ISSN: 2178-7727

ANALYZING THE ROLE OF ETANERCEPT IN FLS CELL LINE IN TREATMENT OF RHEUMATOID ARTHRITIS

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent joint inflammation, leading to pain, swelling, and progressive joint damage. Current treatment options, including nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and biologics, often present side effects and high costs, necessitating the exploration of alternative therapies. Drynaria quercifolia, a medicinal fern known for its anti-inflammatory and antioxidant properties, has shown potential in alleviating symptoms associated with RA. This study investigates the bioactive compounds of Drynaria quercifolia and their therapeutic efficacy in modulating inflammatory pathways involved in RA. Phytochemical analysis and in vitro assays were conducted to assess its anti-inflammatory activity, followed by in vivo studies to evaluate its effectiveness in an RA-induced FLS (Fibroblast-Like Synoviocytes) cell line. Preliminary findings suggest that extracts of Drynaria quercifolia exhibit significant inhibition of pro-inflammatory cytokines and oxidative stress markers, indicating its potential as a natural therapeutic agent for RA. Further research is warranted to elucidate its mechanism of action and validate its clinical applicability.

Keywords: Rheumatoid Arthritis, Joint Inflammation, Drynaria quercifolia, Natural Therapeutic Agent.

1. INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an autoimmune chronic disease that affects the joints, leading to chronic inflammation, pain, swelling, and eventually joint deformity. In contrast to osteoarthritis, which is the result of mechanical wear and tear, RA develops when the immune system of the body mistakenly attacks the synovial membrane the joint's protective lining causing inflammation and ongoing damage. This autoimmune process can spill over into other organs, including the heart, lungs, and eyes, making RA a systemic illness with far-reaching health consequences.

RA occurs in millions of individuals all over the globe, more often in women compared to men. Although RA's cause has yet to be pinpointed, it is the general consensus of experts that RA occurs due to the interplay between genetic factors, environmental stimuli, and dysfunction within the

immune system. Some of the more frequent risk factors are family history of RA, smoking, endocrine disturbances, and past infection with a range of causative agents triggering faulty immune response. The onset is usually seen in the range 30 to 50 years of age (Newman & Fitzpatrick, (1995)), although it may affect any life period.

Rheumatoid arthritis begins with the inflammation of the synovial membrane of the joints, especially in the small ones of the fingers and feet and is mostly bilateral. The inflammatory cells, if in inappropriate large amount, destroy body tissue. The synovial fluid accumulates and the joints swell in time and thicken into a pannus (abnormal tissue). Over time the pannus erodes the joint's cartilage and, possibly, scar tissue will be formed, connecting bone ends. Later this scar tissue can ossify wherewith the joints get immobilized and deformed (Majithia & Geraci, (2007)).

The surrounding structures of the inflammatory joints, as tendon sheaths, bursas and origins of muscles are often involved supporting joint deformations as well. These strains are generally irreversible. In seldom cases the vertebral column, vasculatures and several organs as skin, heart and lungs are also inflamed by RA. The disease gets diagnosed by several methods: ultrasound and MRI (Magnetic Resonance Imagery) which detects inflammation marks in the joints and the surrounding structures; radiograph and MRI which detects meanderings in cartilage and bones. The progress of RA differs clearly from person to person.

It goes from an interminable mild devolution, over consecutive periods of active illness and phases free of complaints, to a very progressive illness, which rapidly grand joint damages and loss of functioning. Although the disease pattern is very individual, over time all people concerned have restricting joint movement and extreme pain in common. About 90% of the people with RA suffer from irreparable joint damages and loss of function. However, only pain can be decreased and the progress of joint damages and loss of function narrowed down. This is achieved by several treatments as drugs, physiotherapy, occupational therapy and adjuvants as prostheses.

RA affects not only the body but also impacts the mental condition. Therewith, people concerned fend mostly for themselves because physicians still rarely go into mental discomfort, such as great complaints of fatigue, loss of appetite and energy, stress and social isolation. Through these multifaceted non-physical symptoms related to rheumatoid arthritis, the disease impairs all areas of life and thus the quality of life: The destruction of joints leads to pain and immobilizing which further reduces several activities as walking, playing around with children and personal hygiene to a minimum. (fig.1)

Moreover, the related pain leads to frustration and feelings of losing control of the own situation. Also, fatigue occurs and people aggrieved draw back more and more which can consequently result in restrictions in social lives. These restrictions can assume broad proportions in people's life. It can impair the leisure activities with friends, occupation as the people concerned cannot further fulfill their common workload and the ability to comply social roles which also form restrictions for other family members (Bijilsma, et.al, (2004)).

Pain and fatigue are the two most common and most frequent reported symptoms in rheumatoid arthritis and are strongly associated with people's all over quality of life. Consecutively, these components get enlarged in separate paragraphs.

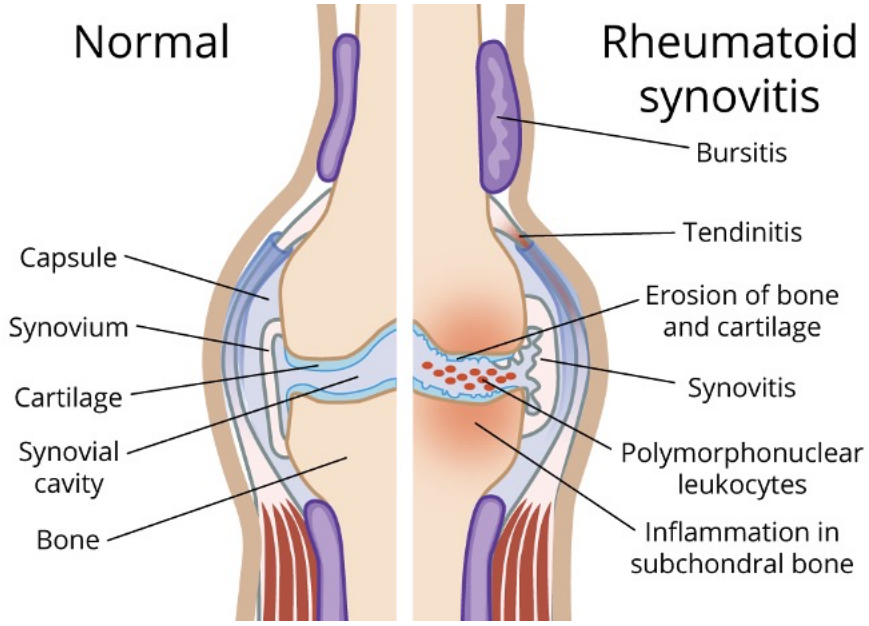

Fig.1: diagram shows knee joint; the left side shows normal structure and the right side shows the rheumatoid arthritis damaging effects.

1.2 PAIN IN RHEUMATOID ARTHRITIS

Pain is the first main factor in this review and is an ailment with serious impact on people's lives. In rheumatoid arthritis, the pain enters aggrieved people's lives as constant companion. In rest periods, the joints are swollen, sensitive to pressure and warm and mostly underlie the so-called morning stiffness. The latter complicates people's movement abilities, especially in the morning. Once the joint structures are partly damaged, the pain also appears during exercising and becomes chronic. This sensation constrains people's activities painfully and the affected joints get conserved by avoiding everyday activities as far as possible. But a relieving posture could in turn lead to disease-unrelated hardening of the joints or to other joints being overly strained, resulting in perceiving more pain.

Although pain severity was perceived individually in several studies, it is generally experienced greater by women than by men (cooper & Denninson, (1998); Affleck, (1999)). Through the permanent presence of pain, it can become the central focus of people with RA. Therefore, the available efforts get predominantly addressed to pain relief and pain coping rather than to family, friends, occupation, health and/or personal encouragement. Thus, the personal and social functioning gets impaired (McCracken & yang, (2006)). Patients end in a vicious circle wherein they invest in methods of decreasing pain for a better quality of life which simultaneously leads to accretive social isolation and hence, heightened attention on pain. However, supportive relationships could be especially beneficial for the experience of symptoms, whereby not all interactions with close social network members are helpful; non-supportive interactions, as a sort help which is not intended by the recipient could further stress people concerned (Revenson, (1991)). This would worsen health condition and end ultimately in a more miserable quality of life

and severe pain sensation. Psychosocial factors which also play a role in RA as depression and stress can hardly be separated from pain because of similar characteristics.i.e. worse sleep; covic, (Adamson & Hough, (2000)). Thus, comorbidity as well as a condition of augmented psychological discomfort according to RA can be on hand in people concerned. In case of comorbidity, if pain is the central focus in the life of people with RA other things can lose in value and could further lead to a depression (Skevington, (1986)). Also states of anxiety are often associated with RA as people concerned feel overstrained with the constrictions accompanying the disease, i.e. the extreme chronic pain the emergence of movement limitations and uncertainty over the further progress.

