Modulation of NFKB ligand RANKL Signaling Lipopolysaccharide Induced Rheumatoid Arthritis Cell Line by *Pithecellobium dulce.*

Thesis Submitted for the Award of the Degree of

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In

Allied Health Sciences

Biochemistry

By

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Dedication

To my beloved mother, ***Smt. Annapoorna S. Tungal***

Behind every achievement stands a mother whose silent prayers, selfless sacrifices, and boundless love shape the very core of our strength. In her presence, I found solacement; in her words, courage; and in her heart, an unwavering light that guided me through every step of this journey.

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INDEX

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

RA	Rheumatoid arthritis
WHO	World Health Organization
ELURA	European Alliance of Association
ACR	American College of Rheumatology
FLS	Fibroblast like Synoviocytes
MTT	3-4 dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide assay.
DMSO	Dimethyl sulfoxide
TNF-α	Tumour necrosis factor-α
IL-1β	Interleukin 1 beta
IL-6	Interleukin 1-6
NSAIDs	Non-steroidal anti-inflammatory drugs
DMARDs	Disease-modifying antirheumatic drugs
NF-kB	Nuclear factor kappa-B
RANKL	Receptor activator of nuclear kappa ligand
LPS	Lipopolysaccharide
MMPs	Matrix metalloproteinases
MMP-1	Matrix metalloproteinase-1
ECM	Extra cellular matrix
RNA	Ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
TIMPs	Tissue inhibitors of metalloproteinases
NCCS	National Centre for Cell Sciences
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
DMEM F-12	Dulbecco's Modified Eagle Medium F-12
FBS	Fetal Bovine Serum
EDTA	Ethylenediaminetetraacetic acid.
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pd	*Pithecellobium dulce*
HPLC	High-performance liquid chromatography

Na	Sodium
Ca	Calcium
K	Potassium
Fe	iron
HCL	Hydrochloric Acid
ADME/T	Absorption distribution metabolism excretion and toxicity
PDB	Protein Data Bank
TPSA	Total polar surface area
WLOGP	Wildman and Crippen's Logarithm of the Partition coefficient
GI	Gastrointestinal absorption
BBB	Blood Brain Barrier
NMA	Normal mode analysis
RMSD	Root mean square deviation
Pd-CuNPs	*Pithecellobium dulce* copper nanoparticles
SAED	Selective area electron diffraction
NaOH	Sodium Hydroxide
CuO	Copper Oxide
ZnO	Zinc Oxide

ABSTRACT

Introduction:

In the world, 1% of people suffer from chronic inflammatory autoimmune diseases like rheumatoid arthritis (RA). The individual with rheumatoid arthritis suffers from synovial inflammation, cartilage degradation, joint destruction, leading to reduced quality of life. The disease pathogenesis involves the activation of pro-inflammatory cytokines and NF-κB ligand RANKL signaling pathway. The research on plant-based drug design and discovery is to target pathways to find promising phytochemical alternatives for relieving symptoms of RA and other chronic diseases. This study aimed to investigate the role of bioactive compounds *Pithecellobium dulce* gallic acid and quercetin, in modulating the NF-κB ligand RANKL signaling pathway by targeting MMP-1 expression through *in silico* and in vitro approaches.

Objective:

To evaluate the effect of isolated bioactive compounds from *Pithecellobium dulce* fruit gallic acid, and quercetin on mRNA expression of MMP-1 in NF-kB ligand RANKL signaling.

Method:

The study involved *In Silico* and experimental in vitro methods. Ligand–protein interaction studies were conducted to assess gallic acids and quercetin's therapeutic potential in targeting the NF-κB ligand RANKL signaling pathway. Additionally, green-synthesized Pd-Cu nanoparticles (Pd-CuNPs) were characterized and evaluated. Pharmacokinetic properties, drug-likeness, gastrointestinal absorption, blood-brain barrier permeability, and solubility were predicted using ADME/T tools. Molecular interaction and molecular dynamics simulation were performed to identify potential interactions. The gene expression of MMP-1 was evaluated through RT-PCR in treated rheumatoid arthritis cells.

Results:

In silico analysis confirmed gallic acid and quercetin have favourable pharmacokinetic and safety profiles. Docking and simulation studies identified strong binding affinities with target proteins. Experimental In vitro results revealed a significant downregulation of MMP-1 mRNA expression in treated RA cells, gallic acid (0.299 ± 0.25, $p < 0.05$), Pd-CuNPs (0.432 ± 0.22), quercetin (0.519 ± 0.01), and crude extract (0.633 ± 0.03), compared to control.

Conclusion:

The study demonstrated that gallic acid, quercetin, and Pd-CuNPs exhibit promising modulatory effects on the NF-κB ligand RANKL signaling pathway. These findings support their potential as plant-based alternative agents for managing inflammatory disease like rheumatoid arthritis.

Keywords: Gallic acid, Quercetin, NF-kB, *Pithecellobium dulce, In Silico,* and in vitro
MMP-1

CHAPTER-I
INTRODUCTION

1.1 Introduction

Rheumatoid arthritis (RA) is a long-term inflammatory autoimmune disease that affects approximately one per cent (1%) of the global population (Kamil Więcek et al.2022). According to the World Health Organization's 2023 update, RA impacts an estimated 18 million people worldwide and remains a leading cause of disability, particularly in low- and middle-income nations where early diagnosis and treatment are often limited. (World Health Organization 2023).

In India, the Indian Rheumatology Association estimates a prevalence of around 0.75%, with higher incidence reported in urban areas. RA is more commonly diagnosed in women than males, with a female-to-male ratio of about 2:1. (Marder et al. 2024) The disease is identified by inflammation of the synovial joints and causes progressive destruction of cartilage and bone as the immune system mistakenly attacks joint tissues. It causes pain, swelling, and stiffness, particularly in the hands, wrists, knees, and feet. In addition to physical symptoms, RA also imposes significant social and psychological burdens on affected individuals (Peng et al. 2024).

The pathogenesis of rheumatoid arthritis shows the involvement of various genetic and environmental factors like obesity, smoking, and infections as potent inducers of rheumatoid arthritis (Croia et al.2019) Based on the pathogenesis of rheumatoid arthritis, various anti-rheumatoid arthritis drugs have been developed for its treatment. These treatments include non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying anti-rheumatic drugs (DMARDs). NSAIDs, like ibuprofen and celecoxib, are effective in reducing inflammation and pain, but long-term use is associated with increased cardiovascular (CV) and gastrointestinal (GI) risks.

DMARDs, including methotrexate and biological agents like TNF-α inhibitors, help suppress disease progression but may lead to increased risks of infections, liver toxicity, and, in some cases, cardiovascular complications. Surgical treatments, such as joint replacement or synovectomy, are considered in advanced stages but carry risks of infection, implant failure, and prolonged recovery, particularly in immunosuppressed RA patients (Zheng et al. 2024)

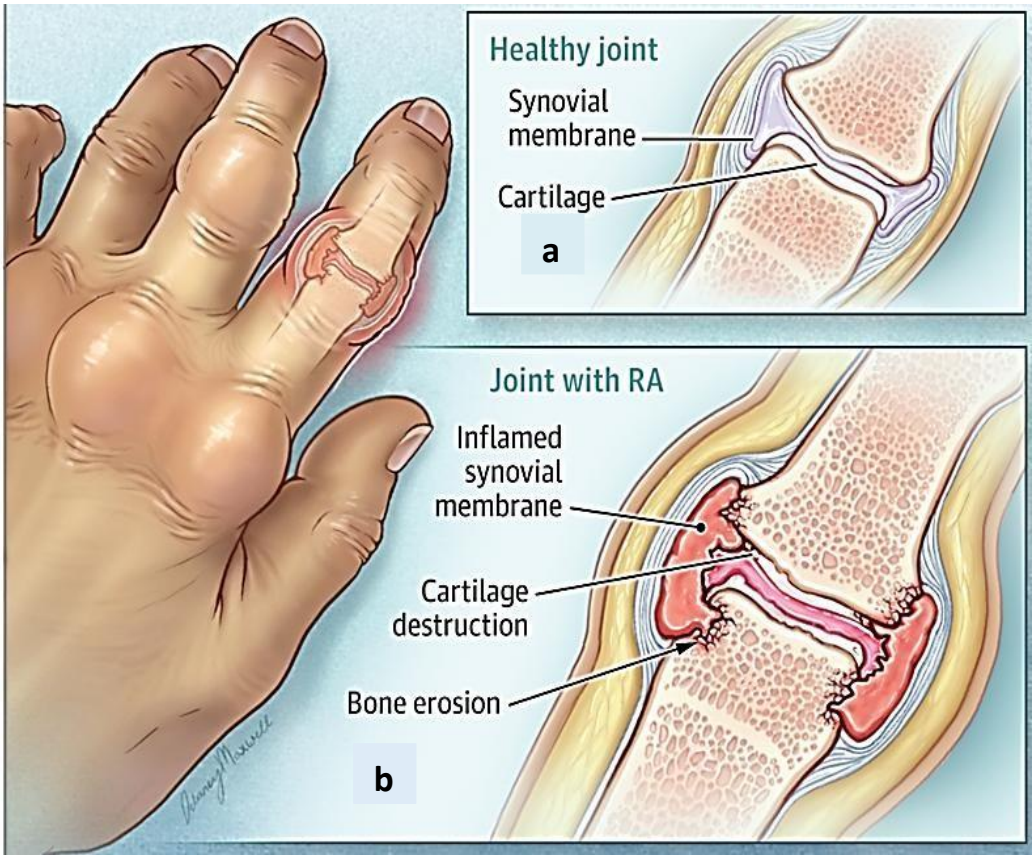

Figure No 1.1.1: **a.** Presents the health joints synovial membrane and cartilage. **b.** Presents joint with rheumatoid arthritis (RA) inflamed synovial membrane, cartilage destruction, and bone erosion. (Smith, M. H., & Berman, J. R. (2022). Jama, 327(12), 1194-1194).

The NF-κB (nuclear factor kappa B) pathway also controls inflammation and the immune response. (Peng et al. 2022) This pathway is responsible for the transcription factor, which activates cytokines, chemokines, and adhesion molecules (Kondo et al. 2021) Moreover, it activates the synovial fibroblast-like cells (FLS), resulting in inflammation and joint destruction (Toghi et al. 2023)

The activation of the pathway (NF-kB) occurs through multiple stimuli, including TNFα, IL-1β, and lipopolysaccharide (LPS), which mainly mimic inflammation in cells derived from gram-negative bacteria like (E. coli) (Taniguchi et al. 2023)

The NF-κB pathway is a major contributor to regulating molecular processes related to inflammation and immune function in RA. It consists of proteins from the

Rel family, such as RelA (p65), c-Rel, RelB, NF-κB1 (p50), and NF-κB2 (p52) (Zhang et al. 2023, Liu et al. 2017). Activation of the NF-κB pathway involves the phosphorylation and proteasomal degradation of the inhibitory protein IκBα, which allows the RelA/NF-κB1 (p65/p50). The NF-κB signalling pathway is key.

This signaling pathway is also responsible for osteoclast differentiation by regulating RANKL expression, thereby contributing to bone erosion. In addition, the NF-κB pathway activates the expression of matrix metalloproteinases, particularly MMP-1, involved in breaking down the extracellular matrix and damaging cartilage in rheumatoid arthritis. (Hayden et al. 2012, Burrage et al. 2006)

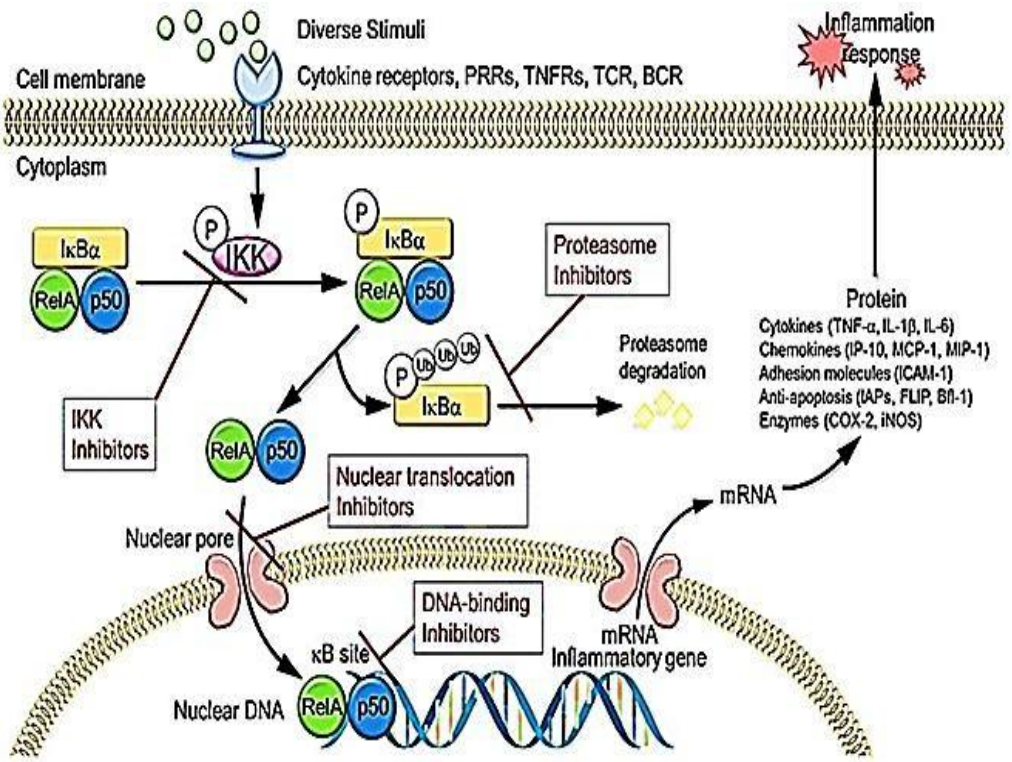

Figure 1.1.2. The NF-κB signaling pathway illustrates the activation of inflammatory transcription factors and mRNA expression stimulated by cytokines such as TNF-α, IL-1β, and IL-6 (Liu et al., 2017).

Naturally, existing phytochemicals have become popular as treatment options for various inflammatory conditions. Conventional drugs used for rheumatoid arthritis (RA) have adverse effects, and there is an urgent need to explore safer alternatives with minimal side effects.

Pithecellobium dulce (Pd) is found throughout the plane of India, native Mexico, and America; it is commonly known as manila tamarind, and in different regions, it is known by different names. The tree is small to medium-sized, spiny, and evergreen in India. Its edible fruits provide protein, fats, carbohydrates, energy, and important vitamins (Venu, et al. 2016, Dhanisha et al. 2022). Because of their nutritional value, these fruits offer many health benefits. The fruits also contain various bioactive compounds, such as flavonoids, phenolics, tannins, and saponins. Studies on the hydroalcoholic extract of the fruit have shown that it contains phenolics and flavonoids. Saponins from the seeds have shown strong anti-inflammatory, antioxidant, and antifungal effects (Murugesan et al. 2019).

It is necessary to explore the new bioactive herbal extract molecules for the treating rheumatoid arthritis. Phytochemicals have become popular as a treatment option for inflammatory diseases. Additionally, research has shown that *Pithecellobium dulce* has anti-inflammatory properties.

There is scope to explore novel therapeutic strategies. NF-kB ligand RANKL signaling plays a key role in lipopolysaccharide induced RA cell lines. Hence, targeting signaling can be sought as a novel therapeutic strategy for RA.

Hence this study aims to investigate the role of NF-κB and RANKL signaling in lipopolysaccharide-induced inflammation in rheumatoid arthritis cells and to evaluate the nutraceutical potential of bioactive compounds isolated from *Pithecellobium dulce* through *in silico* and in vitro approaches. This integrated approach contributes to developing safer, plant-based alternatives for RA.

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CHAPTER-II
LITERATURE REVIEW

2.1 Arthritis

Arthritis originates from the Greek word meaning “joint disease.” It is defined as inflammation in one or more joints, which may be acute or chronic. This inflammation often leads to pain, swelling, and sometimes permanent damage to the joint structure. It is important to note that arthritis is not the same as arthralgia, which refers to joint pain that may occur without any underlying inflammation (Sparks et al., 2019).

Rheumatoid arthritis (RA), osteoarthritis (OA), and psoriatic arthritis (PsA) are three most common and clinically important types of arthritis.

- Rheumatoid arthritis (RA) is a disease responsible for chronic inflammation and progressive joint destruction driven by chronic inflammation.
- Osteoarthritis (OA) is a progressive joint inflammation that leads to cartilage breakdown, tissue damage, and bone remodelling over time.
- Psoriatic arthritis (PsA) is associated with joint pain, swelling, and distinctive features such as enthesitis (inflammation in ligaments or tendons attach to bone) and dactylitis, which causes swelling of the fingers and toes (Ali et al., 2023).

2.2 Rheumatoid Arthritis

RA is a long-lasting autoimmune disease that affects synovial joints and organs like the heart, lungs. RA is an overreactive immune condition that mistakenly target body tissues. Prolonged RA complications may lead to diseases like osteoarthritis (Gao et al. 2024) This disease starts with chronic synovial membrane inflammation, leading to symptoms like swelling, morning stiffness, and fatigue (Guo et al. 2018). The long-term effects of these symptoms damage the cartilage and bone, causing loss of activities in the affected area (Chen et al. 2019).

Global health organization, World Health Organization (WHO) and the European Alliance of Association for Rheumatology (EULAR) have highlighted importance of early diagnosis, life style habits modification, physical managements to overcome RA complications (Murphy et al. 2023, Gwinnutt et al. 2023).

American College of Rheumatology (ACR) guidelines focus on various forms of exercise including aerobic, strength training, to manage pain in RA. These approaches aim to improve physical and mental health of individual affected by RA (Lindéus et al. 2022).

Among the various lifestyle and environmental factors, smoking is one of the most critical risk factors for the development of rheumatoid arthritis (Balasundaram et al., 2024). Obesity causes inflammation, which is responsible for the dysfunction of the immune system. Infection is also an important factor which triggers the various signaling pathways. Another important factor which may lead to impairment in the gut microbiota leading to RA is poor dietary habits (Godha et al., 2023).

2.3 Pathogenesis of Rheumatoid arthritis (RA)

The Pathogenesis of RA is impairment in complex molecular mechanisms with dysfunctions of immune response and proinflammatory cytokine activation. The Pathogenesis of rheumatoid arthritis is complex, involving innate and acquired immune responses that cause chronic inflammation. Important immune cells like macrophages, T-cells, and B-cells join the synovium and interact with fibroblast-like synoviocytes (FLS), these factors help cause the release of inflammation-related cytokines such as IL-1β, TNFα, and IL-6 (Zhang et al. 2024 and Liu et al. 2023). This activation leads to undifferentiated pannus formation, angiogenesis, cartilage destruction and bone erosion. Current treatments aim to modulate these pathological complications of RA (Kim et al., 2023).

2.4 Current treatment approaches of rheumatoid arthritis

Current approaches to managing RA including non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, naproxen, and diclofenac are commonly used to treat inflammation of RA. However, long-term usage may lead to gastrointestinal bleeding, cardiovascular issues, and renal dysfunction. Another common treatment involves conventional drugs disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate, leflunomide and sulfasalazine. Among these, methotrexates are highly used due to their effectiveness and availability (Smith et al., 2024). Surgical treatments, such as joint replacement or synovectomy, are considered

in advanced stages but carry risks of infection, implant failure, and prolonged recovery, particularly in immunosuppressed RA patients (Peng et al., 2022).

Many signaling pathways are involved in RA, which causes chronic inflammation and joint destruction. Among them, the NF-κB pathway plays a central role by activating genes responsible for inflammation and tissue breakdown (Liu et al., 2017). The RANKL–RANK pathway drives bone loss through NF-κB and MAPK signaling (Nedeva, et al., 221), while the MAPK pathway boosts inflammation and joint-lining cell activity (Schett et al., 2000). The JAK/STAT pathway, triggered by cytokines like IL-6, helps regulate immune responses (O'Shea et al., 201s), and the PI3K/Akt/mTOR pathway supports cell survival and angiogenesis (Malemud, 2015). Among these, NF-κB signaling pathway is particularly crucial since it maintains inflammation, connects to other pathways, and targets RA treatment (Sun et al., 2021).

