusion, this study demonstrates that the hydro-alcoholic extract of *Acrostichum aureum* at doses of 200 mg/kg and 400 mg/kg has a significant therapeutic effect on CFA-induced arthritic rats, justifying its traditional use. Further studies are required to investigate the quantitative presence of different phytoconstituents responsible for maintaining the therapeutic efficacy of these formulations.

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