1.3 FATIGUE IN RHEUMATOID ARTHRITIS

Beside pain, chronic fatigue is the second main factor in this review. It is also a very life-impairing factor in rheumatoid arthritis (Silman, (2001)). In various investigations of RA, more than 80% of the samples told to experience fatigue to some degree (Belza, et.al, (1993)) and interferes with physical and mental processes. The form of fatigue which is the main topic in the current thesis refers exclusively to fatigue of rheumatoid arthritis (Belza, et.al, (1993)). Before amplifying it, an established other form of fatigue gets described to prevent that fatigue in RA from getting mixed up with it: the chronic fatigue syndrome (CFS). (fig.2)

CFS is a self-contained chronic illness, characterized by paralyzing exhaustion without any advance after resting, plus a specific combination of further symptoms as headache, sore throat and worse sleep, pain in joints and muscles and problems with concentrating as well as a sustained worsening of fatigue after struggle. But the physical symptoms cannot be explained by acute illnesses of the concerned structures. It is assumed that CFS is a heterogeneous disorder, possibly caused by already cured injuries or inflammations whereby the immune system left chronically active and broaden to the nervous system (Piper, (1989)).

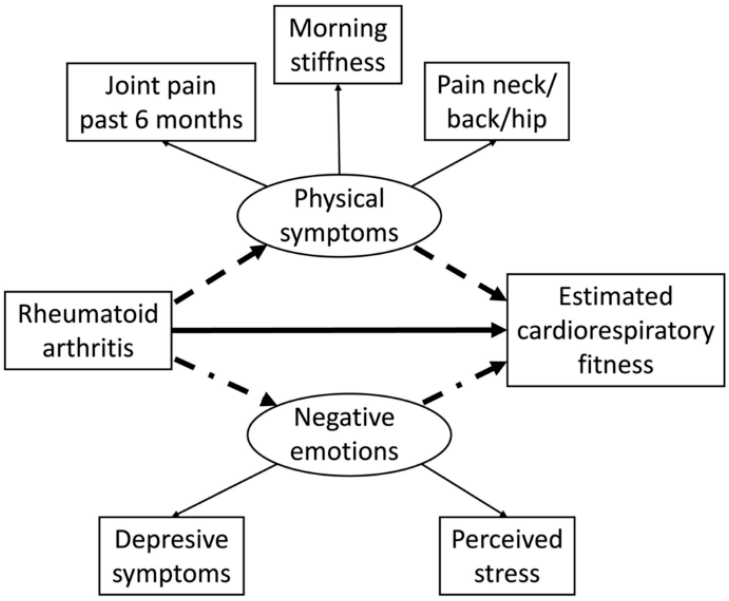

Fig.2: the association between rheumatoid arthritis and estimated cardiorespiratory fitness

Fatigue in RA is also described as a sustained form of exhaustion with no enhancement after sleeping but does not fall in the same category as CFS. Particularly different, are the physical constraints conducting fatigue in RA, as worse sleep and pain in joints and muscles can be related to the inflammations caused by the underlying disease.

Several qualitative studies (Hewlett, et.al, (2005); Repping-Wuts, et.al, (2007)) have asked people with RA to evaluate multiple causes for fatigue in RA. Partially, it is believed that fatigue results from the inflammatory cells in the body and thus from the excessive activity of the immune system. Others credit fatigue with the drugs they have to take or with disturbed and non-restorative sleep. Another factor which people concerned ascribe to fatigue is the increasing energy effort expended to perform normal despite injured joints (Butcher, (2007)).

While there is only little known about the general etiology of fatigue and a unified international definition also lacks, research on predicting factors for fatigue in RA found that physical as well as psychosocial factors are disposed for the degree of fatigue (Englebienner & Meirleir, (2002)). These factors are for instance pain intensity (Huyser, et.al, (2007))., degree of physical disability, worse sleep quality, general health, the presence of depressive and anxiety symptoms the degree of self-efficacy and social support (Morrison & Bennet, (2006)). In addition to the latter, not only the number of social relations is negatively related to RA, also the character of them has an effect on the illness progression. (fig.3)

Thus, good social support is particularly important for coping with symptoms as fatigue, pain and emotions in rheumatoid arthritis, whereby accretive social isolation and problematic social support worsen the experience of disease symptoms. In this sconnection it has to elaborate insistently that problematic social support, i.e. the provider's action (unasked advice) is well intended but not desired by the recipient (Mancuso, et al. (2006)) and is not comparable to social stress, in which for instance someone who provides a great deal of social support (i.e. the partner) may also be a source of significant social stress (i.e. a serious illness). But social stress is also not implicitly inversely related to social support. It rather forms an additional psychosocial construct.

1.4 MOTIVATION FOR THIS STUDY

While the results of the studies according to the field of rheumatoid arthritis are discordant, several topics of the disease are nevertheless of great prominence to fully understand the temporal pattern of pain and exhaustion in rheumatoid arthritis varies from person to person, thus it is essential to consider because information about the variability and degrees of pain and fatigue throughout the disease may be valuable for a more exact definition and understanding of it. Hence, subgroups of people with RA could be formed where upon treatment needs could be matched more individually with decreasing the immense life impact through the symptoms. Furthermore, understanding the interrelation between RA symptoms and psychological mood problems (depression and anxiety) would be crucial because they seem to be the most common related mood problems in RA. (fig.4)

Therefore, information about the strength of relation and causal directions could be utilized for shifting the treatment focus on substantial pathways. This could prevent people with RA experiencing unneeded worse conditions of the disease by itself and comorbidity. Appreciating the

affective modality and effective strength of several psychosocial factors on pain and fatigue as well as their mediating effect on the relation between pain and fatigue and problematic moods would also compose a precious contribution in treating RA. Several confounders or covariates as stress, self-efficacy and social support are gently manageable through improving person's self-management. As combined with pharmaceuticals for the biological markers of RA, disease conditions and the relation between them and psychological problems could be treated more effectively than only medical treatments of the main symptoms. Moreover, this combined therapy could evoke fewer side effects because of lower doses of drugs. A fully understanding of the disease could also provide room for research on developing new therapy models exactly catered to rheumatoid arthritis.