2.5 Role of NF-kB and RANKL signaling Pathways in rheumatoid arthritis.

The NF-kB and RANKL signaling pathways regulates gene expression through transcription factor and contributing in proinflammatory cytokines, chemokines, and adhesion molecules production in inflammation in RA (Barrow et al. 2021). Synovial fibroblast- like synoviocytes (FLS) are targets of NF-kB pathway activation, leading to their proliferation and releasing of inflammatory mediators (Radu et al 2021).

Multiple inflammatory cytokines, such as TNF-α, IL-1β, and bacterial lipopolysaccharide activate the NF-kB pathway (Ding et al 2023).

*Escherichia coli (*E coli*)* is a gram-negative bacteria outer layer covered with LPS, which is *responsible* for activation of immune cells, by mimicking bacterial toxins and triggering inflammatory signaling (Fu et al.2018). Expression of various pro-inflammatory cytokines in the NF-kB pathway by LPS stimulation, which is regulated by transcription factor (Lorenz et al. 2013, Afrasiabi et al. 2023). In rheumatoid arthritis, increased LPS levels stimulate the release of MMPs and pro-inflammatory cytokines, contributing to joint degradation. (Hasan et al 2020).

LPS activation stimulates phosphorylation ubiquitination and degradation of inhibitory protein IkBα, and various gene expression are regulated by NF-kB family proteins mainly the RelA (p65), c-Rel, RelB, NF-kB1(p50) and NF-kB (p52) (Li et al 2020). Among these p65/p50 heterodimer translocate into the nucleus and initiates the transcription of proinflammatory cytokines. Chronic activation of signaling in RA responsible for the inflammation leads joint destruction by activation of RANKL, influences osteoclast there by contributing to bone erosion (Akhter et al. 2022).

2.6 Over view of Matrix Metalloproteinases (MMPs)

MMPs are zinc-dependent enzymes and are group of endopeptidases. They play a vital role in the degradation and remodeling of the extracellular matrix (ECM). MMPs involve several essential physiological processes, such as embryonic development, wound healing, tissue regeneration, and angiogenesis (Visse & Nagase, 2003). They are mainly produced by cells such as synovial fibroblasts, chondrocytes, macrophages, endothelial cells, and neutrophils (Parks et al., 2004). Pathological conditions MMPs can contribute to various diseases such as cancer, cardiovascular disorders, and autoimmune conditions like rheumatoid arthritis (RA) (Kehlet et al., 2021).

MMPs secreted into the extracellular space, activate and degrade ECM proteins like collagen, elastin, fibronectin, and proteoglycans (Malemud, 2006). RA leads to cartilage and bone degradation, influencing inflammation by modulating cytokine activity (Burrage et al., 2006). Because of their central role in both tissue damage and inflammatory pathways, MMPs-especially MMP-1, MMP-3, and MMP are recognized as promising targets for therapeutic intervention in RA (Table No.2.6.1) (Liu et al., 2022).

Table No. 2.6.1 Types of Matrix metalloproteinases and functions in RA. (Singh et al. 2015).

Matrix metalloproteinases (MMPs)	Primary function	Involment in RA
MMP-1	Degrades type I and II collagen protein	Highly expressed in synovial fibroblasts and macrophages, activates proinflammatory cytokines
MMP-2	Degrades type IV collagen and basement membrane remodeling	Plays a role in tissue remodeling and signaling in inflammatory responses
MMP-3	Joint destruction, proteoglycans break down, activation of proinflammatory cytokines	Contributes to joint destruction and activation of pro-inflammatory cytokines
MMP-9	Degrades type IV collagen and gelatin	Angiogenesis and cytokine activation
MMP-13	Degrades type II collagen and degradation of cartilage	Consider for target for preventing cartilage damage

2.7 Role of matrix metalloproteinase-1 (MMP-1) in RA.

Matrix metalloproteinase-1, is belongs to the MMPs family and plays a crucial role in extracellular matrix (ECM) degradation. It primarily cleaves interstitial collagens type I, II, and III, which are essential for maintaining the structural integrity of cartilage and connective tissue (Bian et al. 2023). Activated fibroblast like synoviocytes (FLS) secrete increased levels of MMP-1, leading to degradation of cartilage and bone. Proinflammatory cytokines such as IL-1β and TNF-α increase MMP-1 expression in NF-κB pathway and translocate to the nucleus and binds to the MMP-1 promoter region, increasing transcription and accelerating tissue breakdown (Abdelrahman et al. 2019, Nejatbakhsh Samimi et al.2020).

Further MMP-1 assist angiogenesis by degrades the ECM to promotes new blood vessel formation, and pannus growth leads to chronic inflammation. And

connects with other MMPs like MMP-3 and MMP-13 influences the break down cartilage and bone destruction (Paleolog et al 2009).

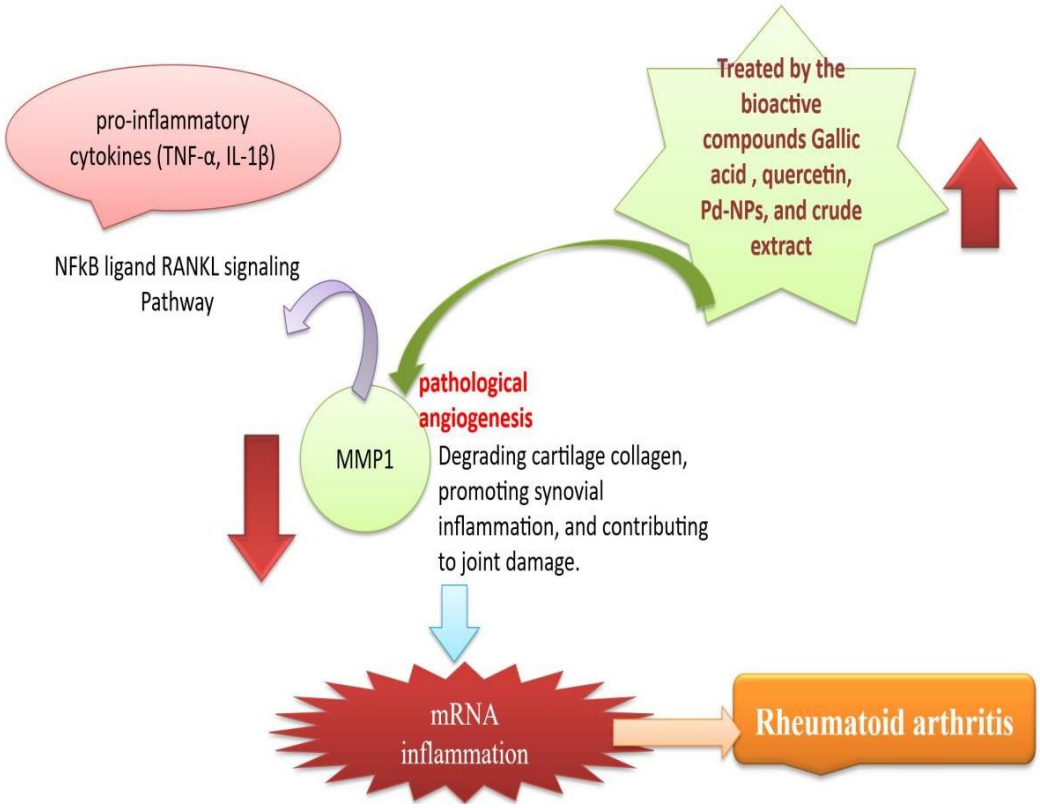

Figure 2.7.1: Modulation of NF-kB-RANKL signaling pathway in rheumatoid arthritis by Bioactive compounds.

This graphical image presents the NF-kB ligand and RANKL signaling pathway in rheumatoid arthritis, activation of cytokines such as TNF-α and IL-1β in the synovial membrane, which stimulates the extracellular matrix degradation (ECM) by elevating MMP-1 expression leads to cartilage degradation and angiogenesis, enhances inflammatory mRNA level in rheumatoid arthritis (RA). Bioactive compounds like gallic acid, quercetin, Pd-NPs and plant extracts can inhibit MMP-1 expression and reduce inflammation. Shows nutraceutical and safer alternatives to manage rheumatoid arthritis (RA).

2.8 Phytochemicals

Phytochemicals and secondary metabolites are diverse groups of physiologically active compounds originating from plants and their parts; these play important roles in various physiological activities. These compounds are classified into alkaloids, flavonoids, terpenes, phenolic acids, tannins, saponins, and glycosides and are known for their anti-inflammatory, antidiabetic, antioxidant, and anticancer properties.

Phytochemical analysis in chemical science is gaining popularity, with researchers focusing on the structure, synthesis and impact of plant compounds on the body (Mosić et al. 2020, Pramanik et al. 2023). Many industries such as pharmaceutical, medical, food and cosmetic make use of naturally occurring compounds due to their medical and health benefits (Rasul MG et al. 2018). Over the years, bioactive compounds have been valued in many traditional medical practices. Due to their phytochemicals and secondary metabolites such as alkaloids, flavonoids, phenolic acids, tannins, saponins, steroids and terpenoids, these plants are recognized for their therapeutic and nutraceutical potential. These bioactive compounds are found in edible and non-edible parts of the plants and trees including fruits, leaves, bark, stem, and root (Boukhatem MN et al. 2024). Their metabolites have several applications as antioxidants, anti-inflammatory, anti-cancer and anti-bacterial properties (Shaikh JR et al. 2020). The increasing demand for natural remedies with health supplements and alternatives have acerated interest in less explored bioactive compounds.

2.8.1. *Pithecellobium dulce (Pd)*

One of the promising and alternative, *Pithecellobium dulce* (p. dulce), commonly known as jungle jalebi, manila tamarind, and vijayati Babula, with many other names in different regions, the tree belongs to the Fabaceae family. Originally native to tropical regions of the Americas, it was found in the plain of the India. The fruits of P. dulce contain wide variety of bioactive compounds with significant therapeutic properties (Dhanisha SS, et al. 2022, Sadasivam S et al. 2025).

2.8.2 Botanical description

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Fabales

Family: Fabaceae

Genus: *Pithecellobium*

Species: *Pithecellobium dulce*

2.8.3 Table Vernacular names of *Pithecellobium dulce* (Sneha et al .2020)

Language	**Name of the fruit**
Telugu	Seema Chinta kaya
English	Quamachil, Madrasthorn, Manila tamrind
Hindi	Vilayati babul, Vilayati imali, jangle jalebi
Malayalam	Korukkapuli
Tamil	Kodukkaapuli
Kannada	Seeme hunase
Sanskrit	Kodukkaapuli
Marathi	Ingraji Chinch
Bengali	Jilapi
Gujarati	Bakhai, Ambli, Goras ambli
Odia	Seema Kaiyan
Chinese	Niutidou
French	Cameche (New Caledonia), Cassiede Manille
German	Camambilarinde
Greek	Pithekos ellobion
Japanese	Huamuche, Guamuche
Philippines	Camachile
Spanish	Gurmukhi, Guama americano, Quamachil

2.8.4 Geographical Distribution

Pithecellobium dulce tree is native Mexico, America, Central Asia found through the plan of India. It is one of 18 species in this genus, commonly grown along road sides, dry land, water land the tree ability to grow any kind of soil (Murugesan et al. 2019)

2.8.5 Morphology

Pithecellobium dulce tree reaching 10-18 meters tall, leaves are bipinnate, has one to two pairs of green, spherical and heart shaped leaflets, with 2-15 mm, barks are grey. Green and white flowers and fruits are edible (Murugesan et al. 2019). The plant grows axillary panicle-like inflorescences, spherical clusters of small, slightly fragrant flowers that are usually white to greenish and around 1 cm across. It produces indehiscent pods that are reddish-pink to greenish-brown in colour. Ten black, glossy, flattened seeds, about 1 cm in diameter, are often found in each pod. The pods are relatively thin, with a width of 1-2 cm and a length of 10-15 cm. (Heuzé et al. 2015). (Figure 2.7.6)

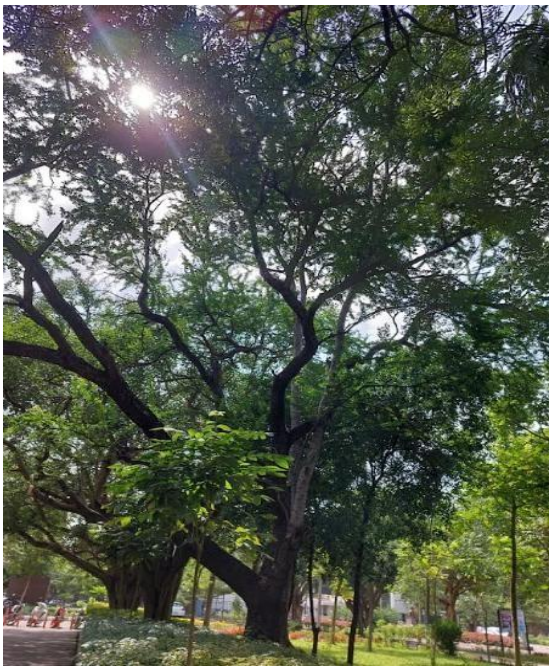

Figure 2.8.5.1 *Pithecellobium dulce* tree

2.8.6 Pithecellobium dulce fruit

The fruits of *Pithecellobium dulce* are easily identified by their distinctive appearance and spirally coiled into one or two loops, showing constrictions between each seed. Initially green with a reddish tinge, the pods gradually turn bright pink or red as they ripen and become reddish-brown after splitting open. When unripe, the pods are fleshy but dry out and take on a papery texture upon maturity. Each pod splits open along both sides to reveal 8 to 12 shiny black seeds, which remain attached by a fleshy aril that can be white, pale pink, or occasionally red. The seeds are flat and lens-shaped, typically 7–13 mm long, 6–11 mm wide, and 2–4 mm thick (CABI. 2022) (Table No. 2.8.7.1).

Table No. 2.8.6.1 Nutritional Value of *Pithecellobium dulce* Reported by (Pío-León et al. 2013, Kulkarni, K et al. 2018, Shukla, M et al. 2024)

Energy	78 Kcal
Water	77.8%
Protein	3%
Fat	4%
Carbohydrate	18.2%
Fiber	1.2%
Ash	6%
Calcium (1.3 % RDI)	13mg
Phosphorous (4.2% RDI)	42mg
Iron (2.7% RDI)	5mg
Sodium	19mg
Potassium (6.3 % RDI)	222mg
Vitamin A	15mg
Thiamine/B1(5.8% RDI)	24mg
Riboflavin/B2 (5.8% RDI)	10mg
Niacin/B6 (3% RDI)	60mg
Vitamin C (221% RDI)	133mg

Fruits contained bioactive compounds like**, 1.** Naringenin **2**. Quercetin **3.** Rutin **4.** Gallic acid **5.** Stigmasterol **6**. Clonazepam **7**. Quinoline **8.** Nootkatone **9.** Juipene **10.** Calarene **11.** Eremophiline **12.** Valencene **13.** Baicaline **14.** 2,5,6-Trimethyl 1,3 oxathiane **15.** Trans 3 methyl, 2N-propylthiophane **16**. D-pinitol **17**. Hexadecenoic acid **18.** Hepatacosanoic acid **19.** Tetraneurin-f **20.** Ethyl 2-bromo-4-methyl-6dimethylsilybenzothiophene-5-carboxylate **21.** 2-Propyl tetrahydropyran-3-ol (Dhanisha et al. 2022).

Figure 2.8.6.1 *Pithecellobium dulce* fruit

2.8.7 Health Benefits of *Pithecellobium dulce* (Pd)

Pithecellobium dulce popular option as growing interest, potential and safer candidate to treat and manage rheumatoid arthritis (RA) The fruits of *Pithecellobium dulce* rich of phytochemicals exhibits a various nutraceutical and pharmacological applications such as useful in diabetes control, inflammation reduction, microbial inhibition, cancer suppression, antioxidant defense, cardiac protection, gastrointestinal support, and larval control (Dhanisha et al. 2022). With this *Pithecellobium dulce* becomes popular option as growing interest potential and safer candidates to treat and manage rheumatoid arthritis RA.

List of health Benefits of *Pithecellobium dulce* (Kulkarni, K. et al. 2018)

- Acts as a natural **antiseptic**
- Helps **lighten skin tone** and **eliminate pigmentation**
- Serves as a **natural skin moisturizer**
- **Removes dark spots**, **treats acne**, and **reduces pimples**
- **Treats oily scalp** and **prevents hair loss**
- Aids in **weight management**
- Considered **beneficial during pregnancy**
- Helps **treat bilious disorders**
- Used in traditional medicine to **reduce fever**
- Assists in the **management of malaria** and **jaundice**
- **Regulates blood circulation**
- **Controls blood sugar levels**; often prescribed for **diabetics**
- **Boosts the immune system**
- Provides **anti-inflammatory benefits** and helps **relieve inflammation**
- Used to **treat mouth ulcers**
- May help **prevent cancer** due to its high antioxidant content
- Traditionally used to **treat venereal diseases**
- **Leaves** are used as a remedy for **indigestion**
- **Bark** is used to relieve **constipation** and support **bowel movement**
- Rich in **vitamin C**, contributing to its **antioxidant properties**

2.9 *In Silico* analysis NF-kB ligand RANKL signaling pathway /*Pithecellobium dulce*

In silico or computational databases have become essential applications in modern drug designing due to their screening compounds interaction like ligand - protein prediction molecular interaction, minimising the experimental costs and speeding up the screening. In recent years, online databases have become popular for identifying and analysing chemical 2D and 3D structure molecular interaction chemical properties and retrieving data. These help in drug design and have broad applications in the medical field.

The study demonstrated the interaction of hemotoxic snake venom peptide and human biomarkers screened by molecular docking and Swiss ADME analysis. The snake venom metalloproteinase (SVMPS) with target biomarkers showed strong binding interaction between protein-protein docking, and drug-likeness without toxicity violation. iMODS analysis screened molecular dynamic simulation of protein flexibility, stability, deformability, B-factor. The study highlights that computational databases predict faster and easily screen the molecules (Honutagi et al. 2023).

Protein Data Bank (PDB) is the designing the high-resolution crystal structure of protein the study has seen virtual matrix metalloproteinase (MMP-1), NF-kB subunit and IkBα, iMODS screening showed Normal Mode analysis (NMA), B-factor, covariance map predicted protein flexibility and dynamic motion of molecules (Brogi et al. 2020).

H-Dock server used to predict the binding energy of natural compounds like gallic acid and quercetin with inflammatory proteins, screened ligand – protein interaction with good binding energy (Yan et al.2017).

Swiss ADME analysis was screened for the toxicity analysis, drug likeness, molecular weight, total polar surface area (TPSA), solubility factor, boiled egg data predicted gastrointestinal absorption (GI), and blood brain barriers crossing (BBB) (Daina et al 2017).

The computational database screen a foundation for identifying potential active compounds. In vitro study provides their effects on inflammatory markers and validates their alternative and nutraceutical applications.

2.10 Invitro analysis NF-kB ligand RANKL signaling pathway /*Pithecellobium dulce*

According to Wang et al. (2023) role of quercetin a natural molecule reduces the inflammation in osteoarthritis (OA) and downregulates the pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, stimulates the chondrocyte catabolism and cartilage degradation. It also supresses the activation of NF-kB signaling, matrix metalloproteinases (MMPs), pro-inflammatory cytokines, and enzymes like iNOS and COX-2 these all influences the cartilage destruction. The author mentioned that quercetin is the potential candidate for the treat of osteoarthritis (OA) (Wang et al. 2023).

Gonçalves et al. (2024) current treatments like NSAIDS and DMARDS, have long term side effects. A recent study has identified natural polyphenolic compounds like gallic acid and quercetin have anti-inflammatory, anti-oxidant, and immunomodulatory properties. Gallic acid can inhibit the NF-kB signaling pathway, supress the MMPs expression by reducing pro-inflammatory cytokines. Quercetin potential flavonoid ability to inhibit the IL-17 activated by the RANKL signaling, osteoclast formation. The targeting molecular pathway like NF-kB, polyphenols show promising downregulation of MMP-1 and MMP-9 expression that contribute joint damage.

The nanoparticle-based study conducted by Wahnou et al. (2024), targeting NF-kB signaling pathway. Supressing MMPs activation and reducing cytokine stimulation TNF-α, IL-6, quercetin showed potential anti-inflammatory role to manage rheumatoid arthritis.

Haleagrahara et al. (2018) in vivo mouse model, collagen-induced arthritis. Linked with NF-kB pathway reduced pro-inflammatory cytokines TNF-α, IL-1β. IL-6, and IL-17, treated by the potential quercetin as natural flavonoid has anti-inflammatory properties.

Behl et al. (2021) studied polyphenols like quercetin epigallocatechin-3-gallate (EGCG) showed anti-inflammatory and anti-oxidant in effects in rheumatoid arthritis, targeting NF-kB and MAPK. Reduce cytokine and inhibit the MMPs activity.

Considering the study on anti-inflammatory properties of gallic acid and quercetin, their nutraceutical application has limited stability and bioavailability with improving targeted delivery, absorption, and therapeutic effects. The nanoparticle delivery method has been employed and has also targeted the NF-kB pathway.

2.11 Green synthesis of *Pithecellobium dulce* copper sulphate nanoparticles (Pd-CuNPs) NF-kB ligand RANKL signaling

Green synthesis is an eco-friendly and sustainable approach. Synthesize nanoparticles using *Pithecellobium dulce* fruit extract and copper sulphate (Pd-CuNPs) as precursors. The bioactive compounds like flavonoids, alkaloids, phenolic, and tannins and acts as natural stabilizing and reducing agents. These molecules help to reduce copper ions (Cu2+) into nanoscale copper particles with a cost-effective, less energy-consuming process and the synthesis of the nanoparticles.

Usman et al. (2013), copper nanoparticle synthesis as cost-effective compared to metals like gold and silver and choice for large-scale production. Copper with a high melting point (approximately 1083-1084) helps to industrial and medical application. The thermal adoptability of Cu nanoparticles is strong and intact during high-temperature techniques like laser treatments and sterilization. The validate the copper by long-term application in the biomedical, drug delivery, implants, wound healing and biosensors.

Ren et al. (2009) and Singh et al. (2018) found that copper nanoparticles have been reported strong antimicrobial, anti-oxidant, anti-cancer activities. Showed lower cytotoxicity to cells synthesized utilizing plant materials. According to Soltys et al. (2021), plant-mediated green synthesis helps sustainable chemistry by avoiding dangerous substances and adverse environments.

Soleiman-Beigi et al. (2025) studied the using the *Pithecellobium dulce* as an environmentally friendly approach to synthesis of nanoparticles and improving the biological and pharmacological properties.

Sepasgozar et al (2021) synthesized CuO and ZnO to evaluate the antioxidant and antibacterial properties utilized Nobilis extract for the first time in their study and screened CuO and ZnO NPs found largely due to small size and large surface area, alters the bacterial membranes and protein for the antibacterial activity. And with other property like antioxidant activity enhancing free radical scavenging ability for metal-polyphenol complex formation. This approach offers promising potential in pharmaceutical and biomedical application.

Hence, the green synthesis of Pd-CuNPs shows a promising alternative eco-friendly approach for synthesizing functional nanomaterials with potential application in the pharmaceutical, medicine and environmental field.

The literature highlights a rheumatoid arthritis condition strongly correlated with the NF-kB ligand RANKL signaling. Shows importance of plant derived bioactive compounds in modulating pathway. With this *Pithecellobium dulce* has been identified for its anti-inflammatory and antioxidant properties.

The bioactive compounds of *Pithecellobium dulce* its specific role in NF-kB ligand and RANKL regulation in RA remains underexplored. Further no comprehensive study is conducted in *In Silico*, Phytochemical isolation and in vitro and green synthesis of nanoparticles from *Pithecellobium dulce* to investigate anti-arthritic potential.

This study aims to bridge this gap by evaluating the Pithecellobium dulce bioactive compounds, by computational, molecular and nanotechnology-based approaches to manage rheumatoid arthritis.

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CHAPTER-III
AIM AND OBJECTIVES

3.1 Aim

To study the role of the NF-kB ligand RANKL signaling in the lipopolysaccharide induced proliferation of rheumatoid arthritis cell line by isolated bioactive molecule from *Pithecellobium dulce*.

3.2 Objectives

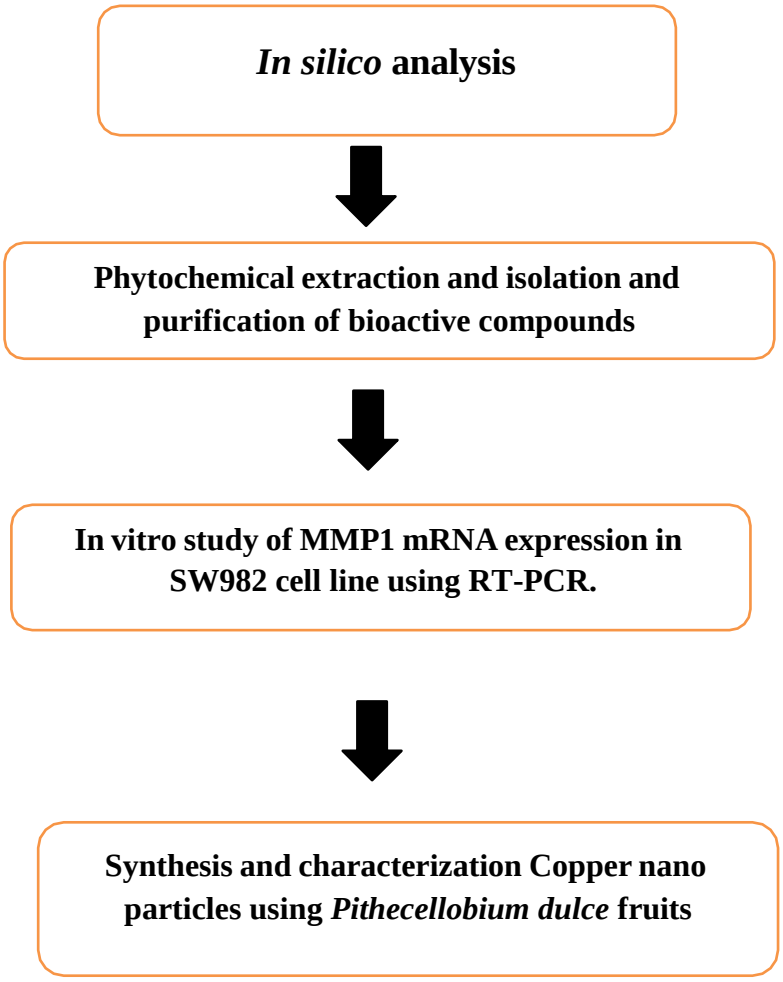
1. Assessment interaction of bioactive molecules from Pithecellobium dulce on NF-kB ligand RANKL signaling by *In silico* studies.
2. Phytochemical extraction, Identification and isolation of bioactive molecules from Pithecellobium dulce fruit.
3. To investigate the effect of isolated bioactive molecule from *Pithecellobium dulce* on NF-kB ligand RANKL mRNA expression in rheumatoid arthritis cell line.
4. Synthesis of nanoparticles using *Pithecellobium dulce* fruit extract and impact on rheumatoid arthritis Cell.

3.3 Hypothesis

The bioactive molecule from *Pithecellobium dulce*, modulates NFKB ligand RANKL signaling pathway in lipopolysaccharide induced proliferation of rheumatoid arthritis cell.

Study Design

A stepwise protocol was followed using standard methods to achieve the study objectives. *In silico* analysis screening various computational tools and databases. This was followed by phytochemical extraction, isolation, and identification of bioactive compounds. The study then progressed to an *in vitro* experimental design using LPS-induced SW982 cells to evaluate gene expression. Finally, the green synthesis of *Pithecellobium dulce* nanoparticles was characterized.



CHAPTER-IV
MATERIALS AND METHODS

4.1 Objective 1. *In silico* analysis

List of data bases tools/ software used *in silico* analysis

The present study investigated in an Intel core is 11th gen DELL Inspiron 15 nvidia laptop. The software and databases used for the current study.

1. Pubchem (Kim, S. et al. 2021).
2. PDB (Shunmuga Priya et al. 2021)
3. Swiss ADME/T (Daina et al. 2017)
4. H-Dock Server (Aldarhami et al. 2023)
5. Discovery Studio (Jejurikar et al. 2021)
6. Molecular Dynamic simulation by IMODs software (Santra et al. 2022)

4.2 Target protein preparation

Retrieval of the target protein from RCBS-PDB

(https://pubchem.ncbi.nlm.nih.gov/)

The NF-kB ligand RANKL signaling pathway protein MMP-1 selected for this study. The two-dimensional (2D) and three-dimensional (3D) crystal structures of targeted protein matrix metalloproteinase-1 (MMP-1) PDB ID-2CLT, and Nuclear Factor kappa-B ligand (NF-κB) proteins were retrieved from the Protein Data Bank (PDB) (Figure No. 4.2.1) (Arda et al. 2024). protein classification, and resolution were recorded. And the protein structure was then imported into Accelrys Discovery Studio, for further analysis removed non-receptor, water molecules and other components for. These structural data were used for molecular docking studies to evaluate the interaction of bioactive compounds from *Pithecellobium dulce* with targeted proteins.

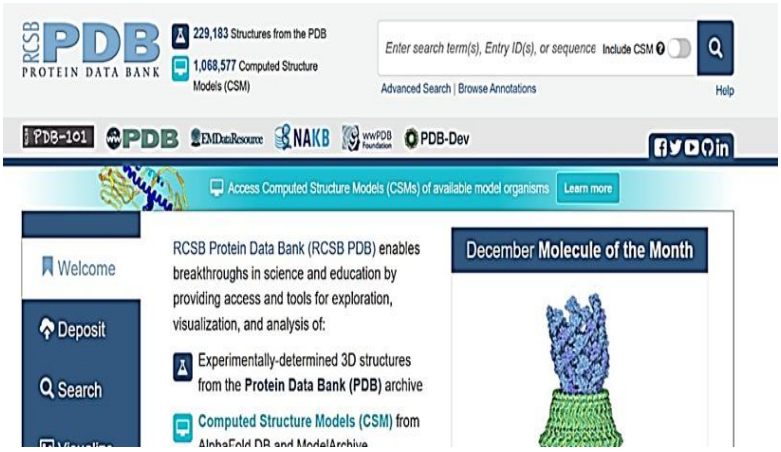


Figure No. 4.2.1 PDB web page (https://www.rcsb.org/)

4.3 Screening of biomolecules **Retrieval of the chemical structure of bioactive compounds from PubChem database.**

The bioactive compounds of *Pithecellobium dulce* fruits identified and selected from the literature review, for study (Kim, et al.2021). PubChem database used to retrieve the 2D and 3D structures of twenty-one (21) bioactive compounds of *Pithecellobium dulce* fruit. Additionally, details such as molecular formula, molecular weight, PubChem Compound ID (CID), compound class, and physicochemical properties (Figure No.4.3.1). For the screening drug likeness property canonical smiles were obtained with geometrical properties refinement by charged groups of hydrogen atoms. Ligands were prepared for further docking analysis

Figure 4.3.1 PubChem web page(https://pubchem.ncbi.nlm.nih.gov/)

4.4 Drug likeness property analysis

Swiss ADME/T (Absorption, Distribution, Metabolism, Excretion, and Toxicity)

The Swiss ADME/T tool evaluated the Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADME/T) (Aldarhami et al., 2023). Swiss ADME analysis evaluated the pharmacokinetic and drug-likeness properties of bioactive compounds derived from Pithecellobium dulce fruit. Key parameters assessed included Lipinski's Rule of Five, TPSA, gastrointestinal (GI) absorption was assessed the active molecule safer to oral availability, and blood-brain barrier (BBB) permeability assed to molecules. Visual predictive models such as the Bioavailability Radar and the BOILED-Egg model were utilized to estimate oral bioavailability and passive diffusion across biological membranes (Rathor et al. 2025). Swiss ADMET tool predicts machine based pharmacokinetic properties of molecules (Figure No. 4.4.1).

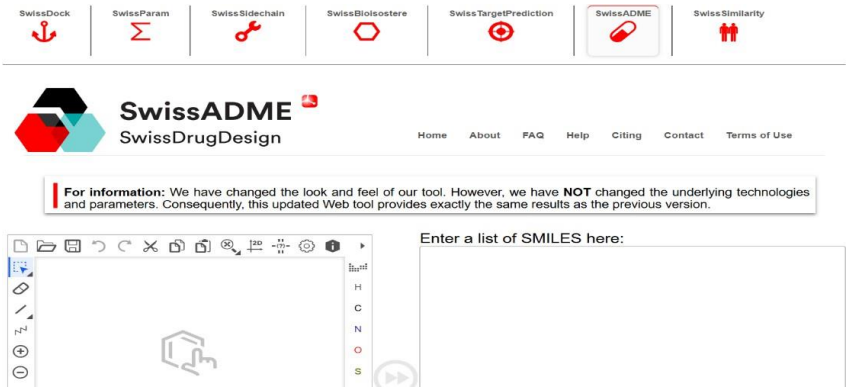


Figure 4.41 Swiss web page (http://www.swissadme.ch)

4.5 Molecular Docking and interaction study (H- DOCK software)

Molecular docking (http://hdock.phys.hust.edu.cn/) was performed to predict the interaction between selected bioactive compounds from *Pithecellobium dulce* fruit and the target protein Matrix Metalloproteinase-1 (MMP1). The docking analysis was carried out using the H-DOCK server, which provided information including binding energy (kcal/mol), confidence score, and ligand root mean square deviation (RMSD in Å). The 3D and 2D docked complexes were retrieved and visualized for interaction analysis. (Mousavi et al. 2024).

4.6 Molecular Dynamics (MD) simulation using iMODs software

iMODS database was used to perform Molecular dynamics simulation. The server (https://imods.iqf.csic.es/) to analyse the structural flexibility and stability of MMP1 protein complex with gallic acid and quercetin (Sumera et al. 2022). Normal mode analysis (NMA) was performed evaluated deformability, B-factor, and covariance of atomic motion with the protein MMP-1. These investigations were used to identify structural dynamic potential confirmational changes of MMP-1 (Figure No. 4.6.1).

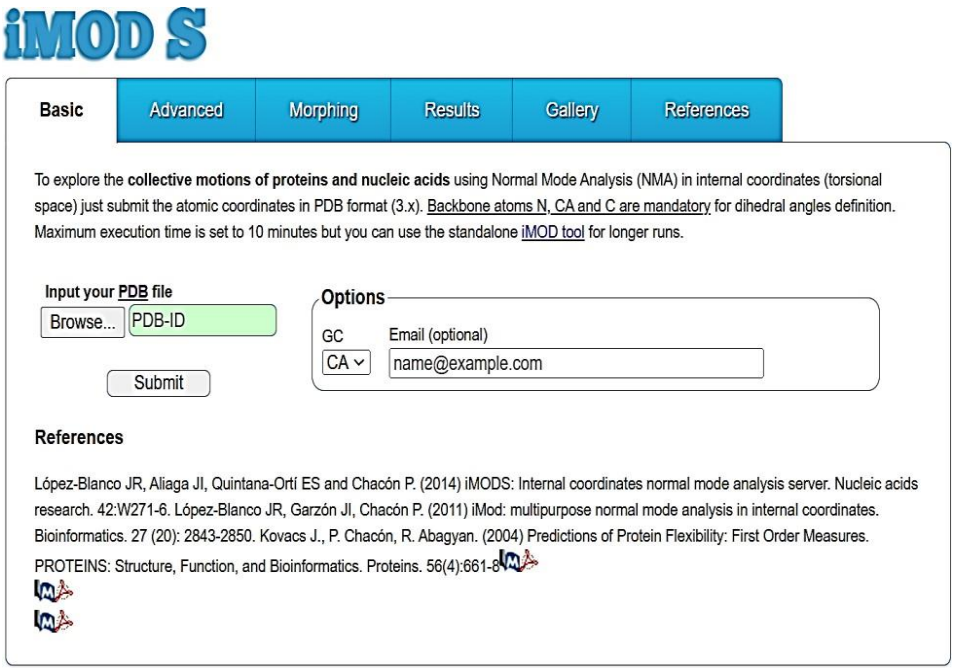


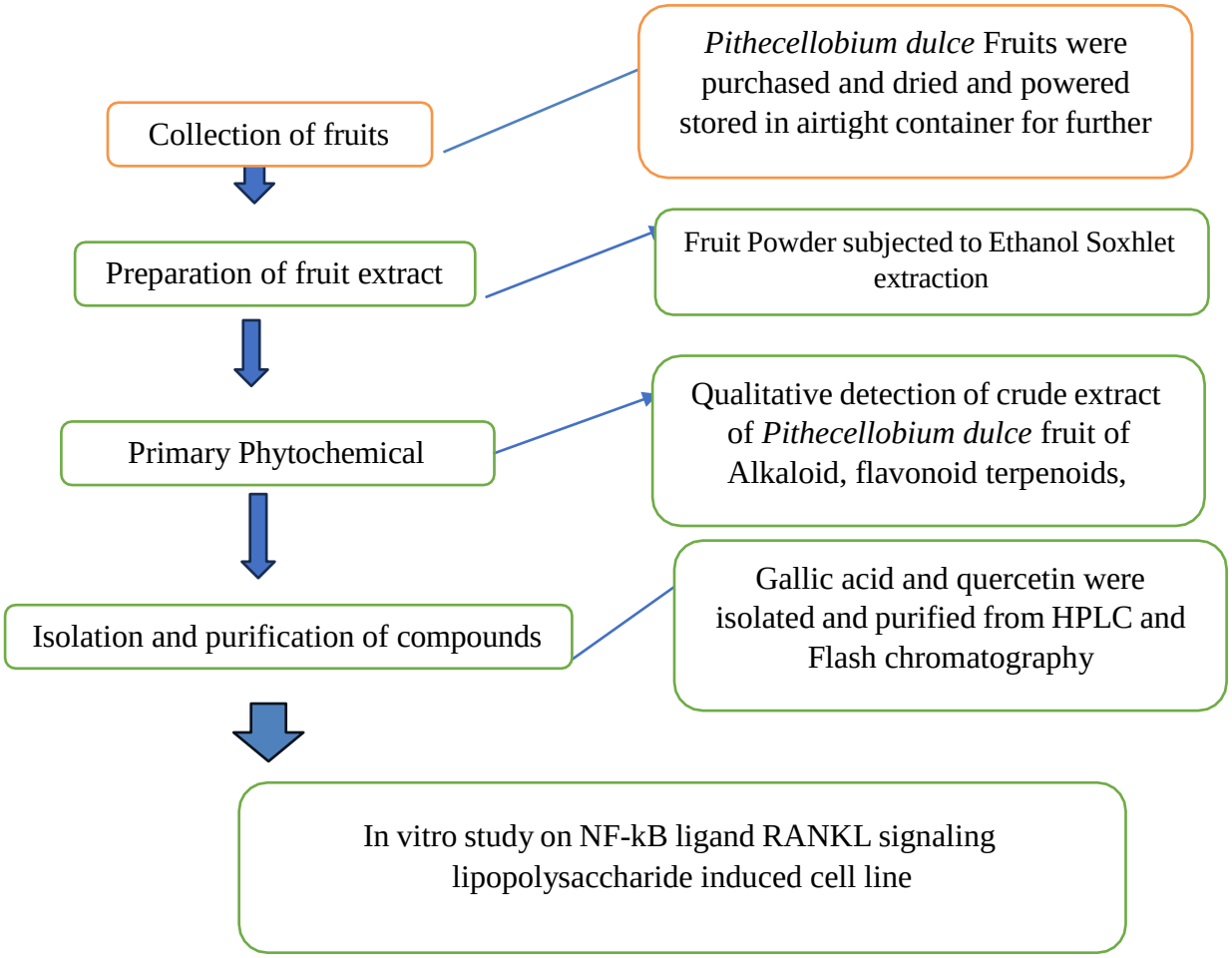
Figure 4.6.1 web page of iMODs (https://imods.iqf.csic.es/)

4.7 Discovery Visualizer

BIOVIA Discovery Studio, a software suite for molecular modeling, computational chemistry, and bioinformatics (https://discover.3ds.com/discovery-studio-visualizer-download (Jejurikar et al. 2021), was used to visualize the 2D and 3D structures of protein-ligand complexes. The software facilitated the identification of amino acid residues involved in ligand binding, generation of Ramachandran plots for structural validation, and prediction of protein structure (Pawar et al. 2021).