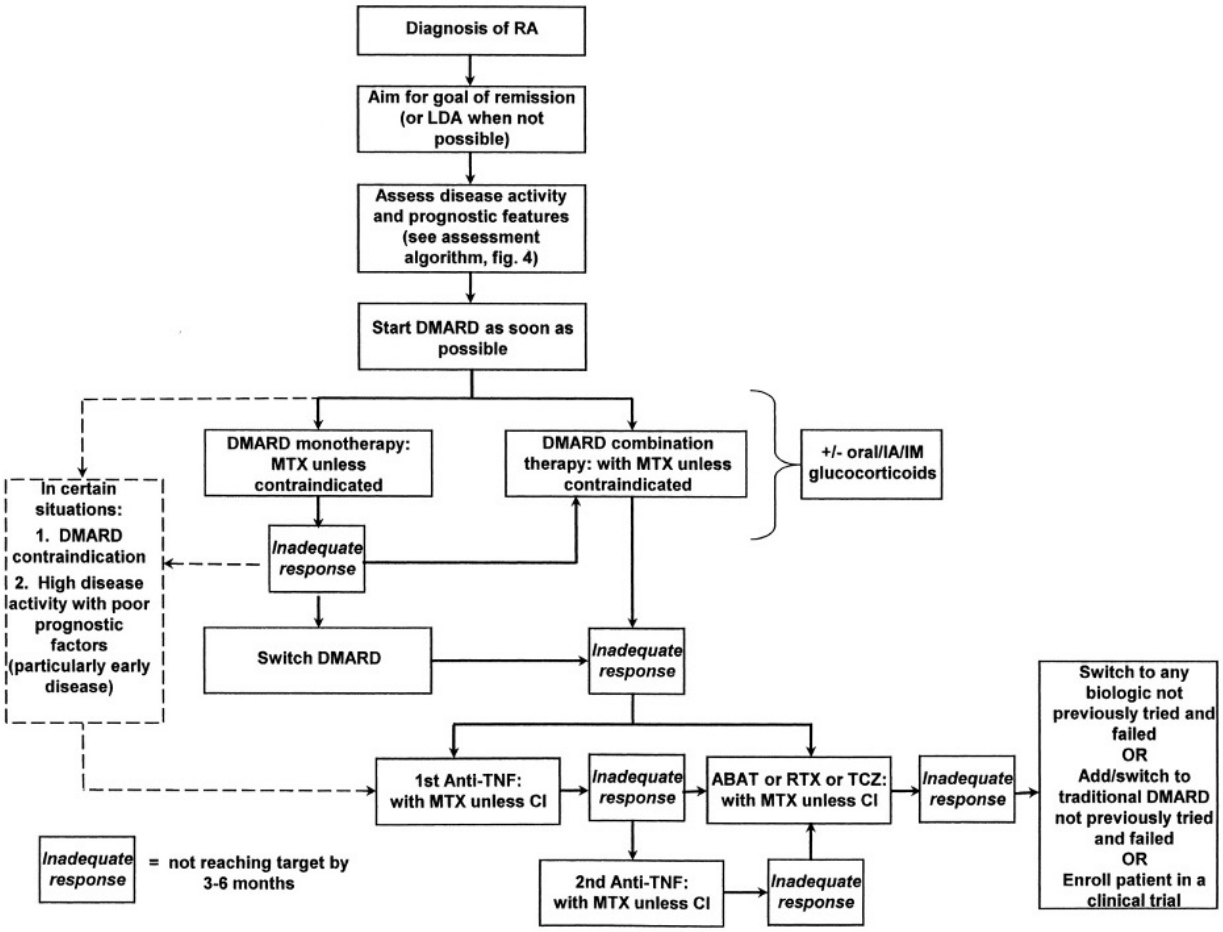

Fig.3: Flowchart showing the DMARD treatment.

The defining feature of RA is progressive cartilage and bone destruction within inflamed joints. Synovial inflammation results in overproduction of inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). These mediators perpetuate a vicious cycle of chronic inflammation, causing joint damage, stiffness, and loss of mobility. Left

untreated, RA may cause permanent joint deformities, excruciating pain, and disability, and it can substantially impact an individual's quality of life.

The current treatments for RA target the reduction of inflammation, the relief of symptoms, and the retardation of disease progression. Traditional drugs involve nonsteroidal anti-inflammatory agents (NSAIDs), corticosteroids, disease-modifying antirheumatic drugs (DMARDs), and biologic drugs that target specific inflammatory pathways. Although these drugs have enhanced disease control, they are accompanied by side effects such as gastrointestinal distress, liver damage, and enhanced susceptibility to infection. Moreover, they are not curative in nature and require the use of alternative treatments with less side effects.

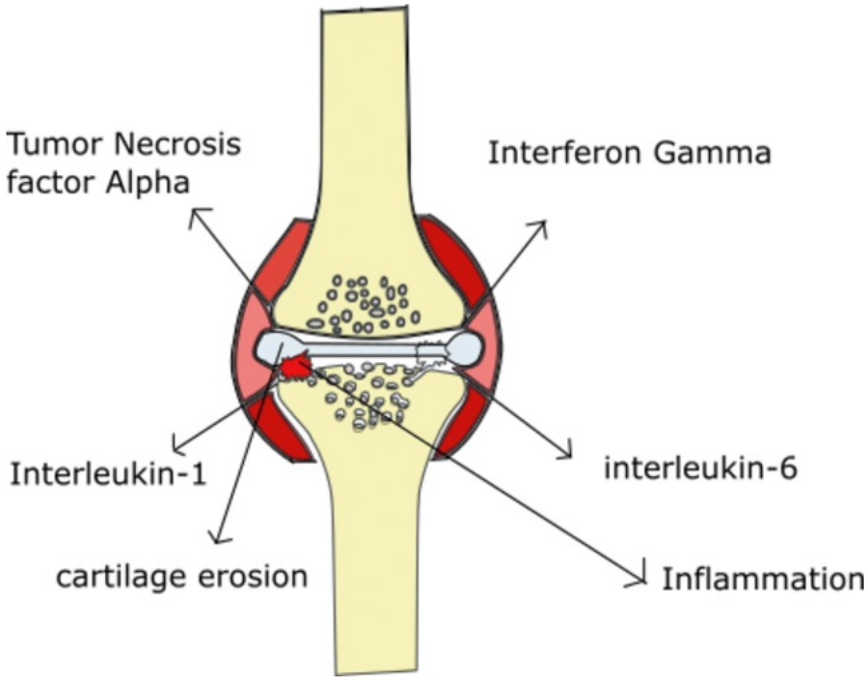

Fig 4: Inflammatory cytokines involved in rheumatoid arthritis

1.5 DRYNARIA QUERCIFOLIA

There has been increasing interest in natural and plant-based therapy for inflammatory diseases such as RA in the last few years. Medicinal plants have been utilized in traditional medicine for many centuries with a potential reservoir of bioactive molecules possessing immunomodulatory and anti-inflammatory activity. Among them, Drynaria quercifolia, a tropical fern species, has drawn interest due to the potential therapeutic use of the plant in RA treatment.

Drynaria quercifolia is traditionally applied in herbal medicine as an analgesic and anti-inflammatory agent. Drynaria quercifolia is bioactive rich in flavonoids, alkaloids, tannins, and saponins, with the latter bioactive compounds proving to have pharmacological activity advantageous for inflammatory disease. Studies propose that Drynaria quercifolia extracts can potentially modulate the immune response as well as reduce the production of inflammatory mediators responsible for promoting RA development. (fig.5)

The possible advantages of Drynaria quercifolia treatment in RA have their basis on the plant's capacity to affect major inflammatory signalling pathways. From research, the plant contains chemicals that can mitigate the activity of TNF-α, IL-1, and IL-6, downregulating the inflammation and shield the joint structures from additional damages. The antioxidants present in the plant could help reverse oxidative stress, which participates in RA disease pathogenesis as a contributor of tissue damage as well as perpetuation of inflammation.

Ensuring efficacy, safety, and standardization has been one of the greatest difficulties faced in coming up with plant therapies. Because of the variables influenced by environment involved in extracting components from plants, standardized methodologies must be introduced to extract as well as form extracts. Impenetrable scientific research like in vitro study, animal tests, and human studies must validate whether Drynaria quercifolia will have medicinal effects for the therapy of RA or not. If effective, this plant may provide a natural alternative to current RA therapy, decreasing dependency on man-made drugs and lessening side effects.

Fig.5 : Drynaria Quercifolia (Mudavattukaal Kizhangu)

1.6 RESEARCH ON DRYNARIA QUERCIFOLIA

Research on Drynaria quercifolia in RA treatment is a promising crossroads between conventional medicine and contemporary pharmacology. By tapping the medicinal potential of this plant, scientists aim to create new therapeutic interventions that are effective and environmentally friendly. The incorporation of plant treatments into mainstream medicine may offer patients additional alternatives for treating RA, especially those who suffer from side effects of conventional medications or prefer alternative methods of healthcare (Scott DL, (2007)).

As research on Drynaria quercifolia progresses, it is important to investigate its mechanism of action, ideal dosing, and potential interactions with current RA drugs. Biotechnology and pharmacognosy advancements can be used to unravel the complete therapeutic potential of the plant, which can lead to novel treatments for better patient outcomes. If effective, Drynaria

quercifolia may revolutionize RA treatment by providing a comprehensive, plant-based remedy that fights inflammation while addressing overall health (Praveen K & Micheal C, (2016)).

The interest in natural treatments for autoimmune disorders represents a larger movement toward the syncretism of traditional and contemporary medicine. As more scientific research becomes available, Drynaria quercifolia has the potential to become a major force in rheumatology, offering a complementary or alternative therapy to traditional RA therapies. Through ongoing research and clinical confirmation, the plant has the potential to revolutionize RA care and provide new hope to millions of patients globally (Baylac S and Racine P, (2004)).

2. MATERIALS AND METHODS

2.1 CHEMICALS

Drynaria Quercifolia, 99.9% Absolute Ethanol, Etanercept, FLS – cell line, TNF – beta, FBS, DMEM medium, Penicillin streptomycin, MTT reagent and DMSO.

2.2 EXTRACT PREPARATION

The plant sample Mudavattukaal Kilangu (Drynaria quercifolia), commonly referred to oak fern, was collected and shade-dried to dehydrate under the sun. Using a mechanical grinder, the dried material was coarsely powdered. 25 grams of the sample powder was macerated in 175 mL of 99.9% ethanol at room temperature for 72 hours. After the extraction period, the mixture was filtered with Whatman No. 1 filter paper. The resulting filtrate was collected and stored for experimental analysis.