4.8 Objective 2 Phytochemical Extraction

Following protocol was used for Phytochemical extraction, identification and isolation of bioactive molecules from *Pithecellobium dulce* fruit.



4.9 Collection of *Pithecellobium dulce* fruit

Approximately 3 kg of *Pithecellobium dulce* fruits were collected from the local Vijayapura Market, Karnataka, India, during March–April. Authenticated by the Department of Dravya Guna, BLDE Association's AVS Ayurveda Mahavidyalaya, Vijayapura, Karnataka, India. The fruits were kept for drying at room temperature. And powered stored in airtight container (Figure No. 4.9.1a).

a. **b.**

Figure No. 4.9.1 a. *Pithecellobium dulce* fruit and b. Dried *Pithecellobium dulce* powder

BLDE Association's AVS Ayurveda Mahavidyalaya, Vijayapur

DEPARTMENT OF DRAVYAGUNA

Authentication of Herb Drugs

Dr.Shashidhar Naik Professor & Head Dr.Vidyalaxmi Pujari Asso.Professor

Ref. No: 12/25.

Date: 28.04.2025

Certificate

This is to certify that, Mrs Soumya Tungal, Ph.D Scholar, Department of Biochemistry, Allied Health Sciences, BLDE (Deemed to University), Vijayapur, working on Research project titled, "Modulation of NFkB Ligand RANKL Signaling Lipopolysaccharide-Induced Rheumatoid Arthritis Cell line by *Pithecellobium dulce*." She has given the raw drug samples for identification and authentication, and the given samples are identified as follows,

Sl no.	Latin name	Part
1.	*Pithecellobium dulce* Benth	Fruit

Authentication Officer

Authentification Officer
Department of Dravyaguna
BLDEA's AVS Ayurveda Mahavidyalaya
Vijayapur-586109.

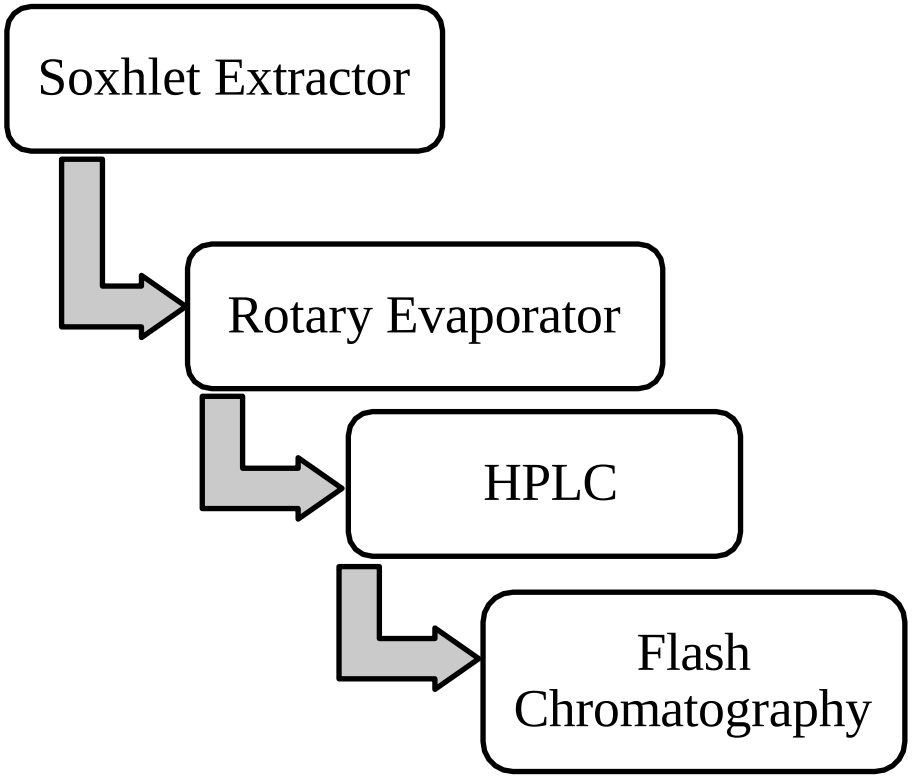
I/c
Head of the Department

Professor and HOD
Dept. of PG Studies in Dravyaguna
BLDEA's AVS Ayurveda Mahavidyalaya
Vijayapur.

Figure No 4.9.1 b Authentication letter

4.10 The following techniques used for the extraction, isolation and purification of *Pithecellobium dulce* fruit.

The *Pithecellobium dulce* dried fruits were subjected to ethanol extraction by using Soxhlet extraction, followed by rotary evaporator to separate solvent from sample, isolation and quantification of bioactive compounds done by high-performance liquid chromatography and purification was done using flash chromatography.



4.11 Soxhlet Extractor

Soxhlet extraction is the separation of bioactive compounds from various parts of plant material by using selective solvents through standard methods. The aim of the extraction is to separate plants soluble metabolites, while the insoluble plant material, like residue. Initial crude extract requires additional processing. There are several commonly used extraction techniques (Azwanida et al. 2015).

Soxhlet extraction is the one of the popular extraction methods because it uses low consummation of solvent. Environmentally friendly and cost-effective, time and energy saving (Vargas-Madriz et al. 2020, Srivastava et al 2021).

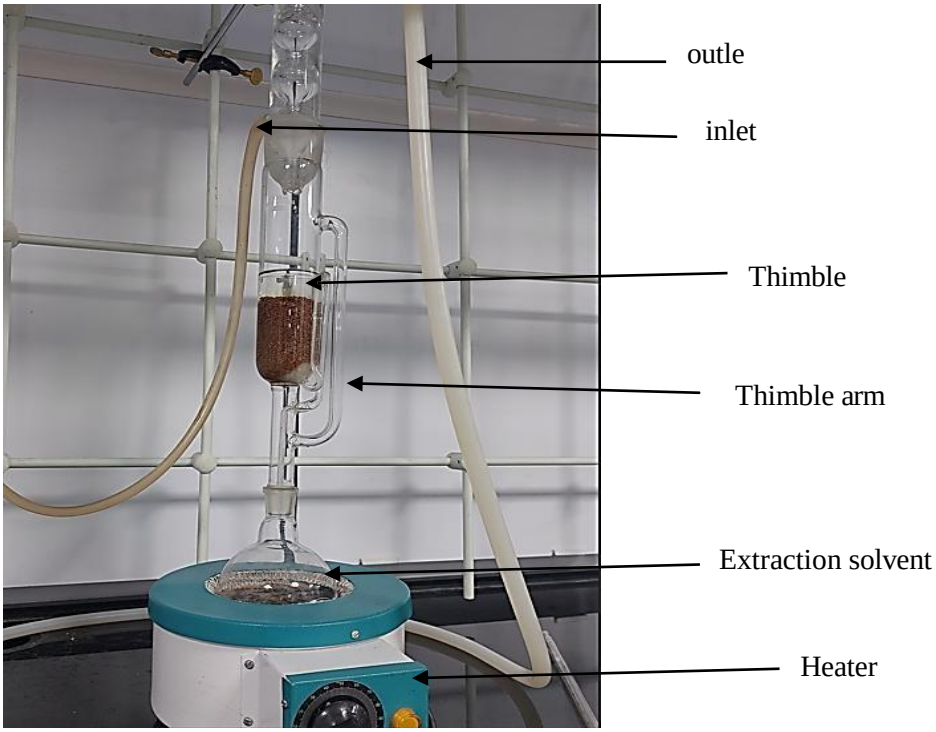


Figure No 4.11.1Soxhlet apparatus

The extraction used a Soxhlet extractor, a standard and simple handling method for processing plant materials. The apparatus is made of glass with a spherical bottom flask, an extraction chamber connected by a siphon tube, and a condenser connected to the extractor (Lavenburg et al. 2021).

100 grams of dried *Pithecellobium dulce* fruit powder taken, packed it in filter paper, and placed it in a thimble in the extraction chamber. About 500ml of ethanol

was poured into a 500ml round bottom flask. The apparatus was seated properly; solvent was heated by providing 70-80°C temperature the ethanol was evaporated and pass through the condenser. The condensed solvent dropped into the extraction chamber, where it came into contact with the plant material and reached the siphon tube; the solvent and extracted compounds flowed back into the round bottom flask. This cycle was repeated continuously at the same temperature ((Sindhu et al.2021). The process was conducted over 48 hrs, and approximately 20 cycles were processed until the extraction was complete and all soluble compounds were extracted from the plant material (Banu et al. 2015) (Figure No. 4.11.1).

4.12 Rotary evaporator

The crude sample separation was conducted using a rotary evaporator to separate ethanol from the extract under vacuum conditions. The vacuum system allowed ethanol to evaporate at a lower boiling point, which was then collected as the vapour condensed back into liquid form (Nortjie et al. 2022). This sensitive method focused on the concentration of the plant's importance without applying high temperatures (Figure No.4.12.1). The rotary evaporator operated at 50°C and 80 revolutions per minute (rpm) to separate the ethanol from the plant sample (Kumari et al. 2017).

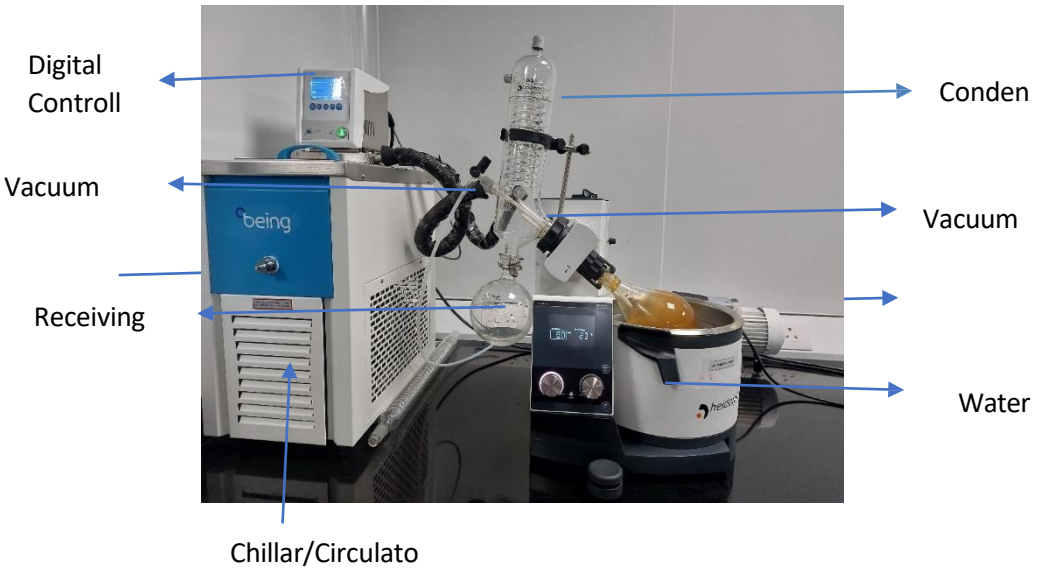


Figure No.4.12.1 Rotary evaporator

4.13 Phytochemical Screening of *Pithecellobium dulce* fruit extract

The preliminary phytochemical analysis was conducted using aqueous extract and ethanol extract utilised various qualitative tests to identify the primary components.

Table 4.13.1 Photochemical qualitative analysis of *Pithecellobium dulce* fruit

Name of the Test	Observation
Alkaloids: 0.4 g of plant extract was stirred with 8 ml of 1% HCl, and the mixture was warmed and filtered. 2 ml of filtrate was treated separately with (a) few drops of Mayer's reagent and (b) few drops of Dragendorff's Reagent	formation of yellow PPT
Flavonoids (Ferric chloride test): About 0.5g of each extract was boiled with 5 ml of distilled water and then filtered. To 2 ml of this filtrate, a few drops of 10% ferric chloride solution was added	Yellow, Orange or red
Tannins: The test sample of each extract was taken separately in water, warmed and filtered. To a small volume of this filtrate, a few drops of 5 % w/v solution of ferric chloride prepared in 90 % alcohol were added. Appearance of	Bluish-black or greenish-black ppt
Saponins:1 g of each extract was boiled with 5 ml of distilled water and filtered. To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 minutes	Frothing indicates presence of Saponins
Terpenoids -: Dissolve the plant extract in chloroform and add a few drops of conc. Sulfuric acid	Red, pink or purple

4.14 Identification and isolation of bioactive compounds from *Pithecellobium dulce* by High-Performance Liquid Chromatography (HPLC)

Quantification of bioactive compounds using High-Performance Liquid Chromatography (HPLC) is increasingly recognized as one of the most important analytical techniques for herb quality control and fingerprinting research (Banu KS et al.2015) Plants are primarily analysed for non-volatile chemicals such as higher terpenoids, various phenolics, alkaloids, lipids, and sugars (Nortjie et al. 2022).

HPLC works best for substances detectable in the ultraviolet or visible ranges of the spectrum. Natural products are typically separated after evaluating a relatively crude extract in biological experiments to accurately characterize the active component (Sasidharan S et al. 2011).

HPLC results are interpreted by examining chromatograms that display the chemical separation in a sample. This approach involves comparing the chromatogram of a standard to that of a plant extract to quantify gallic acid. In our study, we have used the "JASCO AUTOSAMPLER" instrument with a reversed-phase C18 column (silica powder as the stationary phase).

Analytical procedures were performed at 20 μL sample was injected, and measurements were taken at a wavelength of 280 nm. The mobile phase used was... solvents consisted of HPLC-grade methanol and water. This setup allowed for precise measurement of flow rate, retention time, and wavelength. The retention time (RT) for both the standard and the extract was 2.8 minutes. This indicates that the chemicals were detected based on their travel time across the chromatographic column, which facilitated their identification by comparison with the standard (Figure No. 4.14.1).

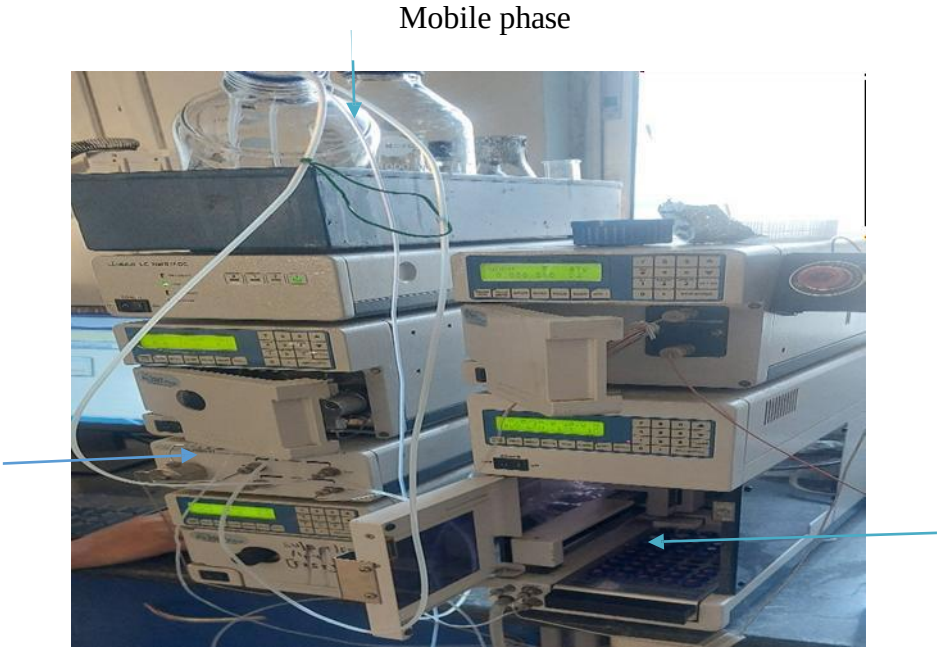


Figure No.4.14.1 Instrument: JASCO Autosampler

Instrumental details

Instrument JASCO AUTOSAMPLER.

Reversed-Phase Column- C18 (Silica powder stationary phase)

Solvent System- Methanol & Water (mobile phase)

Wave length – 280nm

Injection Volume - 20µl

4.15 Flash Chromatography

Flash chromatography combines medium and short-column chromatography with air pressure to achieve quick separation (De Silva GO et al. 2017). It is commonly used to separate molecular mixtures into distinct components, making it valuable for drug discovery and plant metabolite purification (Ingle KP et al. 2017, Fernando GSN). Flash chromatography improves on traditional methods in two key ways. First, it uses slightly smaller silica gel particles (250–400 mesh), which helps with better separation. However, since these finer particles slow down the flow of solvent, a gentle pressure (around 10–15 psi) is applied using gas to help push the solvent through the column more efficiently. This process results in fast and high-

resolution chromatography, commonly referred to as “flash” chromatography (Compton DL et al. 2020, Roge AB, et al. 2011, Agatonovic-Kustrin S et al. 2022)

Our study used the Combi Flash RF^{+} Lumen instrument to purify gallic acid and quercetin. The ethanol extract was used of solid silica powder and loaded into the flash chromatography column. A solvent mixture of methanol and water was used, and the wavelength was set to 280 nm to detect gallic acid and quercetin based on standard references. (Figure No. 4.15.1).

Elution occurred when the mobile phase was pushed through the column under pressure, causing the compounds to move at different speeds through the stationary phase, depending on their interaction with both phases. Less polar compounds moved faster, while more polar compounds moved slower. Fractions were collected at predetermined intervals as the compounds eluted from the column.

Figure No. 4.15.1 Combi Flash RF+ Lumen

Column and Conditions

Column: Silica 4g column.

Flow Rate: 18 ml/min.

Solvent System: Methanol-Water (mobile phase)."

Equilibration Volume: 33.6 ml.

Air Purge: 0.5 minutes.

Loading Type: Solid.

This technique we have applied for separation and purification of bioactive compounds from *Pithecellobium dulce* fruits, the crude extract and targeted compounds, gallic acid and quercetin were purified using different chromatographic techniques like HPLC and flash chromatography.

4.16 Extracted gallic acid purification and column condition.

4g of silica powder was used in column for purification of gallic acid. The mobile phase consists of solvents like methanol and water with flow rate 18ml/min. The system was equilibrated with 33.ml of solvent and crude extract mixed with silica powder and loaded in solid form.

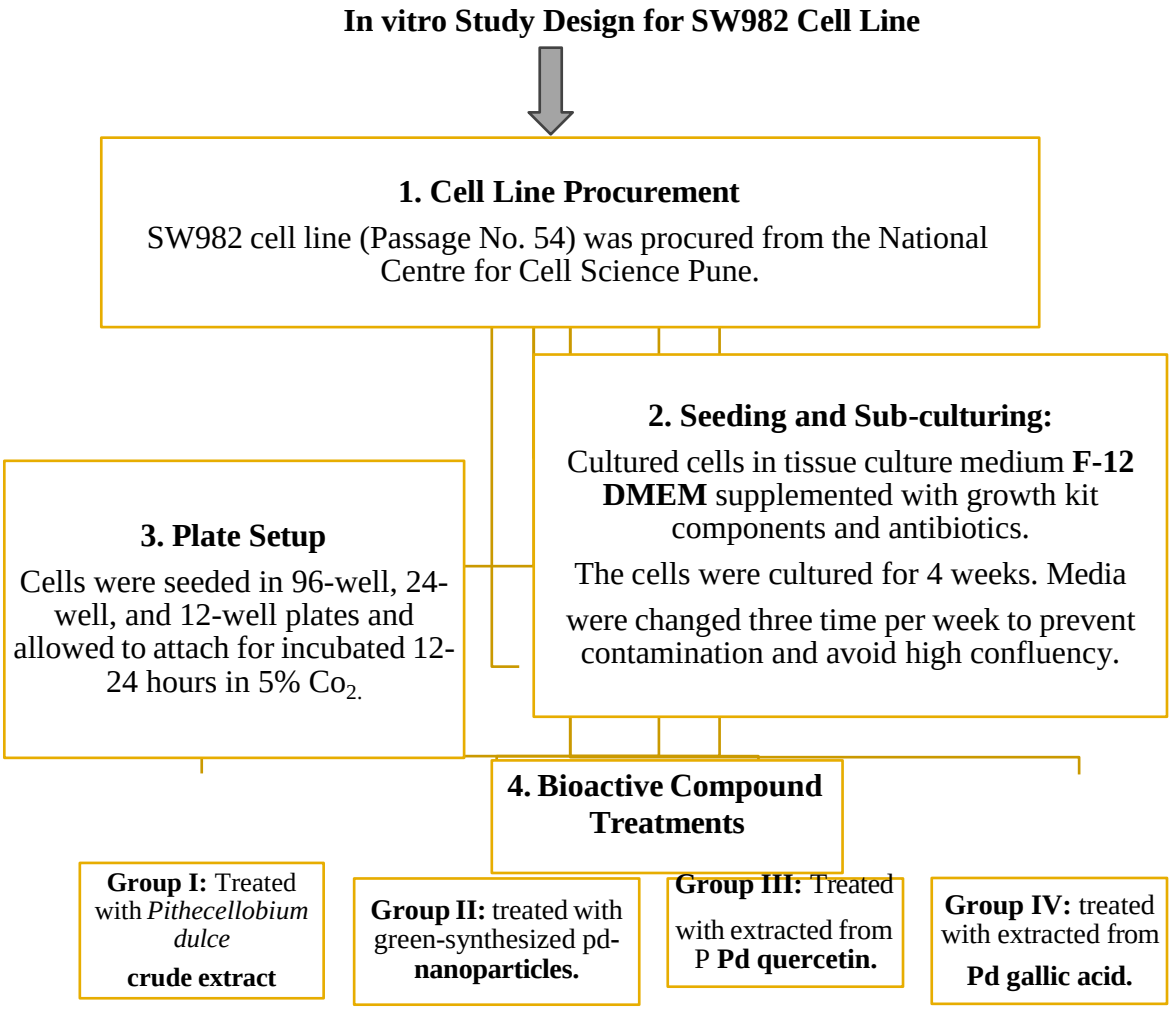
Analysis was conducted using dual wave length detection, 270nm (red) and 280nm (purple) with a peak width of 30 second a threshold of 0.2. AU. The air purge tie was at 0.5 Minutes. The chromatographic run was set to monitor a full wavelength range from 200-600 nm, suitable for phenolic compounds.

4.17 Extracted quercetin purification column condition.

12g of silica powder was used in column and processed, the mobile phase was made up of two solvents—hexane (solvent A) and ethyl acetate (solvent B) and was run through the system at a steady flow rate of 5 mL per minute. The system was equilibrated with 100.8mL of solvent, and the sample was loaded in solid form. Dual wave length detection was used at 254 nm (red) and 280 (purple), peak width 1 minutes, threshold 0.20 AU, air purge 0.5 minutes. The operated spectral range of 200-500 nm. Specific for flavonoid.

4.18 Objective 3. In vitro study design

To investigate the effect of isolated bioactive molecule from *Pithecellobium dulce* on NF-kB ligand RANKL mRNA expression in Rheumatoid arthritis cell line.

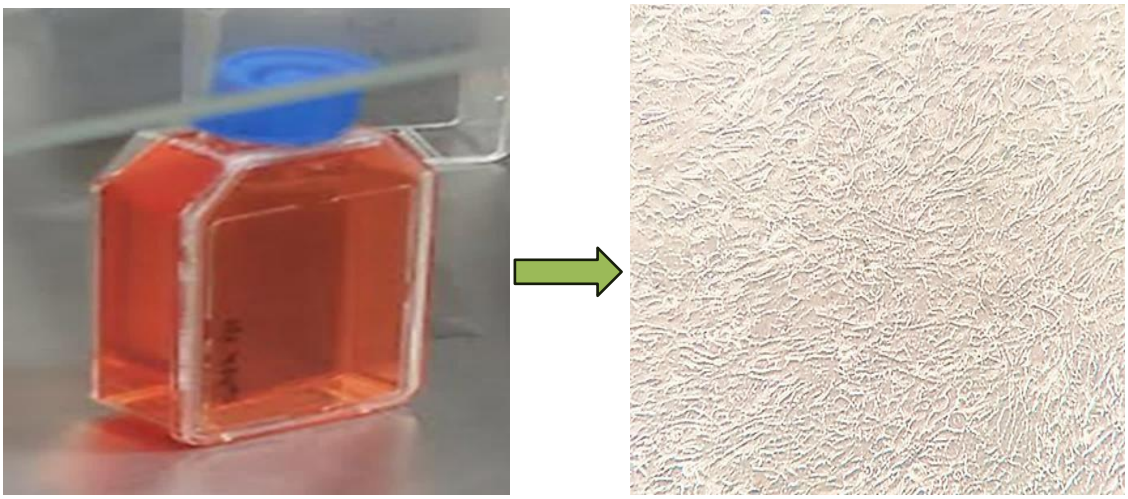


4.19. Gene expression analysis of MMP-1 was performed on treated cells using RT-PCR, following RNA extraction and cDNA synthesis, with GAPDH as the housekeeping gene.

4.19 Description of SW982 rheumatoid arthritis cell line

SW982 cell lines was purchased from NCCS Pune. and cultured in Dulbecco's Modified Eagle Medium F-12, tissue culture medium and supplemented with nutrients (Figure No. 4.19.1a, b).

4.19.a MH7A is a widely used alternative to SW982 for RA studies, offering stable growth and inflammatory response. Primary HFLS-RA cells provide higher relevance but are limited by variability and passages (Kawahito et al., 1999, Bresnihan et al., 2002) U-937 cells suit macrophage studies, while HC-RA and HOb-RA are used for cartilage and bone. However, SW982 was chosen for its ease of culture, consistent proliferation, and fibroblast-like characteristics relevant to RA synovial inflammation (Kupfer et al., 2011).



a b

Figure No 4.19.1 **a.** SW982 cell line **b.** microscopic view of cell confluency

4.20 Preparation of media for SW982 cell line culture

Components

- DMEM-F12: 44.5 mL
- Fetal Bovine Serum: 5 mL
- Antibiotic: 0.5 mL
- Storage: Prepared in a T-25 flask

Preparation of Bioactive Compounds for inducing inflammation in SW982 cell line using LPS

- *Pithecellobium dulce* Crude Extract: 10 mg in 1 mL Dimethyl Sulfoxide (DMSO)
- Nanoparticles (*Pithecellobium dulce*): 10 mg in 1 mL Dimethyl Sulfoxide (DMSO), Quercetin: 50–56 µM, Gallic Acid: 201 µM
- Lipopolysaccharide (LPS) Preparation: 5.8 mg in 1.5 mL Phosphate-Buffered Saline (PBS)-20 µL used for induction of inflammation.

4.21 Cell Culture and sub culture

The cultured SW982 cell line in DMEM/F12 medium, 10% fetal bovine serum and 1% antibiotics supplemented and Ethylenediaminetetraacetic Acid (Więcek, et al. 2022)

- Cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide to support optimal growth conditions.
- Seeded cells in 96-well and 24-well plates, allowed to adhere to the surface for 12–24 hours, and incubated under 5% CO_2.
- After incubation, cells were washed with PBS.
- Cells were treated with 1 mL of ethylenediaminetetraacetic acid trypsin-EDTA and incubated in a 5% CO_2 incubator until detachment was confirmed under a microscope.
- 1 mL of DMEM/F12 medium was added to the trypsinized cells to neutralize the enzyme.
- The cell suspension was centrifuged at 1500 rpm for 3 minutes.
- The resulting cell pellet was carefully collected and resuspended in fresh medium.
- 50 µL of trypan blue dye was mixed with 50 µL of the resuspended cell solution in a 1:1 ratio. The mixture was gently mixed and loaded onto a hemocytometer. Cell viability and total count were determined under a microscope.

4.22 MTT assay for cell viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay)

- Seeded 10,000 cells in the ninety-six (96) well plate and 24 hours incubated at 37°C with 5% CO_2 to allow the cells to adhere.
- Added 20 µL of lipopolysaccharide (LPS) to all wells in the 96-well plate to induce inflammation.
- Treated the cell with different concentration of bioactive compounds stock solution 10 mglmL by making groups Group I - crude extract, Group II- *Pithecellobium dulce* nanoparticle, group III- extracted quercetin, Group IV- extracted gallic acid.
- Added 50 µL of MTT reagent to each well. 3-4 hours incubate at 37°C in a 5% CO_2 incubator.
- Metabolically active cells reduce the yellow MTT dye into insoluble purple formazan via mitochondrial enzymes. The number of viable cells correlates with the intensity of the purple colour (Figure No. 4.22.1).
- Removed media carefully without disturbing formazan crystals.
- Added 150 µL DMSO to dissolve crystals.
- Absorbance was calculated by 570 nm with a microplate reader.
- Normalize readings to controls and estimate IC_{50}. Value (Ghasemi et al 2023).

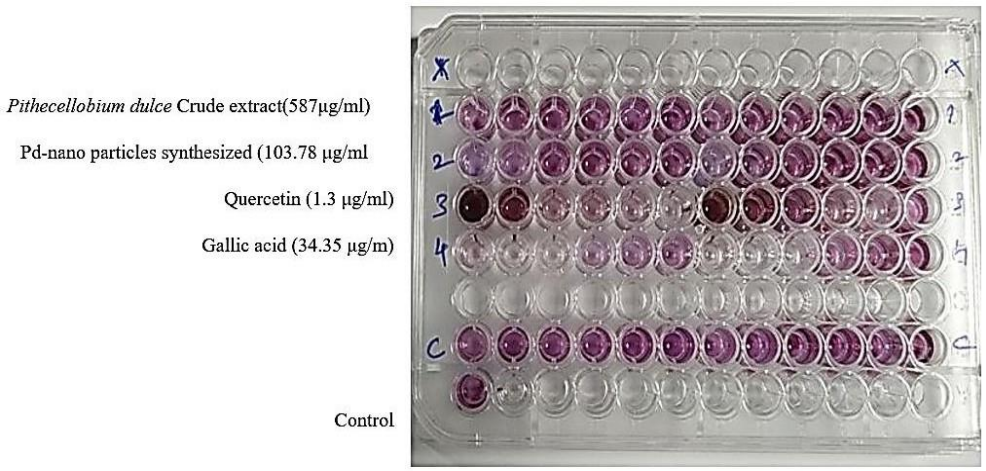


Figure No 4.22.1 MTT assay plate yellow colour to purple color corelate number of viable cells.

4.23 Gene expression study design

- RNA isolation
- RNA quantification
- RT-PCR
- Data analysis

4.24 Isolation of RNA by Trizol reagent

The following steps were used to isolate RNA from the culture SW982 cell line. Isolate the cells by centrifugation at 5000rpm for 5 minutes, after removed the culture media and add 55ml of trizol reagent to the pelleted cell. After homogenization of lysis in trizol sample can be stored at -70 ^{o}C for up to 1 month or store at 4^{0}C for a week and extract the RNA (Kim et al. 2024).

- To ensure complete dissociation of nucleo-protein complexes, incubation 5 minutes at room temperature.

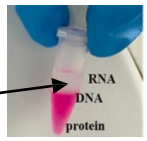
Add 250 µL of Trizol reagent to the sample tube (total volume: 750 µL). Vortex the sample vigorously for 2–5 minutes.

Phase Separation

Added 150 µL of chloroform. Shake thoroughly for 15 seconds and allow the sample incubation at 10 minutes. Centrifuge 15 minutes at 4 °C 10,000 rpm ,to separate into three phases..

RNA Precipitation

Carefully transfer the upper, colorless aqueous phase to a fresh tube. Add 750 µL of 2-propanol and mix gently. Let it stand at room temperature for 5–10 minutes. Centrifuge at 10,000 rpm for 10 minutes at 4 °C.



RNA Washing and Resuspension

750 µL of 75% ethanol wash the RNA pellet , centrifuge 5 minutes 4 °C at 8,000 rpm for, and supernatant discard . 5–10 minutes air-dry the pellet for and resuspend in 30 µL of nuclease-free water. Dissolve by gentle tapping and incubate at room temperature for 30 minutes.

StorageStore the RNA at –20 °C or –80 °C for long-term use.

4.25 RNA Quantification

RNA quantification was performed using a Biorad Nanodrop spectrophotometer. Sample purity was checked at absorbance 260/280nm. It is a fast and rapid method to quantify the purity of RNA.

4.26 cDNA Synthesis

Complementary DNA was synthesized using High-Capacity Reverse Transcription Kit (**Applied Biosystems, Cat. No. 4368814**). The master mix composition for each reaction is 10 µL and 10 µL of RNA sample (Table 4.26.1). The reaction was incubated in a thermal cycler with a temperature profile 25^{o}C for 10 minutes (primer annealing), 37^{o}C for 120 minutes (reverse transcription), 85 ^{o}C for 5 minutes (enzyme inactivation), followed by a hold 4^{o}C. Synthesized cDNA was stored at -20^{o}C for future use.

Table 4.26.1 Master mix composition cDNA

S. No	Composition	1 Reaction	12 Reaction
01	10X RT Buffer	2.0 µL	24 µL
02	25X dNTP Mix	0.8 µL	9.6 µL
03	10X RT Random primer	2.0 µL	24 µL
04	Reverse-transcription Enzyme	1.0 µL	12 µL
05	Nuclear-free water	4.2 µL	50 µL

Added 10 µL of the master mix and 10 µL of the isolated RNA sample were performed for the Polymerase chain reaction (PCR) followed with PCR thermal cycle setting

PCR thermal Cycle Settings

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	25^{0}C	37^{0}C	85^{0}C	4^{0}C
Time	10 Min.	120 Min.	5 Min.	Hold

4.27 Real –Time PCR (qRT PCR)

Gene Expression study

SYBR Green-based real-time PCR was used to measure the levels of gene expression. Prepared master mix composition each reaction contains 18 µL of master mix and 2µl of cDNA template following (Table 4.27.1) has shown details of composition with volume. And also, primers designed MMP-1 gene (F&R) with sequence 5’ to 3’, and housekeeping gene (F&R) with sequence 5’ to 3’. And thermal cycle set with different time interval and program was run and calculated the cycle threshold value (CT) (Maren et al. 2023).

Material and Reagents

Table 4.27.1 Master Mix composition qRTPCR

S. No	Composition	Volume Per reaction
01	SYBR Green (Thermo Fisher Scientific, Cat no. 4344463)	10 µL
02	Forward primer	1.0 µL
03	Reverse Primer	1.0 µL
05	Nuclear-free water	6.0 µL
06	cDNA templates	2.0 µL
Total volume		20 µL

Primer details: Order Details: Primer synthesis Order ID: 11400089667

Gene	**Primer**	**Sequence 5' --> 3'**	**Length MW/Ig/mol**
MMP1	F	AAAGGGAATAAGTACTGGGC	206239.12
	R	CAGTGTTTCCTCAGAAAGAG	216445.21
GAPDH	F	TGGTATCGTGGAAGGACTCAATGAC	247432.84
	R	ATGCCAGTGAGCTTCCCGTTCAGC	247304.72

Thermal Cycling setting

Steps	Temperature (°C)	Time	Cycles
Step1. Denaturation	95 °C	10 min.	1
Step2. Annealing	95 °C	15 Sec.	
Step3. Extension	60 °C	45 Sec.	42

4.28 Statistical analysis

- Statistical analysis for gene expression was conducted using SPSS version 27. Descriptive statistics, including mean and standard deviation (SD), were calculated along with 95% confidence intervals (CI). Non-parametric tests were employed to assess differences between groups, specifically the Mann-Whitney U test for two groups and the Kruskal-Wallis test for more than two groups. A p-value < 0.05 was considered statistically significant.

4.29 Synthesis of nano particles using *Pithecellobium dule* fruit extract and impact on rheumatoid arthritis cells.

Nanotechnology is gaining importance in the development of nanomaterials and nanoparticles. These are utilized in various fields, such as catalysis, electrochemistry, biomedicine, cosmetics, and pharmaceuticals.

Nanoparticles are with a diameter of less than 100 nm. The synthesis of transition metal nanoparticles using plant extracts is known as green synthesis. This method is simple, cost-effective, eco-friendly, and sustainable, using natural resources to reduce metal ions (Sepasgozar et al., 2021).

In this study, we attempted to synthesize nanoparticles using *Pithecellobium dulce* fruit extract. The following materials and protocols were used:

Materials

1. *Pithecellobium dulce* fruits (15g)
2. Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$, 0.1 M)
3. Distilled water
4. 1 M NaOH (2 g)
5. Whatman filter paper
6. Centrifuge tubes
7. pH indicator strips

4.30 Protocol for the nano particle synthesized from the *Pithecellobium dulce* fruit extract.

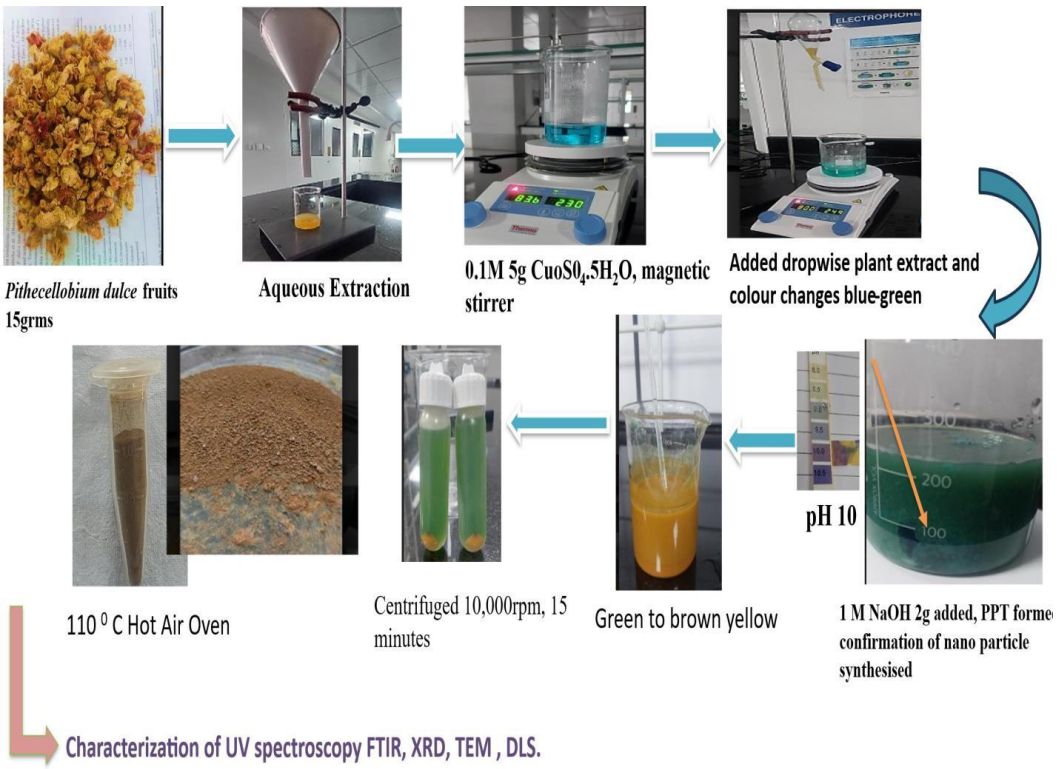


Figure: 4.30.1 *Pithecellobium dulce* fruit nanoparticle synthesis (CuSo4 .5H2O)

- 15 grams of dried *Pithecellobium dulce* fruits were taken, and an aqueous extract was prepared.
- 5 g of $CuSO_4 \cdot 5H_2O$ (0.1M) was dissolved in a beaker, and the solution was stirred using a magnetic stirrer at 80°C, along with the respective standard references.
- The aqueous fruit extract was added dropwise into the beaker containing the copper sulfate solution, and stirring was continued for 2 hours.
- After 2 hours, 2 g of NaOH (1M) was added as a capping agent while maintaining continuous stirring.
- The formation of a precipitate confirmed the synthesis of nanoparticles, with the pH reaching 10.
- A color change from green to yellow was observed after overnight incubation, followed by centrifugation at 10,000 rpm for 15 minutes.