2.3 PHYTOCHEMICAL ANALYSIS

Appropriate bioactive constituents found in the ethanolic extract of Drynaria quercifolia (Mudavattukaal Kilangu) was identified through preliminary phytochemical analysis. Standard qualitative methods were employed to test for major classes of phytochemicals. These include: alkaloids, flavonoids, phenols, tannins, saponins, glycosides, terpenoids, and steroids. Each of the tests was done based on established chemical processes that exhibit the presence of certain compounds by colour changes or formation of precipitates. Various reagents confirmed the assumption of the respective alkaloid and theorems corresponding to capillary action theory detection were performed on lead acetate. Phenols and tannins were confirmed with the use of ferric chloride while saponins were assessed with the foam test. This tested the correctness of assumptions as well as the composition of chemical mixtures which in result, determines the efficiency of the plant extract's attributed therapeutic benefits.

2.4 GCMS ANALYSIS OF MUDAVATTUKAAL KILANGU

The bioactive constituents available in the sample collected were analysed using Gas Chromatography-Mass Spectrometry (GC-MS) after the ethanolic extract of Drynaria quercifolia (Mudavattukaal Kilangu) was prepared. The GC-MS was set up to standard settings with helium

as the carrier gas. Then, a filtered extract was further processed to yield a 0.5 µL aliquot which was to be injected into the system. The processes the samples underwent included: separation by volatilization and retention time in the GC column, followed by identification through mass spectral fragmentation pattern comparison to the NIST library database. The GC-MS analysis was able to identify a variety of phytochemicals such as: L-Valine N-ethoxycarbonyl, dodecanoic acid (lauric acid), n-Hexadecanoic acid (palmitic acid), Linoelaidic acid, 5,7-dimethoxycoumarin, and myristic acid. These phytochemicals are vital due to their possible pharmacological importance. These purposes include: skin and tissue inflammation, infection remediation, and cellular wear and tear mitigation. The outcomes obtained per GC-MS runs affirm the medicinal capabilities posed by Drynaria quercifolia, while also justifying its longstanding use in traditional medicine.

2.5 ANTIOXIDANT ASSAY OF MUDAVATTUKAAL KILANGU

The assessed Mudavattukaal Kilangu (Drynaria quercifolia) ethanolic extract antioxidant activity using the DPPH radical scavenging test, also known as DPPH assay. It is based on the ability of the extract's antioxidant components to convert the DPPH radical with its deep purple color to a lighter yellow color. Different concentrations of an extract were prepared and mixed with freshly prepared DPPH solution in methanol. The reaction was allowed to proceed for 30 minutes at room temperature in the dark. Following incubation, the solution's absorbance was read on a UV–Vi's spectrophotometer at 517 nm. The blank DPPH reaction without sample extract was used as a control consisting of methanol and DPPH. The percentage of activity was measured to evaluate antioxidant potential of the extract. This method is effective in assessing plant extracts for their ability to scavenge free radicals because of its speed and sensitivity the results achieved.

2.6 ANTI INFLAMMATORY ACTIVITY OF CYTOKINE SIGNALLING MOLECULE

The in silico molecular docking study involved looking at the binding affinities and interactions of some chosen phytocompounds from Drynaria quercifolia and the major proteins in the anti-inflammatory pathway signalling for cytokines using docking simulations. Specific 3D protein structures pertinent to cytokine interaction were downloaded from the Protein Data Bank and AlphaFold. Phytocompounds obtained from GC-MS analysis were fetched from PubChem and their chemical structures were processed into appropriate formats using Open Babel. Using AutoDock Tools, the protein structures were processed to remove water molecules, add polar hydrogens, and optimize geometry. Docking was done in PyRx software where ligands and proteins were first converted to PDBQT format, and binding poses and estimation of interactions were calculated with AutoDock Vina anchored to the signalling proteins. The Discovery Studio program was used for analysing and viewing the interaction of ligands with the proteins, assisting in defining the binding sites and the molecular associations responsible for anti-inflammatory action.

2.7 CYTOXICITY ASSAY

The MTT assay was performed on RA-FLS (Rheumatoid Arthritis – Fibroblast-Like Synoviocyte) cell lines to determine the cytotoxicity and cell viability of Drynaria quercifolia ethanolic extract (Mudavattukaal Kilangu). The cells were grown in DMEM medium containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO2. Once the cells reached the appropriate confluency, they were split into 96-well plates. The cells were treated with various proportions of the plant extract from 5µg/ml to 500µg/ml for 24 and 48 hours. There was a control group that had TNF-α administered to them, to induce an inflammatory response. At the end of each incubation period, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to every well and incubated for 4 hours. The viable cells were shown to reduce yellow MTT salt and form insoluble purple formazan crystals. These crystals were then dissolved by DMSO to receive a harmless solution which had a consistent colourful appearance. The absorbance could then be assessed at 540 nm using a microplate reader. The percentage of viable cells was determined in comparison to the untreated cell count of the control group.

2.8 INSILICO MOLECULAR DOCKING

Stopping the inflammation processes may be accomplished by molecular docking of selected phytocompounds from Drynaria quercifolia. We fetched the 3D structure of the target proteins-Resistin (PDB ID: Q9HD89) was collected from AlphaFold while Galectin-3 (PDB ID: 1A3K) was downloaded from the Protein Data Bank's website. We then gathered the five bioactive compounds containing: tetradecanoic acid, dodecanoic acid, linolelaidic acid, hexadecanoic acid methyl ester, and 5,7-dimethoxy-2H-1-benzopyran-2-one from the PubChem database in SDF format. Open Babel was employed to convert the ligands to PDB format in order to comply with the docking software. The docking process was executed with the help of PyRx software where AutoDock Vina was utilized as the docking engine. Before docking, the water molecules in the proteins had to be removed together with the addition of Hydrogen bonds. Finally, the proteins and ligands had to be placed in PDBQT format. Interaction analysis and post docking interaction-visualization utilizing Discovery studio was done hence key binding sites and interaction patterns of the ligands and aim proteins could be determined

3. RESULTS AND DISCUSSION

3.1 Phytochemical Analysis of Mudavattukaal Kilangu

The qualitative phytochemical screening of the liquid extract of Mudavattukaal Kilangu was carried out using standard chemical tests. The analysis revealed the presence or absence of key phytoconstituents, as summarized below:

Table 1: Phytochemical analysis of Mudavattukaal kilangu

S. No.	Phytochemical	Test Used	Result

1	Alkaloids	Mayer's Test	Present
2	Carbohydrates	Fehling's Test	Present
3	Phytosterols	Liebermann–Burchard Test	Absent
4	Glycosides	Legal's Test	Present
5	Tannins	Lead Acetate Test	Present
6	Saponins	Foam Test	Present
7	Reducing Sugars	Fehling's Test	Present
8	Flavonoids	$ZnCl_2$ Reduction Test	Present
9	Proteins	Ninhydrin Test	Present

These findings confirm that the extract is rich in multiple classes of secondary metabolites that are often associated with medicinal properties such as antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities. The only phytochemical group found to be absent was phytosterols.