- The residue was collected and dried in a hot air oven at 110°C to ensure complete moisture removal.
- The dried powder was sent for characterization - Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), X-ray Diffraction (XRD), UV-Visible Spectroscopy, and Fourier-Transform Infrared Spectroscopy (FTIR) (Shahid et al.2022, Amin et al. 2021, Ramasubbu et al. 2023).

  - Morphology analysis: **TEM (Transmission Electron Microscopy)**
    Revealed, particle size, shape and surface features.
    DLS (Dynamic light Scattering)
    Confirmed average particle size
  - Structural analysis: **XRD (X ray Diffraction)**
    Identified crystalline structure with Peaks.
  - Optical properties: **UV spectroscopy**
    Surface plasmon resonance.
    FTIR (Fourier Transform infrared spectroscopy)
    Verified functional groups.

- The synthesized Pd-Cu nanoparticles (Pd-CuNPs) were evaluated for their effect on MMP-1 mRNA expression in LPS-induced SW982 cells."

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CHAPTER-V
RESULTS AND DISCUSSION

Result

Bioinformatics databases and *in silico* approaches were performed to support the experimental findings by predicting the molecular behaviour and therapeutic potential of selected bioactive compounds from *Pithecellobium dulce* fruit.

The molecular data and chemical structures of the compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/), the corresponding protein structures were obtained from the RCSB Protein Data Bank (PDB) (https://www.rcsb.org/). SwissADME (http://www.swissadme.ch/) was used to assess drug-likeness and predict pharmacokinetic and toxicity profiles. HDOCK (http://hdock.phys.hust.edu.cn/) was carried out for molecular docking to evaluate the binding interactions between the compounds and target protein MMP-1 involved in the NF-κB ligand RANKL signaling pathway. Furthermore, iMODS (https://imods.iqf.csic.es/) was used to perform normal mode analysis, helping to assess the stability and flexibility of the protein–ligand complexes. The results presented below summarize the docking scores, pharmacokinetic profiles, and structural interactions of selected compounds with key target protein MMP-1 involved in NF-kB ligand RANKL- signaling, offering insights into their possible therapeutic role in rheumatoid arthritis.

5.1 Protein crystal structure retrieved from the protein data bank (PDB) data base

The NF-κB signaling pathway involves several proteins, which were identified through a literature review, including NF-kB (P50), NF-kB (P52), RelA (P65), RelB, IkBα, and MMP-1. Matrix metalloproteinase-1 (MMP-1) was selected as the target protein due to its reported anti-inflammatory properties.

The (Table No 5.1.1) represents the list of NF-kB proteins, of which were retrieved form the Protein Data Bank (PDB) **(**https://www.rcsb.org/)**.**

The NFkB1(P50) protein PDB- id 1SVC, resolution 2.60 Å, molecular weight 47.01 kDa, and Class Homo-sapiens, followed by NFkB2 (P52), PDB-id 1A3Q, resolution 2.10 Å, molecular weight 71.12 kDa, class- Homeo- sapiens. Rel A (P65) PDB-id 4kv4, resolution 2.50 Å, molecular weight 37.98 kDa, Class- Homo-sapiens. Rel B PDB-id 1ZKA, resolution 2.20 Å, molecular weight 12.32 kDa, and class Mus

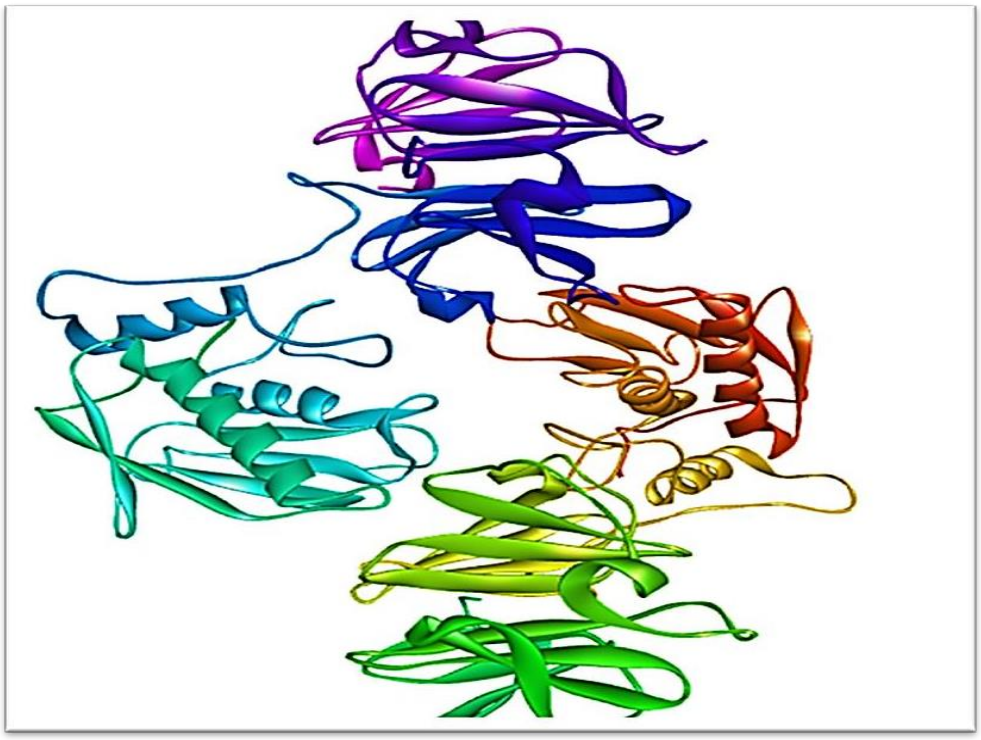
musculus. IkB α PDB-id 1NFI, resolution 2.70 Å, molecular weight 140.6 kDa and class- Homo-sapiens.

Targeted MMP-1 protein PDB-id 2CLT, resolution 2.67 Å, molecular weight 85.04 kDa, and class Homo-sapiens.

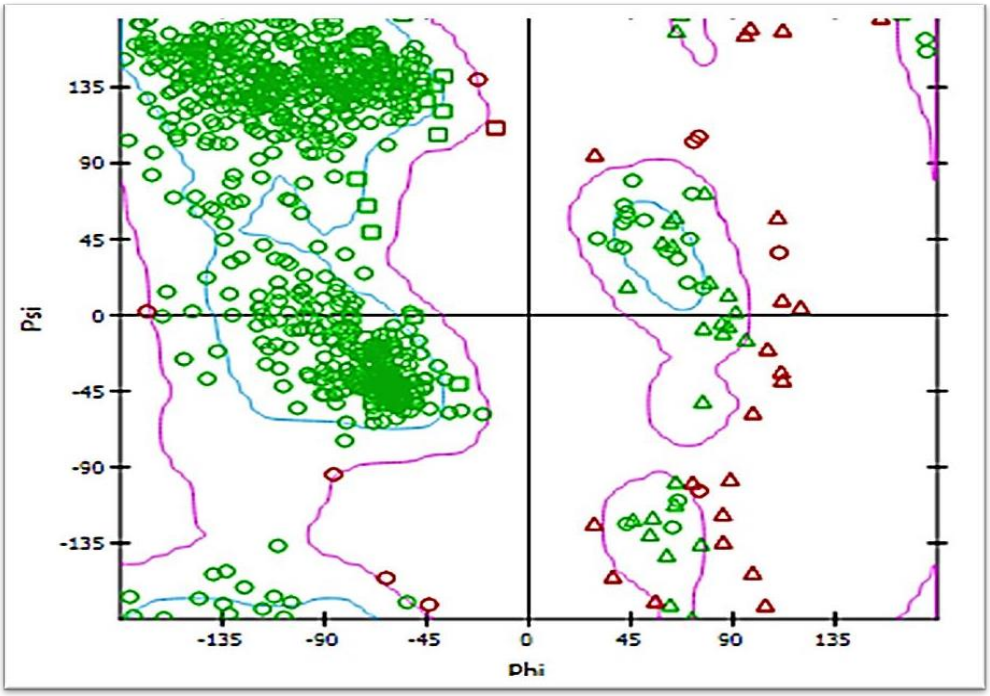
Table No 5.1.1. List of NF-kB pathway proteins

SI NO.	Name of the Proteins	Protein Data Bank- ID (PDB-ID)	Resolution In Å	Molecular weight (KDa)	Class
1	NFkB1(P50)	1SVC	2.60 Å	47.01 kDa	Homo-sapiens
2	NFkB2 (P52)	1A3Q	2.10 Å	71.12 kDa	Homo-sapiens
3	Rel A (P65)	4kv4	2.50 Å	37.98 kDa	Homo-sapiens
4	Rel B	1ZKA	2.20 Å	12.32 kDa	Mus musculus
5	IkB α	1NFI	2.70 Å	140.6 kDa	Homo-sapiens
6	MMP1	2CLT	2.67 Å	85.04 kDa	Homo-sapiens

3D crystal structure of the Matrix metalloproteinase 1 (MMP1) protein retrieved from the PDB database. Ramachandran plot analysis confirmed the presence of favourable amino acid conformations shown in the (Figure No. a & b 5.1.2). The protein structure was further processed for molecular docking analysis with the selected ligand.



a. Crystal structure of MMP1 Protein



b. Ramchandran plot

Figure No 5.1.2 **a.** Crystal structure of MMP1 protein, **b**. Ramchandran Plot Ramchandran plot represents X axis Phi and Y axis Psi, protein active potential structure with region that energetically stable (Green colour) favourable, inactive region (Red colour) unfavourable.

5.2. Screening of Bioactive compounds from *Pithecellobium dulce*

Retrieval of chemical structure of bioactive compounds from the PubChem data bases.

A total of 21 bioactive compounds from *Pithecellobium dulce* fruits were identified based on the literature review. These compounds were selected for their reported biological properties, including anti-inflammatory, anti-oxidant, antidiabetics, anti-cancer, and immunomodulatory properties.

Bioactive compounds details retrieved from the PubChem Data base.

The bioactive compounds diverse chemical classes and their properties retrieved from the PubChem database. For each compound, the PubChem ID, molecular formula, molecular weight, and chemical classes retrieved and tabulated (Table 5.2.2). According to literature molecular weight should be less than 500 (<500), and most of the compounds belongs to flavonoids, phenolic compounds and terpenoids and chemical structure of each compound retrieved from the PubChem database shown in the (Figure 5.2.1).

List of chemical structure retrieved from the PubChem database

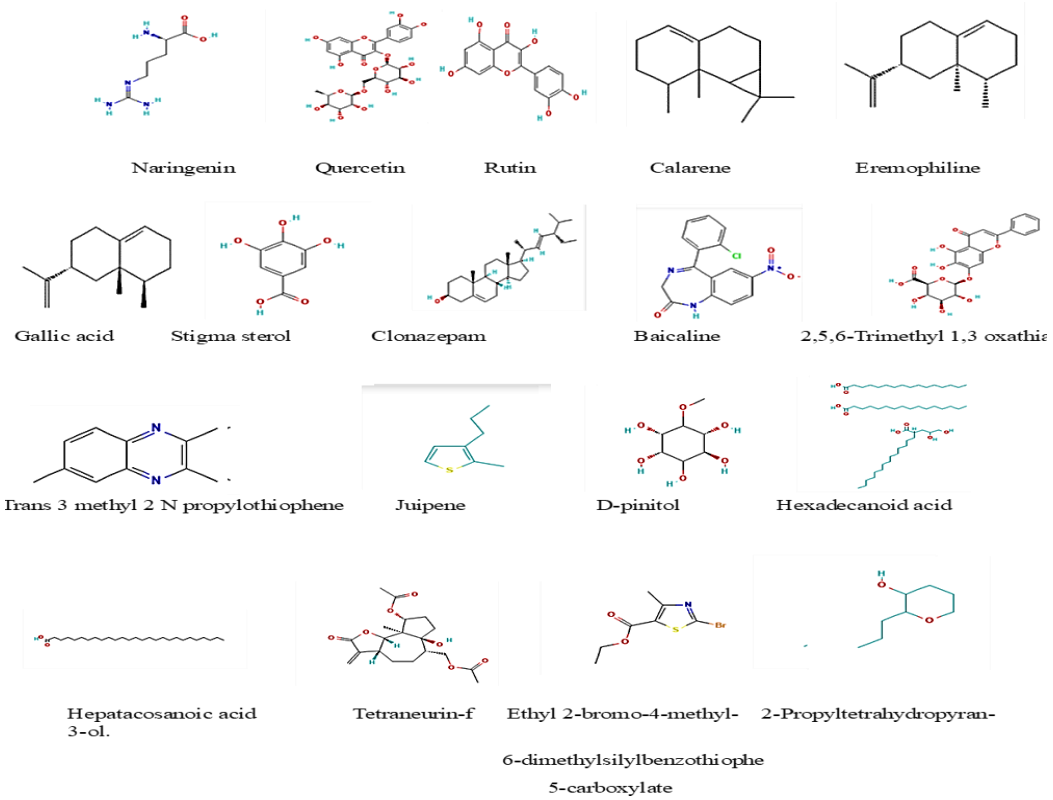


Figure 5.2.1Chemical structure of bioactive compounds.

Table 5.2.2 PubChem details of Bioactive compounds of *Pithecellobium dulce*

SI NO.	Name of the Compounds	PUB Chem ID	Molecular formula	Molecular Weight	Class
1.	Naringenin	932	$C_{15}H_{12}O_5$	272.25	Flavonoid
2.	Quercetin	5280343	$C_{15}H_{10}O_7$	302.23	Flavonoid
3.	Rutin	5280805	$C_{27}H_{30}O_{16}$	610.5	Flavonoid
4.	Gallic acid	370	$C_7H_6O_5$ o	170.12	Phenolic acid
5.	Stigmasterol	5280794	$C_{29}H_{48}O$	412.7	Phytosterol
6.	Clonazepam	2802	$C_{15}H_{10}ClN_3O_3$	315.71	Benzodiazepine
7.	Quinoline	7047	C_9H_7N	129.16	Heterocyclic Aromatic Compound
8.	Nootkatone	1268142	$C_{15}H_{22}O$	218.33	Sesquiterpene
9.	Juipene	1796220	$C_{15}H_{24}$	204.35	Sesquiterpene
10.	Calarene	28481	$C_{15}H_{24}$	204.35	Sesquiterpene
11.	Eremophiline	12309744	$C_{15}H_{24}$	204.35	Sesquiterpene
12.	Valencene	9855795	$C_{15}H_{24}$	204.35	Sesquiterpene
13.	Baicaline	64982	$C_{21}H_{18}O_{11}$	446.4	Flavonoid
14.	2,5,6Trimethyl1,3 oxathiane	548225	$C_7H_{14}OS$	146.25	Heterocyclic
15.	Trans 3 methyl, 2N-propylthiophane	6429953	$C_8H_{12}S$	140.25	Heterocyclic
16.	D-pinitol	164619	$C_7H_{14}O_6$	194.18	Cyclitol
17.	Hexadecanoid acid	87282186	$C_{51}H_{102}O_8$	843.3	Fatty acid
18.	Hepatacosanoic acid	23524	$C_{27}H_{54}O_2$	410.7	Saturated Fatty Acid
19.	Tetraneurin-f	101306773	$C_{19}H_{26}O_7$	366.4	Polyketide
20.	Ethyl2-bromo-4-methyl-6dimethylsilybenzothiophene-5-carboxylate	2824057	$C_7H_8BrNO_2S$	250.12	Organosilicon
21.	2-Propyl tetrahydropyran-3-ol	541755	$C_8H_{16}O_2$	144.21	Aliphatic

5.2 Swiss Absorption, Distribution, Metabolism, and Excretion (ADME/T) – Drug likeness properties analysis of bioactive compounds of *Pithecellobium dulce*

Swiss ADME/T analysis was performed for the bioactive compounds of *Pithecellobium dulce* using the canonical SMILES of the compounds retrieved from the PubChem database. All 21 bioactive compounds were subjected to ADME/T evaluation. Each compound was found to have less than 10 rotatable bonds, which showed within range. The total polar surface area (TPSA) for all compounds were also within the acceptable range of <140 Å^2.

Gallic acid and quercetin showed high gastrointestinal (GI) absorption but did not cross the blood-brain barrier (BBB). Similar observations were made for the other compounds, as detailed in (Table No. 5.2.1). Furthermore, all compounds obeyed Lipinski's Rule of Five, indicating good drug-likeness.

Table No. 5.2.1 ADME/T analysis of bioactive compounds of Pithecellobium dulce

SI NO.	Name of the compounds	No. rotatable bonds <10	TPSA <140 Å^2	Class	GI absorption	BBB penetration	Lipinski rule	violation
1.	Naringenin	1	86.99	Soluble	High	No	Yes	0
2.	Quercetin	1	131.36	Soluble	High	No	Yes	0
3.	Rutin	6	269.43	Soluble	Low	No	No	3
4.	Gallic acid	1	97.99	Very Soluble	High	No	Yes	0
5.	Stigmasterol	5	20.23	Pure soluble	Low	No	Yes	1
6.	Clonazepam	2	87.28	Soluble	High	No	Yes	0
7.	Quinoline	0	12.89	Soluble	High	High	Yes	0
8.	Nootkatone	1	17.07	Soluble	High	High	Yes	0
9.	Juipene	0	0.00	Moderately soluble	Low	No	Yes	1
10.	Calarene	0	0.00	Moderately soluble	Low	No	Yes	1
11.	Eremophiline	0	0.00	Moderately soluble	Low	No	Yes	1
12.	Valencene	0	0.00	Moderately soluble	Low	No	Yes	1
13.	Baicaline	5	176.12	Moderately soluble	Low	No	Yes	1
14.	2,5,6Trimethyl1,3 oxathiane	0	34.53	Soluble	High	High	yes	0
15.	Trans 3 methyl, 2N-propylthiophane	2	28.24	soluble	High	High	yes	0
16.	D-pinitol	0	110.38	Highly soluble	Low	No	yes	0
17.	Hexadecanoid acid	45	152.36	Insoluble	Low	No	No	2
18.	Hepatacosanoic acid	25	37.30	Poorly soluble	Low	NO	Yes	1
19.	Tetraneurin-f	5	99.13	Soluble	High	NO	yes	0
20.	Ethyl2-bromo-4-methyl-6dimethylsilybenzothiophene-5-carboxylate	3	67.43	Soluble	High	yes	Yes	0
21.	2- Propyl tetrahydropyran-3- ol	2	144.21	Very soluble	High	yes	Yes	0

5.3 Boiled egg data retrieved from the Swiss ADME/T data base to check GI absorption and BBB permeability.

The BOILED-Egg plot performed the gastrointestinal absorption and blood-brain barrier permeability of 21 candidate molecules based on their WLOGP and TPSA values. The analysis revealed that 18 out of 21 molecules were within the white region, indicating a high probability of passive gastrointestinal absorption. Among them, 11 molecules also fell within the yellow area, suggesting the potential to cross the blood-brain barrier (BBB) and investigate the central nervous system, as shown in (Figure No 5.3.1)

Additionally, gallic acid and quercetin were positioned within the white region (GL), confirming their high predicted gastrointestinal absorption. Notably, three molecules (Molecule 5, Molecule 13, and Molecule 16) were positioned outside the white region, implying suboptimal oral bioavailability.