3.2 GC-MS Analysis of Mudavattukaal Kilangu

The GC-MS analysis of Mudavattukaal Kilangu extract identified several bioactive chemical constituents based on retention time (RT), peak area, and molecular structure. The major compounds identified are summarized below:

Table 2: GC-MS analysis of Mudavattu kaal kilangu

RT (min)	Compound Name	Molecular Formula	Peak Area %	Reported Bioactivity
7.38	L-Valine, N-ethoxycarbonyl	C8H15NO4	10.94%	Antioxidant, amino acid derivative
9.23	Dodecanoic acid (Lauric acid)	C12H24O2	2.25%	Antimicrobial, antifungal

9.31	11H-Dibenzo[c,f][1,2] diazepin-11-one, 3,8-dichloro	C13H8Cl2N2O	3.37%	Antidepressant analog, CNS active agent
9.48	Bis(2-ethylhexyl) phthalate	C24H38O4	14.67%	Plasticizer, potential endocrine disruptor
10.02	Tetradecanoic acid (Myristic acid)	C14H28O2	1.87%	Emollient, antibacterial
10.67	Hexadecanoic acid, methyl ester (Methyl palmitate)	C17H34O2	1.46%	Antioxidant, antimicrobial
10.87	n-Hexadecanoic acid (Palmitic acid)	C16H32O2	23.60%	Anti-inflammatory, antioxidant
11.28	5,7-Dimethoxy-2H-1-benzopyran-2-one (Limettin)	C11H10O4	7.56%	Antioxidant, anticancer
11.85	Linoelaidic acid (trans-Linoleic acid isomer)	C18H32O2	29.97%	Hypocholesterolemic, anti-inflammatory
11.99	Eicosanoic acid (Arachidic acid)	C20H40O2	4.30%	Emollient, fatty acid

The GC-MS analysis reveals a chemically diverse profile rich in fatty acids, esters, phenolic compounds, and bioactive aromatics with total peak area of 100%, aligning with traditional medicinal uses of Mudavattukaal Kilangu in treating inflammatory and metabolic conditions. The most abundant compound identified was Linoelaidic acid (29.97%), a polyunsaturated fatty acid known for its cardiovascular benefits and anti-inflammatory properties. Similarly, n-Hexadecanoic acid (23.60%), widely reported for anti-inflammatory and antimicrobial activity, supports the extract's therapeutic relevance. The presence of Limettin (7.56%), a methoxylated coumarin, contributes to antioxidant and potential anti-cancer properties. Its detection confirms the plant's suitability for oxidative stress-related disorders (Fig 6). L-Valine derivative presence (10.94%) suggests nutritional or metabolic significance, potentially supporting muscle recovery and immune modulation. Although Bis(2-ethylhexyl) phthalate is often considered an environmental contaminant, its presence may reflect environmental uptake; however, its consistent detection

necessitates purification and formulation care. The diverse chemical makeup highlights the medicinal importance of Mudavattukaal Kilangu, especially for formulations aimed at:

- Anti-inflammatory therapies
- Antioxidant and metabolic regulation
- Antimicrobial or skin protective uses

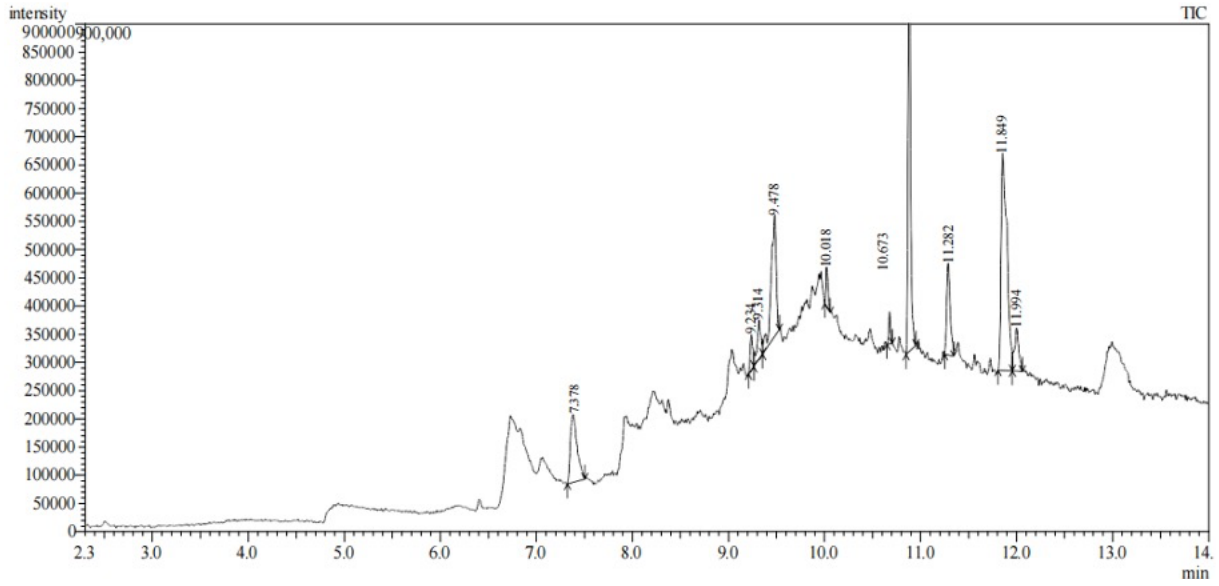

Fig 6: GC-MS result

3.3 Antioxidant Activity

The antioxidant potential of Mudavattukaal Kilangu extract was investigated using three in vitro assays: DPPH radical scavenging, Nitric Oxide (NO) scavenging, and Hydrogen Peroxide (H_2O_2) scavenging. All three assays demonstrated a dose-dependent increase in free radical scavenging activity, indicating the presence of bioactive compounds capable of neutralizing reactive species (Hovenkamp, (2017)).

3.3.1 DPPH Radical Scavenging Activity of Drynaria quercifolia

The extract exhibited strong antioxidant activity in the DPPH assay. At the lowest tested concentration (25 µg/mL), the radical scavenging activity was 20%, which steadily increased to 88% at the highest concentration (500 µg/mL). This indicates the extract's efficient hydrogen-donating ability, reducing the stable DPPH radical to a non-radical form. The color change from deep violet to yellow confirmed the reduction process, supporting the extract’s potential as a free radical quencher. (Fig.7)

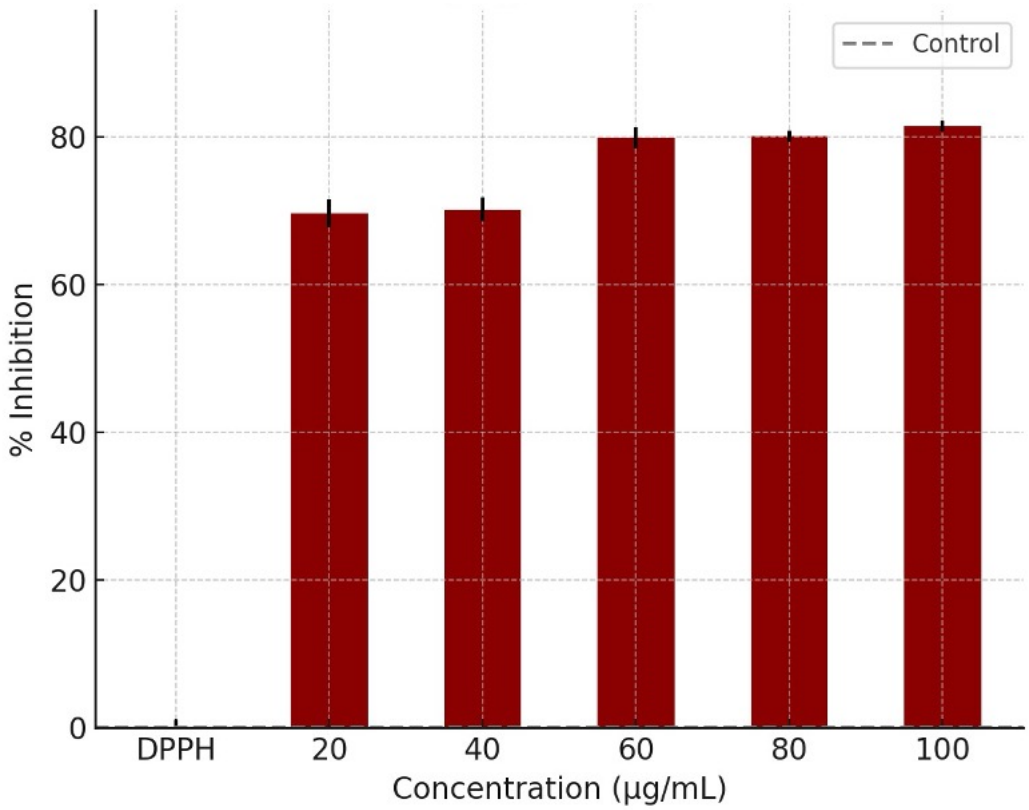

Fig. 7: DPPH Radical Scavenging activity.

3.3.2 Nitric Oxide (NO) Scavenging Activity

In the NO scavenging assay, the extract effectively inhibited nitric oxide formation in a concentration-dependent manner. The scavenging activity ranged from 18% at 25 µg/mL to 83% at 500 µg/mL. Since nitric oxide contributes to oxidative stress and inflammation, its inhibition reflects the extract's capacity to reduce nitrosative damage. This suggests that the extract may be beneficial in managing inflammation-related oxidative conditions(Fig.8).