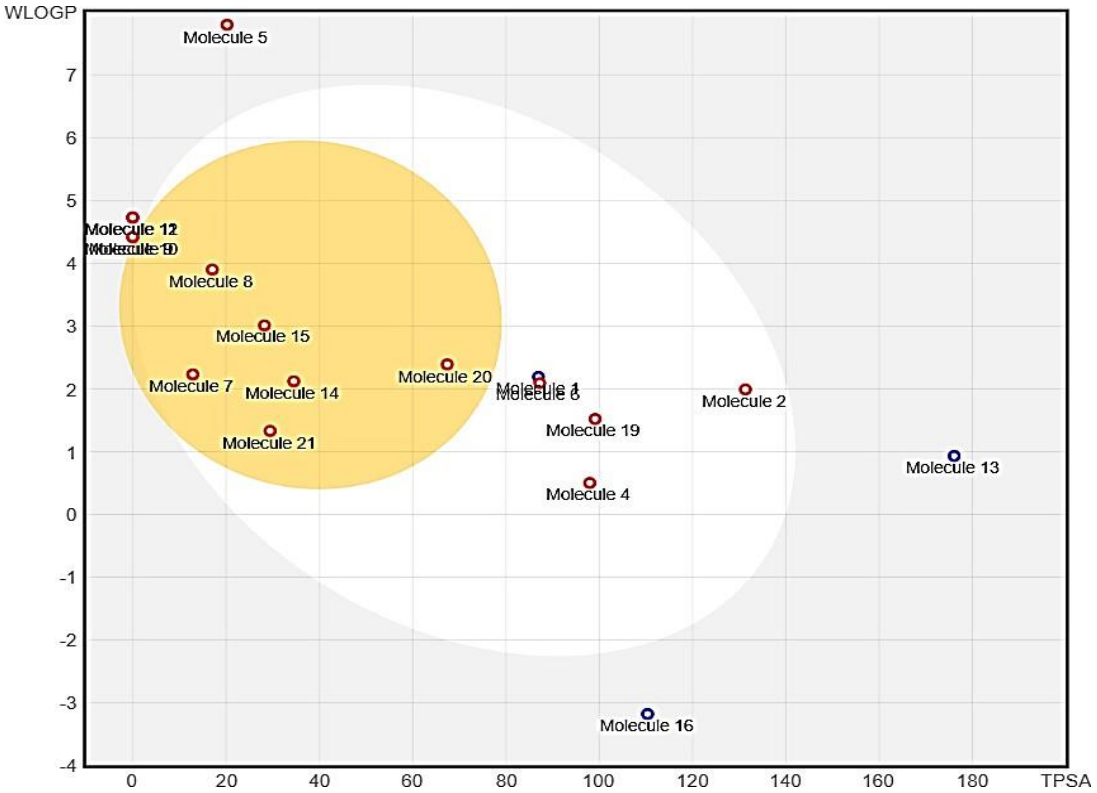
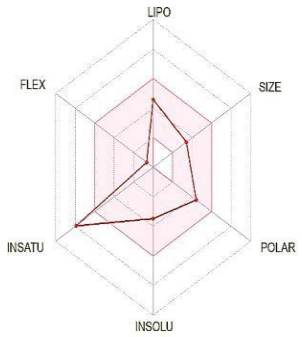


Figure No 5.3.1 Boiled egg data represent the White region: GI (Gastrointestinal absorption) Yellow region: BBB (Blood Brain Barrier Penetration)

5.4 Bioavailability Bio radar of Bioactive compounds form Pithecellobium dulce

Bio-radar is graphical presenting data which was retrieved from the Swiss ADME/T to show a compound's drug-likeness. The bio-radar graphic shows various pharmacokinetic and physicochemical properties, including lipophilicity, size, polarity, solubility, flexibility, and saturation, represented in the (Figure No 5.4.1). Compounds in the "pink area" of the radar appear to have favourable drug-like characteristics, indicating possible safety and efficacy.



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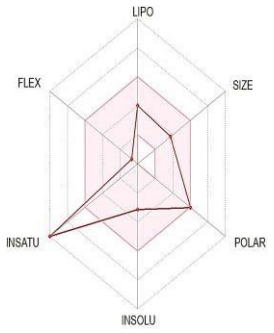

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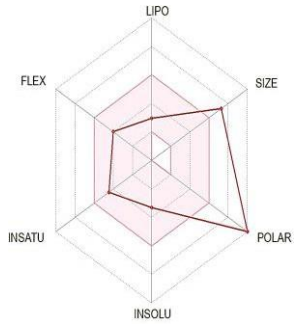

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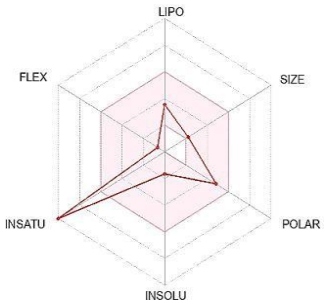

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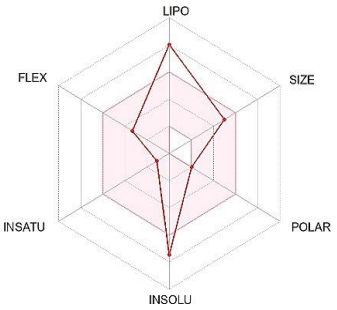

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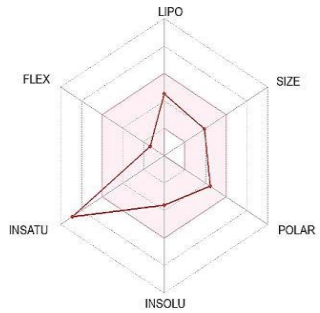

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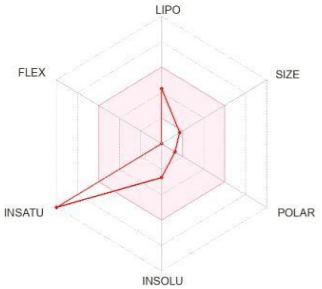

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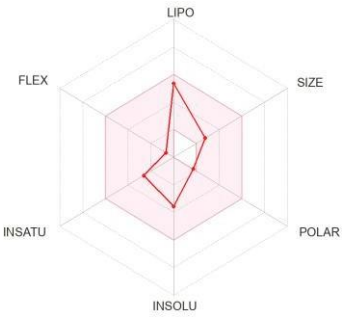

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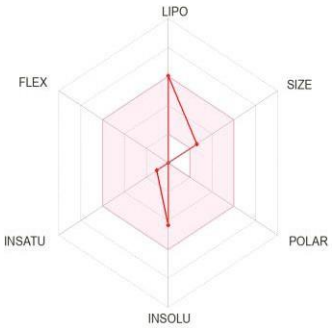

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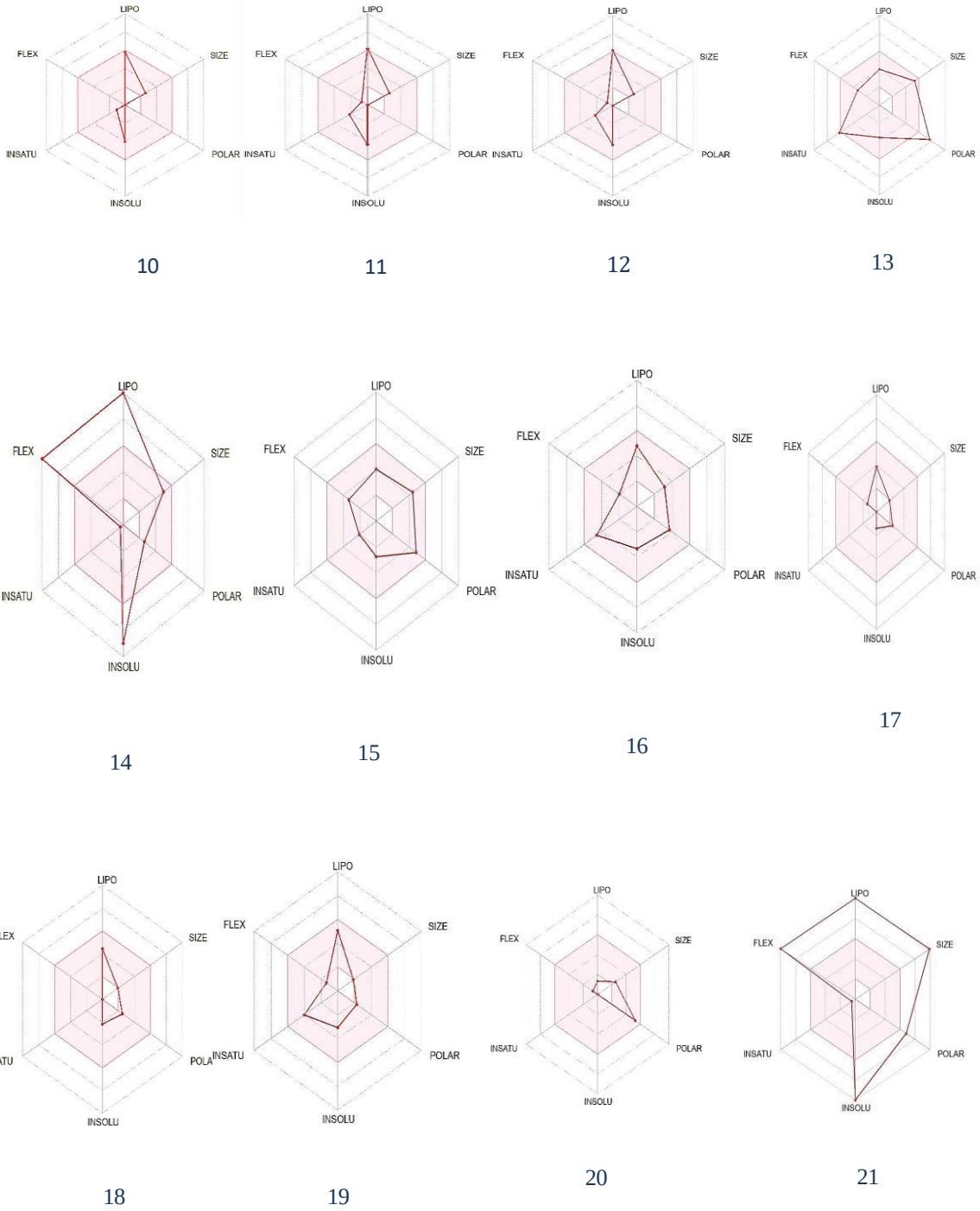


Figure No 5.4.1 *Pithecellobium dulce* fruits Bioavailability Radar (Bio radar) a. red area: This indicates the optimal range for each parameter. B. Blue polygon: Represents the properties of the molecule. 1.Naringenin, 2.Quercetin, 3. Rutin, 4. Gallic acid, 5. Stigma sterol, 6. Clonazepam, 7. Quinoline, 8. Nootkatone, 9. Juipene, 10. Calarene, 11. Eremophiline, 12. Valencene, 13. Baicaline, 14. 2,5,6-Trimethyl 1,3 oxathiane, 15. Trans 3 methyl, 2N-propylthiophane, 16. D-pinitol, 17. Hexadecanoid acid, 18. Hepatacosanoic acid, 19. Tetraneurin-f, 20. Ethyl 2-bromo-4-methyl-6-dimethylsilylbenzothiophe-5-carboxylate, 21. 2 Propyltetrahydropyran-3-ol.

A total of 21 bioactive compounds from *Pithecellobium dulce* fruits were selected based on a literature survey. All 21 compounds were subjected to ADME/T (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis. Among them, nine bioactive compounds met the pharmacokinetic criteria, including toxicity, Lipinski's rule, gastrointestinal absorption, blood-brain barrier permeability, and solubility.

Those compounds that did not meet these criteria were excluded from further analysis. The selected nine compounds were processed (Table No.5.5.1) for molecular docking and molecular dynamics simulation.

5.5 Molecular Docking analysis was performed by (H-Dock server)

Molecular docking was performed to investigate the interaction between selected bioactive compounds and targeted protein. Each compound was analysed for its binding energy (Kcal/mol), confidence score and ligand root mean square deviation in A^0 (RMSD).

The 3D and 2D docked structured retrieved from the H-DOCK server shown in the (Figure No. 5.5.2). Among the tested compounds gallic acid and quercetin showed favourable binding energy, and required less energy for stable interaction.

Gallic acid interacted with 12 amino acids residues of Matrix metalloproteinase-1 (MMP1), showed binding energy -9.10 Kcal/Mol, confidence score 0.511 and ligand RMSD 53.11A^0.

Quercetin interacted with 11 amino acids residues, binding energy -7.52 Kcal/Mol, confidence score 0.313 and ligand RMSD 27.37 A^0 (Table No. 5.5.1).

Table No 5.5.1 Docking analysis of MMP1 with Bioactive compounds from *Pithecellobium dulce*

SI. No	Name of the compounds	MMP1		
		Binding energy (kcal/mol)	**Confidence Score**	**Ligand Rmsd A^0**
1	Quercetin	-7.52	0.511	53.11
2	Gallic acid	-9.10	0.313	27.37
3	Quinoline	-8.43	0.212	82.68
4	Nootkatone	-8.17	0.203	53.94
5	2,5,6-Trimethyl1,3 oxathiane	-6.46	0.153	62.69
6	Trans 3 methyl, 2N-propylthiophane	-1.08	0.303	67.59
7	Tetraneurin-f	-9.53	0.251	54.76
8	Ethyl 2-bromo-4-methyl-6-dimethylsilylbenzothiophe 5-carboxylate	-6.17	0.146	55.01
9	2-Propyltetrahydropyran-3-ol.	-1.36	0.43	54.84

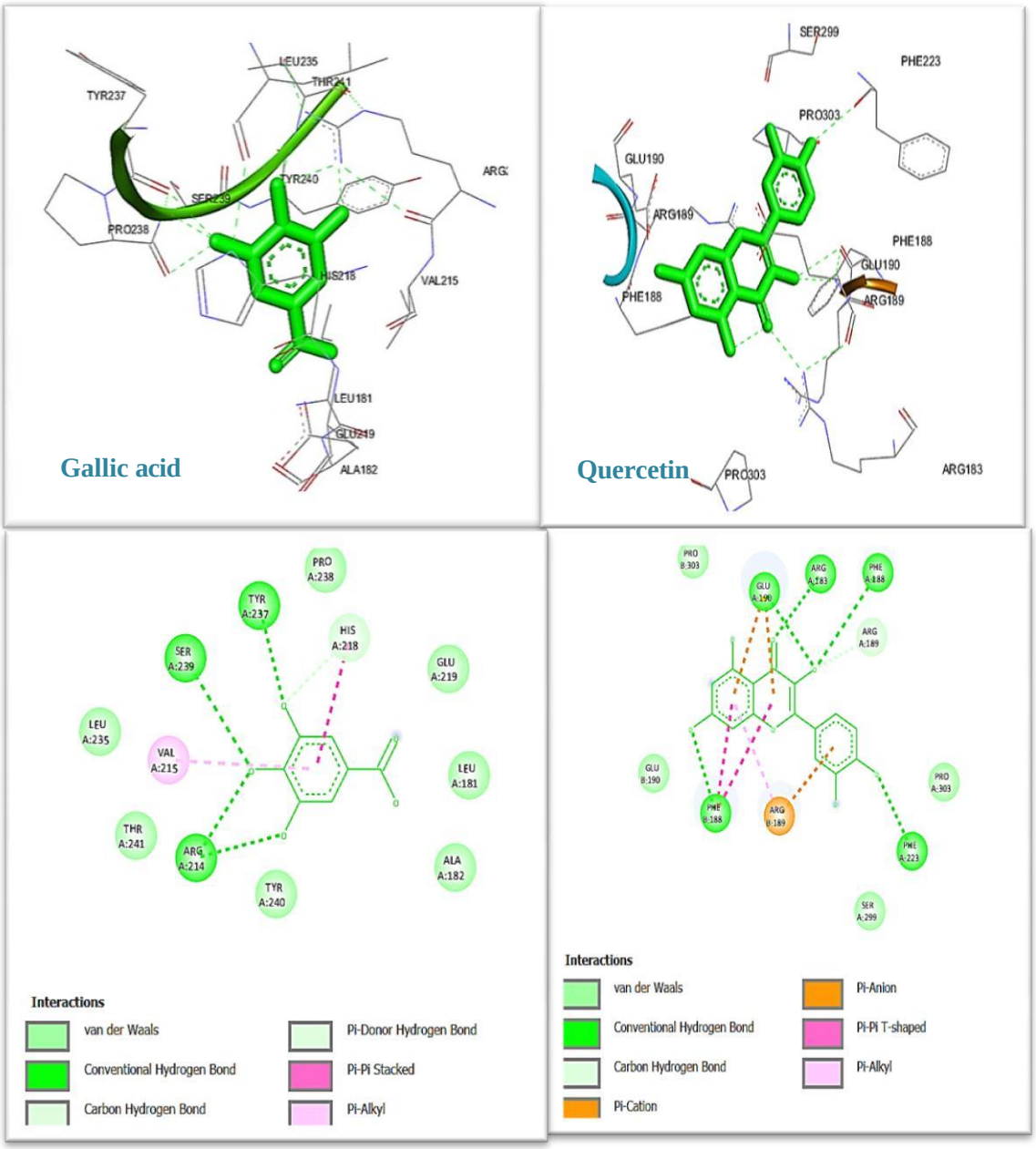


Figure No 5.5.2 Docked image of gallic acid, quercetin and 2D structures.

Based on the molecular docking results, molecular dynamics simulations were carried out to evaluate the protein-ligand complexes' stability, flexibility, and dynamic behaviour.

5.6 Molecular dynamic simulation (MD simulation) by iMODs

The molecular dynamics simulation was performed using the iMODS free software. The simulation provided deformability, B-factor, Normal mode analysis and residue motion correlation through covariance analysis.

(I) Deformability analysis

The MMP1-Gallic acid complex with ID 0105195501532 shows deformability along the residue index, where the X- axis represents the atom index or residue position in the molecule, and the Y- axis represents deformability indicating the flexibility and mobility of each residue. The multiple high peaks across the residue index, suggest potential hinge regions and areas of high flexibility, Lower region indicate rigid, less mobile areas.

The analysis helps identify structurally important regions related to molecular function, binding, and protein dynamics graphically shown in the (figure 5.6.1a. and figure5.6.1.c). MMP1-Quercetin complex with ID 0406095852785 similarly exhibited deformability, notably the peaks were X-axis atom index, Y-axis deformability, peaks indicate high deformability regions identifying potential hinge points. Molecular flexibility and structural dynamics represented graphically in the (Figure No 5.6.1 b.and Figure No 5.6.1.d).

(II) B-factor analysis

The B-factor graph provides normal mode analysis (NMA) and protein data bank (PDB) information. MMP1- Gallic acid NMA B-factor showed frequent fluctuations with peaks with high flexibility. And PDB value was observed indicating differences in dynamic behaviour prediction. MMP1- Quercetin complex the B-factor peaks more flexibility than the gallic acid. In the central and C-terminal regions. The suggests that quercetin may induce more flexibility.

(III) Covariance Matrix analysis

The covariance graph showed movement of residue. MMP1-Gallic acid presented a mixed pattern of correlation and anti-correlation, pattern indicating a balanced and targeted interaction between the ligand and protein domains. The constant internal mobility inside domain is shown by a few localised red clusters along the diagonal. In MMP1- Quercetin showed red clusters, indicated a wider and

more consistent motion across residues. In both complexes correlated motions, residues moving in the same direction, where red correlated motions (residues move together), white uncorrelated motions. Blue anti-correlated motions (residues move in opposite directions), diagonal line represents self-correlation of residues.

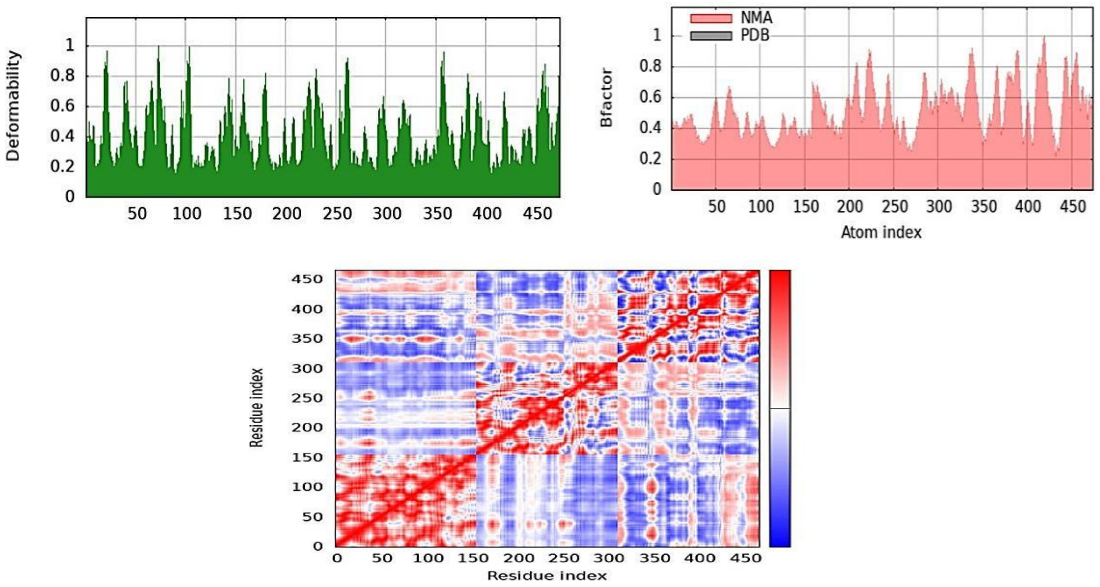


Figure No 5.6.1 a. Molecular dynamics simulations in iMODS for MMP1 with gallic acid deformability, B-factor plot and covariance map.