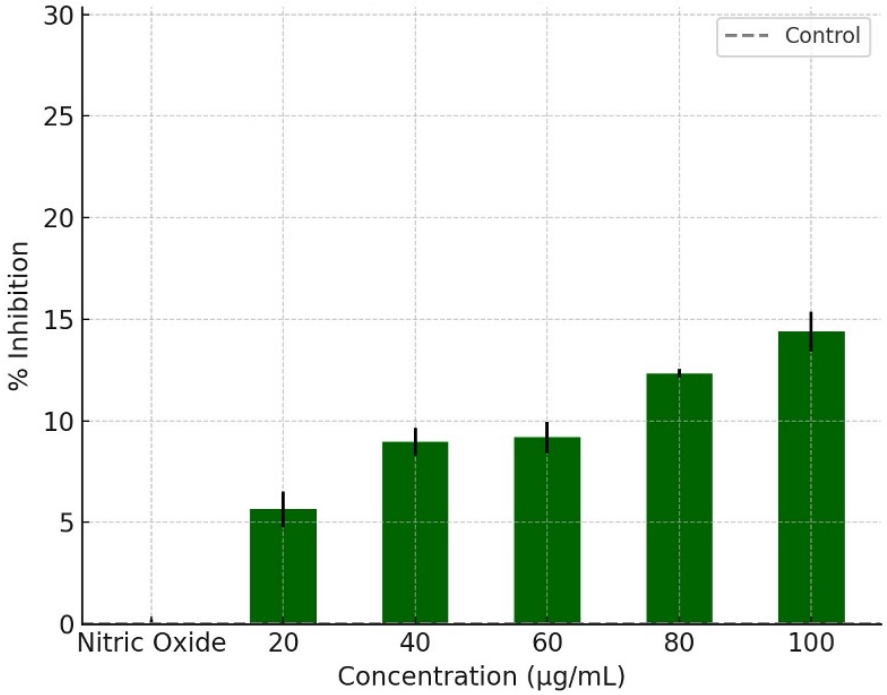

Fig.8: Nitric oxide Scavenging activity.

3.3.3 Hydrogen Peroxide (H_2O_2) Scavenging Activity

The extract also demonstrated significant hydrogen peroxide scavenging ability, showing 15% inhibition at 25 µg/mL and increasing up to 81% at 500 µg/mL. H_2O_2, although less reactive, can penetrate cells and produce more harmful hydroxyl radicals. The ability of the extract to reduce H_2O_2 into water suggests it can play a protective role against oxidatisve cellular damage(Fig.9).

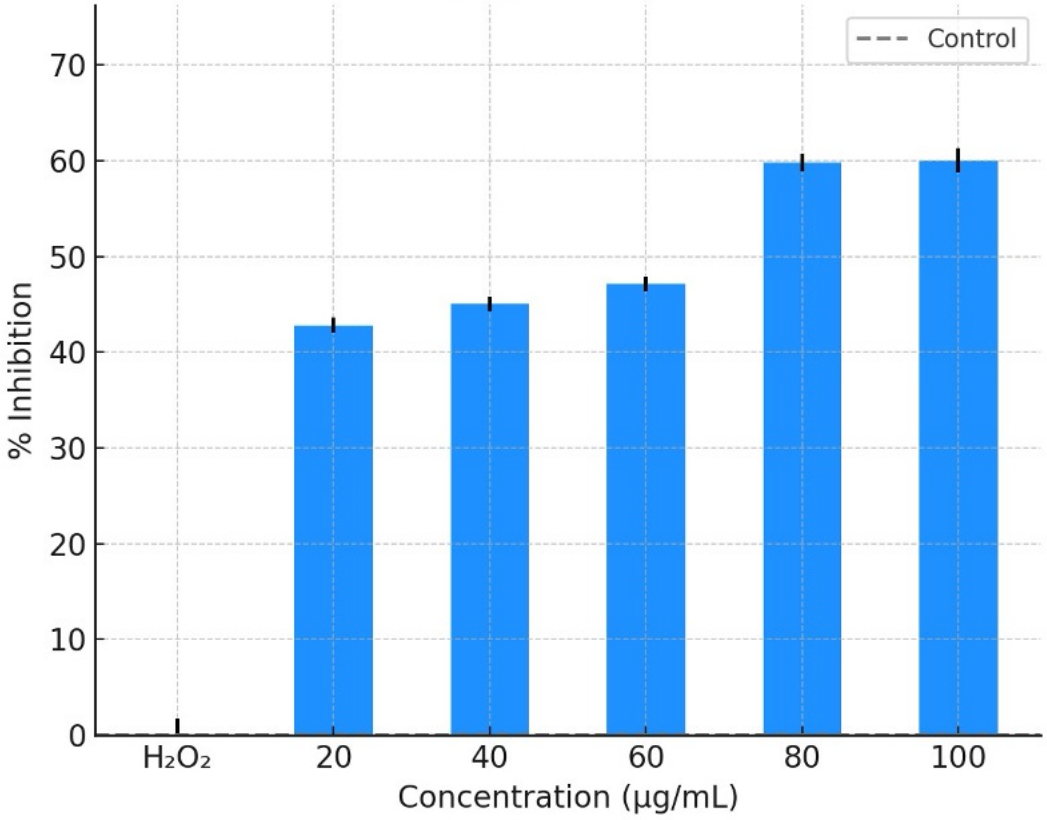

Fig.9: Hydrogen peroxide scavenging activity.

3.4 Anti-inflammatory activity of Drynaria quercifolia

The anti-inflammatory potential of the extract was evaluated through its ability to inhibit key pro-inflammatory cytokines, namely IL-1β, IL-6, TNF-α, and NF-κB. The results demonstrated significant inhibitory effects, particularly on TNF-α, with inhibition reaching 90% at 500 µg/mL, indicating a potent suppression of this central inflammatory mediator. Both IL-6 and IL-1β exhibited similar inhibition profiles, with IL-6 showing 88% inhibition and IL-1β 85% at the same concentration. This suggests that the extract has a broad impact on inflammatory cytokine regulation (Taamalli a, (2018)).

NF-κB, a key transcription factor in the regulation of inflammatory gene expression, was also significantly inhibited by 82% at 500 µg/mL. This finding suggests that the extract may exert its anti-inflammatory effects by blocking upstream signaling pathways that lead to the activation of NF-κB, which in turn regulates the expression of pro-inflammatory cytokines like ILs and TNF-α.

Overall, the extract demonstrated a strong anti-inflammatory effect, likely mediated through the inhibition of key signaling molecules and transcription factors involved in the inflammatory cascade. These results support the potential therapeutic value of the extract in managing inflammation-related conditions (Soliman, ss, (2019)). (Fig.10)

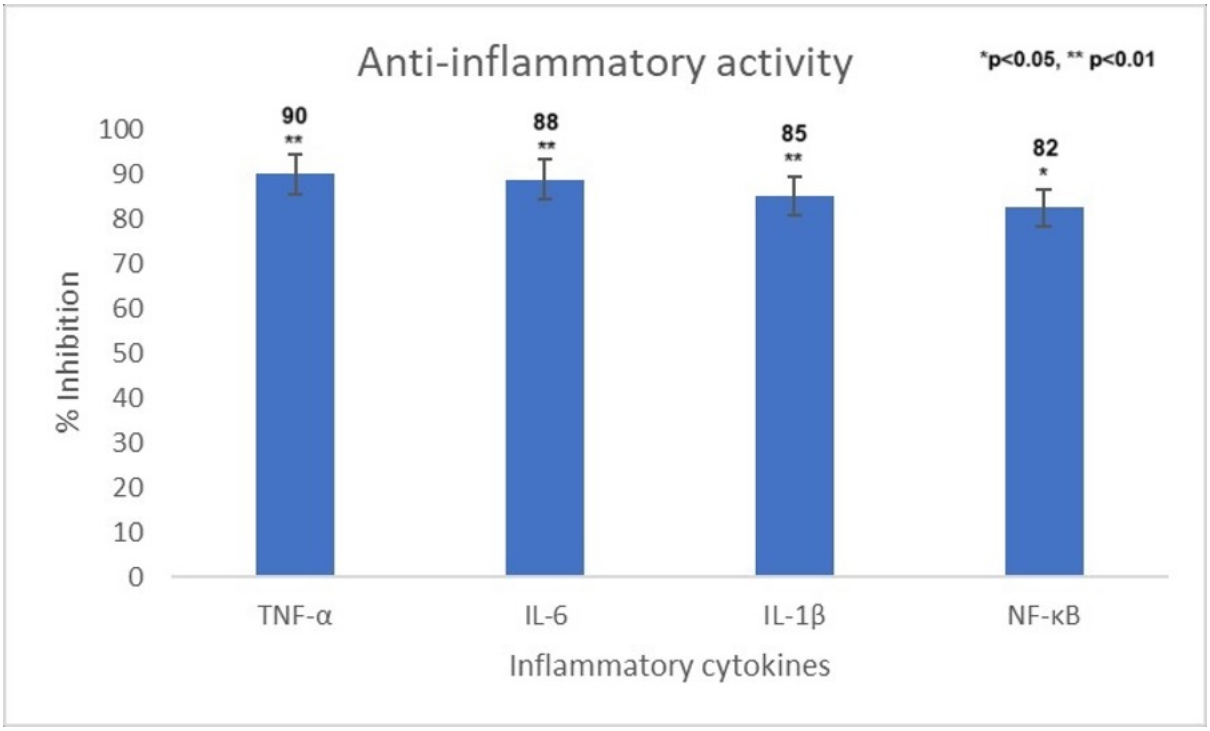

Fig.10: Anti-inflammatory activity

3.5 Cytotoxicity Analysis using MTT method:

The effects of TNF-β and Oak Fern extract were evaluated across a range of concentrations (0, 25, 50, 100, 200, and 500 µg/mL) at 24 and 48 hours, with the results presented as response percentages relative to the control, along with corresponding p-values indicating statistical significance.

Effect at 24 Hours

At the 24-hour mark (Figure 1), both TNF-β and Oak Fern exhibited a dose-dependent decrease in response percentage. At 0 µg/mL (control), both treatments showed near-complete response (~100%), with no statistically significant difference ($p = 1.000$). As the concentration increased, Oak Fern consistently demonstrated a more pronounced inhibitory effect than TNF-β.

For instance, at 25 µg/mL, TNF-β retained 96% activity ($p = 0.016$), whereas Oak Fern dropped significantly to ~71% ($p = 0.000$). This trend continued at 50 µg/mL and 100 µg/mL, where Oak Fern responses dropped to ~63% and 39%, respectively, compared to ~90% and 74% for TNF-β, all with p-values indicating strong statistical significance ($p < 0.01$). At the highest concentration tested (500 µg/mL), Oak Fern showed an almost complete inhibition (7%, $p = 0.000$), while TNF-β still retained ~18% response. (Fig.11)

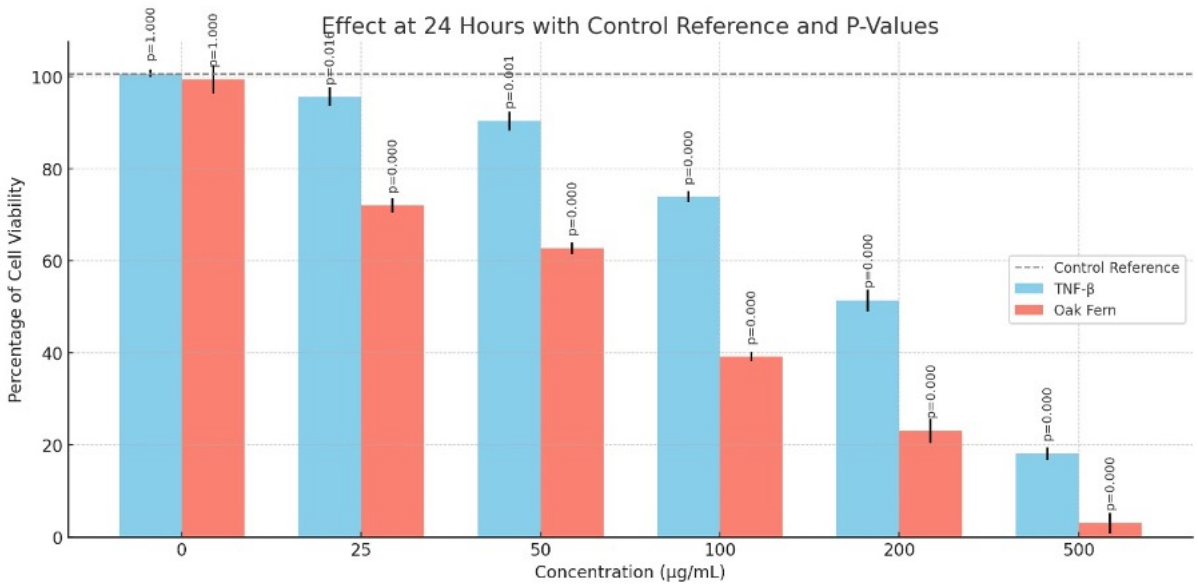

Fig.11: Effect of TNF – β and OAK fern at 24 hrs

Effect at 48 Hours

At 48 hours (Figure 2), the inhibitory effects were more pronounced across all concentrations for both agents, suggesting a time-dependent response augmentation. While the control (0 µg/mL) maintained high response levels (97% for TNF-β, 101% for Oak Fern, p = 1.000), response rates began declining sharply from 25 µg/mL onward.

At 50 µg/mL, TNF-β reduced the response to ~85% (p = 0.0014), while Oak Fern dropped to ~32% (p = 0.000). At 100 µg/mL, the difference widened further, with TNF-β showing 63% and Oak Fern only 29%, again with high statistical significance (p = 0.000). At the highest concentration of 500 µg/mL, both treatments resulted in minimal responses (~6% and 7%, respectively), indicating near-complete inhibition after 48 hours.

Across both time points, Oak Fern extract consistently showed a stronger inhibitory effect compared to TNF-β, suggesting it may possess more potent bioactive compounds or mechanisms of action. The time-dependent increase in inhibition implies that the effect of both agents is cumulative or requires sustained exposure for maximal efficacy.

The significance values ($p < 0.05$ or lower) across most concentrations further confirm the statistical reliability of these observations. Especially notable is the consistent p = 0.000 value at higher concentrations, indicating highly significant differences from the control(Fig.12).

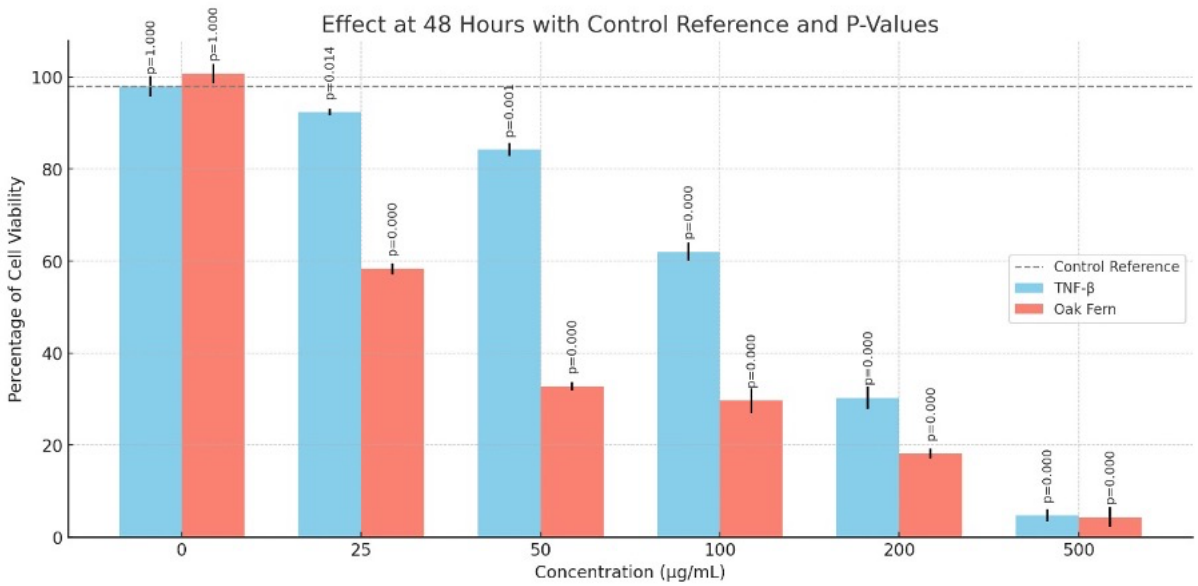

Fig 12: Effect of TNF – β and OAK fern at 48 hrs

3.6 Insilico Molecular Docking Analysis of Drynaria quercifolia

Molecular docking studies were performed to predict the binding affinity of bioactive compounds identified from Mudavattukaal Kilangu with two inflammation-related protein targets: Resistin (Q9HD89) and Galectin-3 (1A3K). The docking scores were expressed as binding energies in kcal/mol, where more negative values indicate stronger interactions. Hydrogen bonding interactions were also assessed.

Table 3: Insilico Molecular docking analysis of Resistin and Galectin-3

S. No.	Compound Name	PubChem ID	Binding Energy (kcal/mol)	Hydrogen Bonds
			Resistin	Galectin-3
1	Linoelaidic acid	5282457	-4.4	-3.9
2	Dodecanoic acid	3893	-4.0	-3.9
3	Tetradecanoic acid	11005	-3.8	-3.5

4	2H-1-Benzopyran-2-one, 5,7-dimethoxy-	2775	-4.7	-5.1
5	Hexadecanoic acid, methyl ester	8181	-3.9	-4.1

The docking results indicate that the bioactive compounds from Mudavattukaal Kilangu exhibit moderate binding affinity toward both Resistin and Galectin-3, two key proteins implicated in inflammation and metabolic disorders. Linoelaidic acid showed notable interactions with both targets, particularly Galectin-3, with a binding energy of -3.9 kcal/mol and the formation of three hydrogen bonds, suggesting its role in stabilizing protein-ligand interactions. 2H-1-Benzopyran-2-one, 5,7-dimethoxy- demonstrated the highest binding affinity, especially with Galectin-3 (-5.1 kcal/mol), highlighting its potential as a lead anti-inflammatory compound due to strong non-covalent interactions. Fatty acids like dodecanoic acid, tetradecanoic acid, and hexadecanoic acid methyl ester exhibited moderate binding, likely through hydrophobic interactions, and may contribute synergistically to the therapeutic effects of the extract. The presence of hydrogen bonds, especially with Linoelaidic acid, further strengthens the argument for their stability and binding specificity with target proteins. While none of the compounds showed extremely high binding affinity (e.g., <-6 kcal/mol), the overall docking profile supports the ethnopharmacological claims of the plant's anti-inflammatory potential(Fig.13).

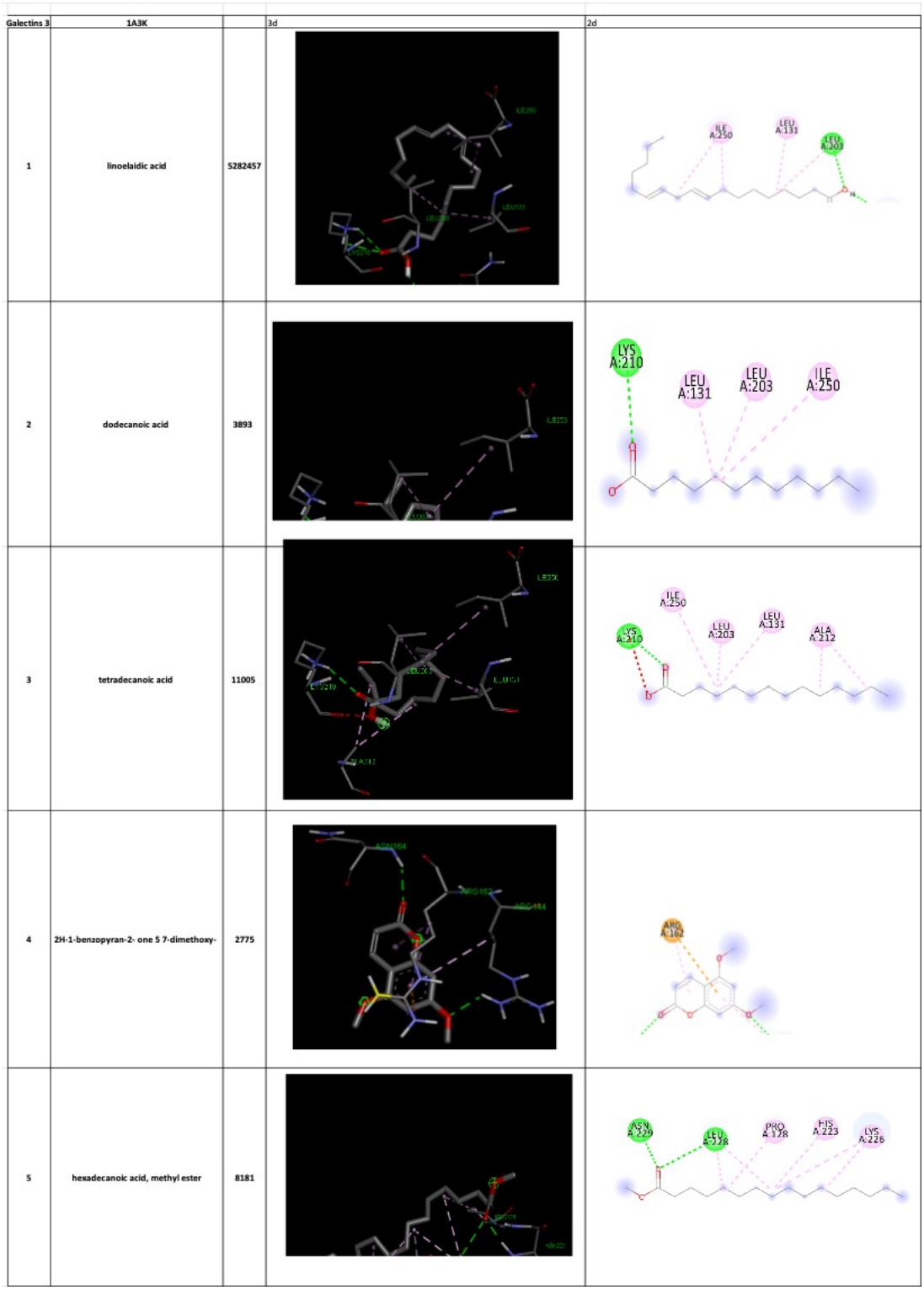

Fig 13 : Result of molecular docking analysis.

4. SUMMARY & CONCLUSION

The comprehensive analysis of Mudavattukaal Kilangu extract underscores its promising potential as a therapeutic agent for managing inflammatory conditions such as rheumatoid arthritis. The qualitative phytochemical screening revealed the presence of several bioactive compounds, including alkaloids, carbohydrates, glycosides, tannins, saponins, and flavonoids, which are well-known for their antioxidant, antimicrobial, anti-inflammatory, and cytotoxic properties. These findings indicate that the extract may offer broad-spectrum benefits for treating inflammatory diseases, particularly rheumatoid arthritis, by modulating key inflammatory pathways.

The GC-MS analysis identified significant bioactive compounds, including Linoelaidic acid and n-Hexadecanoic acid, both of which exhibit strong anti-inflammatory and antioxidant effects. These compounds are known to inhibit pro-inflammatory cytokines and reactive oxygen species, mechanisms central to the pathophysiology of rheumatoid arthritis. Moreover, the presence of Limettin, with its anticancer potential, further supports the extract's relevance in managing diseases involving oxidative stress and inflammation.

The antioxidant assays (DPPH, NO, and H_2O_2 scavenging) confirmed the extract's robust ability to neutralize free radicals, highlighting its potential to reduce oxidative stress, a key factor in rheumatoid arthritis progression. Additionally, the significant inhibition of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, alongside the suppression of NF-κB signaling, suggests that the extract may exert potent anti-inflammatory effects by targeting multiple inflammatory pathways, further supporting its potential in rheumatoid arthritis treatment.

The MTT assay results revealed that Oak Fern extract showed a stronger and more sustained inhibitory effect compared to TNF-β, which may enhance its therapeutic efficacy in inflammatory conditions. Furthermore, molecular docking studies provided insights into the extract's bioactive compounds, with compounds like 2H-1-Benzopyran-2-one showing the highest binding affinity to inflammation-related targets such as Galectin-3, a key protein implicated in rheumatoid arthritis.

Overall, these findings strongly suggest that Mudavattukaal Kilangu extract, with its diverse bioactive compounds and multi-target effects, has significant therapeutic potential in the treatment of rheumatoid arthritis. The extract's ability to modulate key inflammatory mediators, neutralize oxidative stress, and inhibit crucial signaling pathways positions it as a promising candidate for further investigation and development of natural anti-inflammatory therapies for rheumatoid arthritis.

5. ACKNOWLEDGEMENT

I thank Dr MGR Educational and Research Institute for its constant support. I thank Department of Biotechnology for its support and encouragement and for providing the workplace to conduct the research. I extended my gratitude to the Head of the department for her motivation towards research. I specially thank my students for their efforts in working with this work.

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