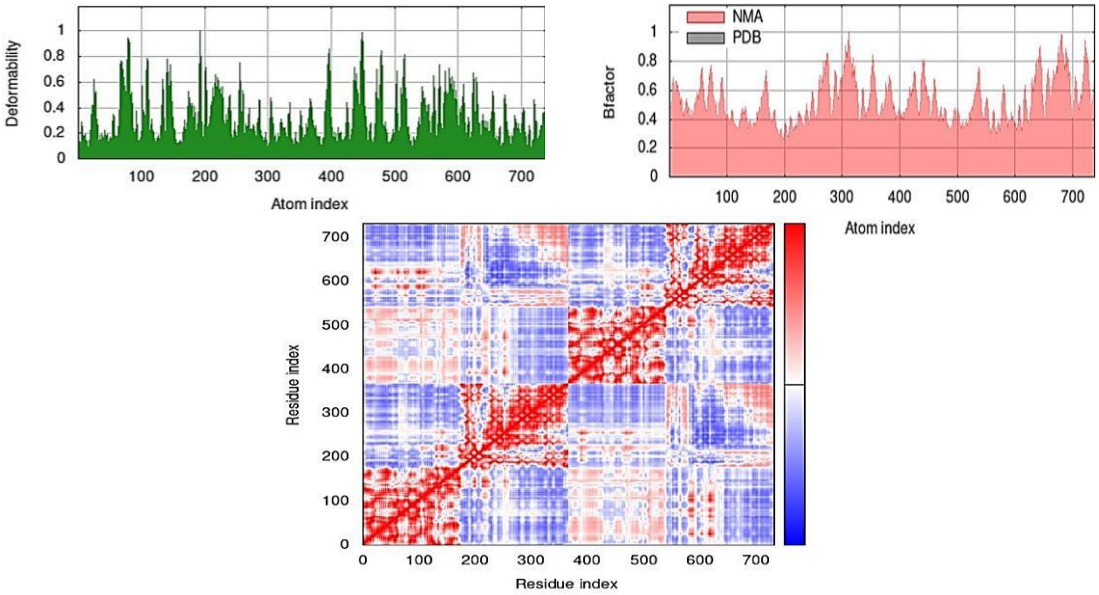


Figure No 5.6.1 b. Molecular dynamics simulations in iMODS for MMP1 with quercetin deformability, B-factor plot and covariance map

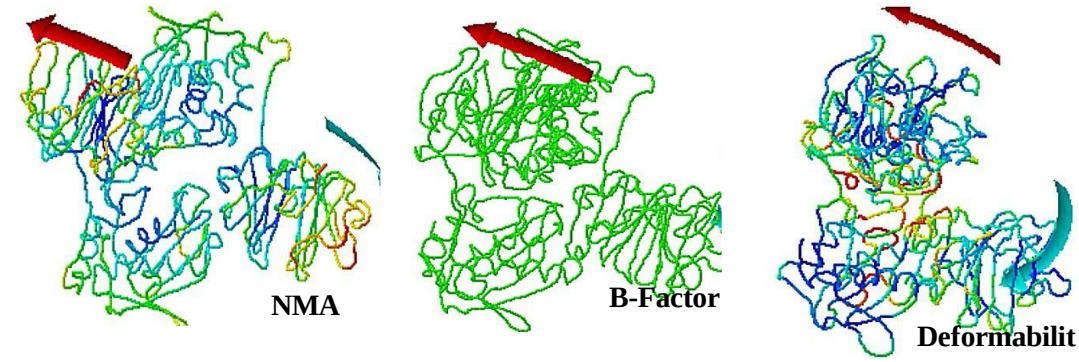


Figure No 5.6.1 c. Molecular mobility evaluated by NMA of the docked complexes: MMP1 with Gallic acid

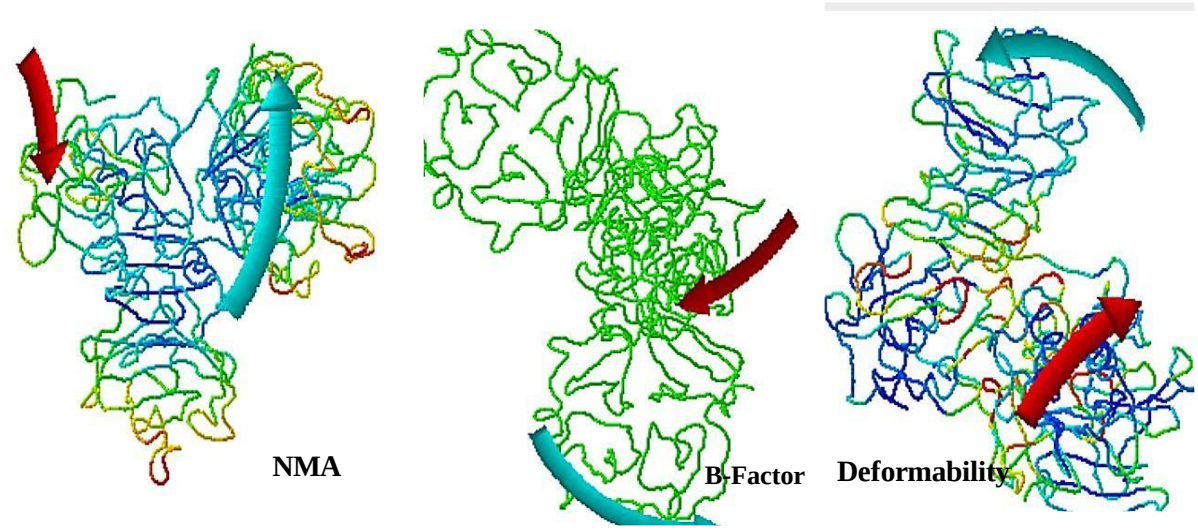


Figure No 5.6.1.d. Molecular mobility evaluated by NMA of the docked complexes: MMP1 with Quercetin

5.7 Phytochemical Extraction result

Phytochemical extraction, Identification and isolation of bioactive molecules from *Pithecellobium dulce* fruits.

a. Soxhlet extraction and rotary evaporator yield

Soxhlet extraction was carried out using ethanol as a solvent to isolate compounds from *Pithecellobium dulce* fruits. This method effectively extracted ethanol-soluble compounds and those mixed with insoluble substances. From 100 grams of fruit material, around 31% of the sample was successfully extracted.

After extraction, the ethanol was evaporated using a rotary evaporator, obtained brown-coloured liquid extract that amounted to roughly 31% of the original sample. The extract was then stored at 4°C to preserve its components for later phytochemical analysis.

b. Phytochemical Screening

The qualitative analysis of phytochemicals from *Pithecellobium dulce* fruits extracts confirmed the presence of various bioactive compounds as shown in the (Table No 5.7.1). The analysis reaction was recorded as absent (-), present (+), moderately present (++), or highly present (+++).

The tannins found in the both aqueous and ethanol extract confirmed solubility in polar solvents. Alkaloids were moderately present in the aqueous extract (++) but less in ethanol (+). Flavonoids moderate (++) in both, soluble in the polar and semi polar solvents. Saponins found in the ethanol extract (+), indicating ethanol extraction is suitable. Terpenoids were moderately present (++) in the both equally soluble. Phenolic compounds present in the high (+++) in the both extractions indicated solubility in polar solvents.

These results confirmed that the *Pithecellobium dulce* extract contained multiple classes of phytochemicals, with ethanol extraction shown a broader application for extract compound.

Table No 5.7.1 Phytochemical qualitative analysis of Pithecellobium dulce fruit

SI No.	Test	Aqueous	Ethanol
1	Tannins	+	+
2	Alkaloids	++	+
3	Flavonoids	++	++
4	Saponins	-	+
5	Terpenoids	++	++
6	Phenols	+++	+++

Note: High +, Highest +++, Note present -

5.8 Isolation and Identification of bioactive compounds by High-Performance Liquid Chromatography (HPLC) Analysis

The HPLC analysis was performed to identify and quantify the presence of phytochemicals in the *Pithecellobium dulce* fruit extract, analysis was carried out focusing on two major bioactive compounds like gallic acid and quercetin.

(I) Quantification of gallic acid from *Pithecellobium dulce* fruit

Standard gallic acid solution was prepared in different concentration from 12.5 µg/mL to 200 µg/mL. The chromatogram showed presence of gallic acid confirmed for the standard at about 2.8 minutes retention time shown in the (Table No 5.8.1). A calibration curve was plotted based on the peak area versus concentration showing a strong liner relationship with an R^2 value 0.9924.

Analysis of the plant extract showed peaks (Figure 5.8.2), at a retention time of 2.7 minutes, confirmed presence of gallic acid. The peak area was recorded as 211061 µV. Sec, around 34.28 µg/mL (Table 5.8.1a). Concentration was obtained based on the calibration curve (Figure No 5.8.3).

Table No 5.8.1 Quantification of standard gallic acid by HPLC

SI NO.	Gallic acid conc. in (µg ml)	Intensity (Area µV.sec)	Retention time (tR) minutes
1	12.5	13997	2.8 min
2	25	105209	
3	50	346113	
4	100	859005	
5	200	1714707	

Table No 5.8.1 a. Quantification of extracted gallic acid from *Pithecellobium ducle*

SI NO.	Retention time(tR) minutes	Intensity (Area µV.sec)	Quantity Conc.
1	2.7 min	211061	**34.28(ug/ml)**

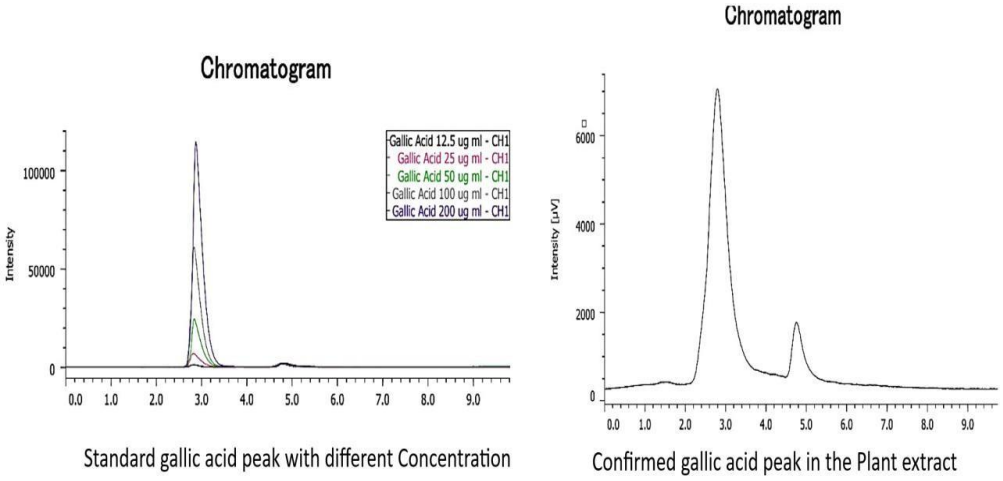


Figure No 5.8.2. Chromatogram Peaks of Pd extracted gallic acid

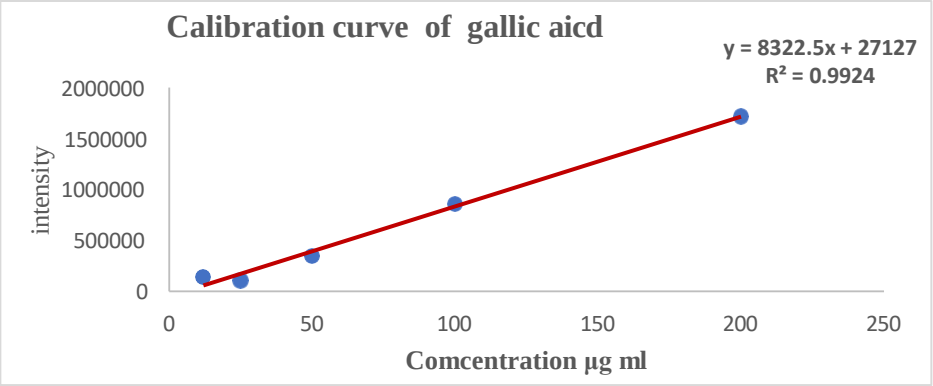


Figure No 5.8.3 Calibration curve of Pd extracted gallic acid

(II) Quantification of quercetin from *Pithecellobium dulce*

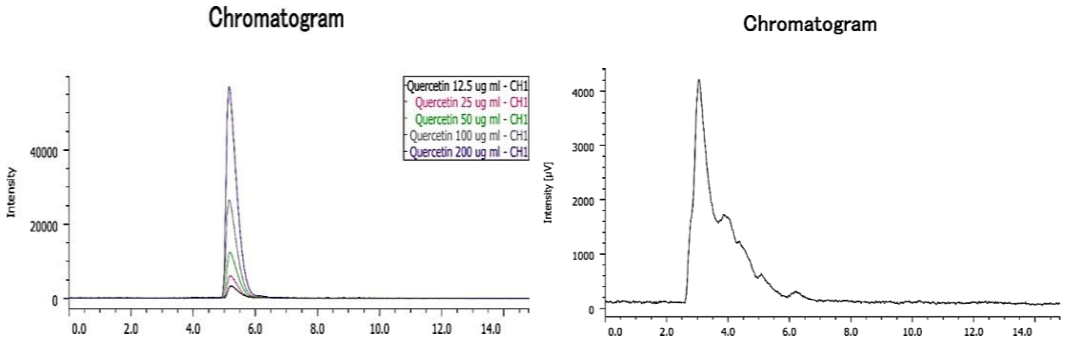
Similarly, standard quercetin solution was prepared in different concentration from 12.5 µg/mL to 200 µg/mL, with a retention time 3.5 minutes (Table 5.8.4). The calibration curve for quercetin showed linearity with an R^2 value 0.9979 (Figure No 5.8.6). For the plant extract showed a peak (Figure No 5.8.5) at 6.1-minute corresponding to standard with peak area 45465 µV. Sec, and concentration 8.64 µg/mL (Table No 5.8.4a).

Table No 5.8.4 Quantification of standard quercetin

SI NO	Concentration of Quercetin in µg ml	Intensity Area µV.sec
1	12.5	75424
2	25	139118
3	50	286973
4	100	616858
5	200	1347247

Table No 5.8.4.a Quantification of *Pithecellobium dulce* extracted quercetin

SI NO.	Retention time	Intensity (Area µV.sec)	Quantity Conc.
1	6.1 minute	45465	8.64(ug/ml)



Standard quercetin peak with different concentration

Confirmed quercetin peak in plant extract

Figure No 5.8.5 Chromatogram peaks of pd extracted quercetin

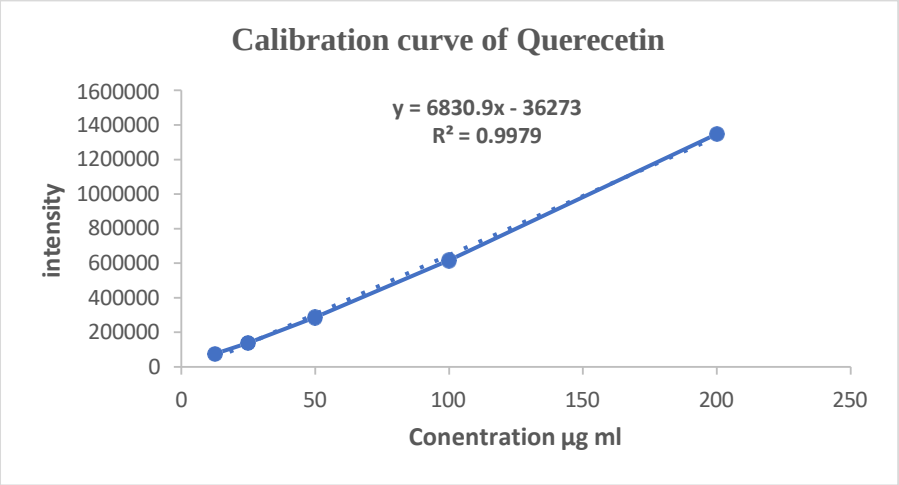


Figure No 5.8.6 Calibration curve of Pd extracted Quercetin

5.9 Flash chromatography purification of Gallic acid and Quercetin

a. Extracted gallic acid Purification

Flash chromatography was performed to analyse gallic acid chromatographic profile showed three peaks in the first 5 minutes of the run (Figure No 5.9.1), the total run time was 31.5 minutes.

I. Peak 1. was observed at retention time 3.0 minutes with an absorbance value of around 1.9 AU at 270 nm (red).
II. Peak 2. Observed at retention time 2.75 minutes, with a maximum absorbance of 1.8 AU at 280 nm (purple).
III. Peak 3. Was detected at 3.25 minutes, showed an absorbance of 1.7 AU across 200-600 nm (orange)
IV. Among the three peaks peak 2 was indicated higher concentration of gallic acid, under given chromatographic conditions. While peak1 and peak 2 represents less concentration fractions.

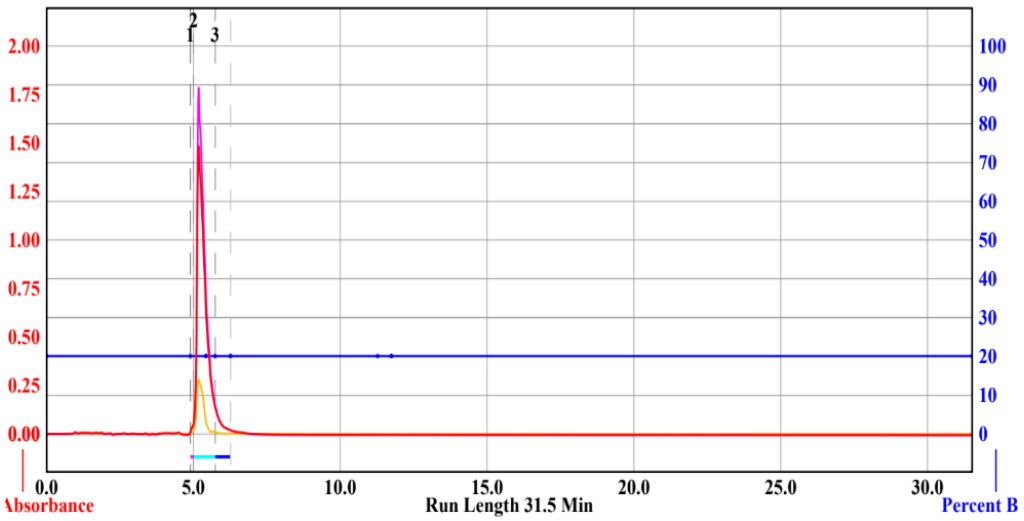


Figure No 5.9.1 Flash chromatogram of extracted gallic acid peaks

b. Extracted quercetin Purification

Similarly flash chromatograph was performed for the quercetin obtained clear peaks (figure 10) run time 60 minutes. Peak width was 1 minute, absorbance at 254 nm (red) and 280 nm (purple). Detection was based on a threshold of 0.20 AU. An absorbance for orange peak 200-500 nm confirmed the presence of quercetin. These findings were confirmed the successful purification of quercetin shown in the (Figure No 5.9.20).

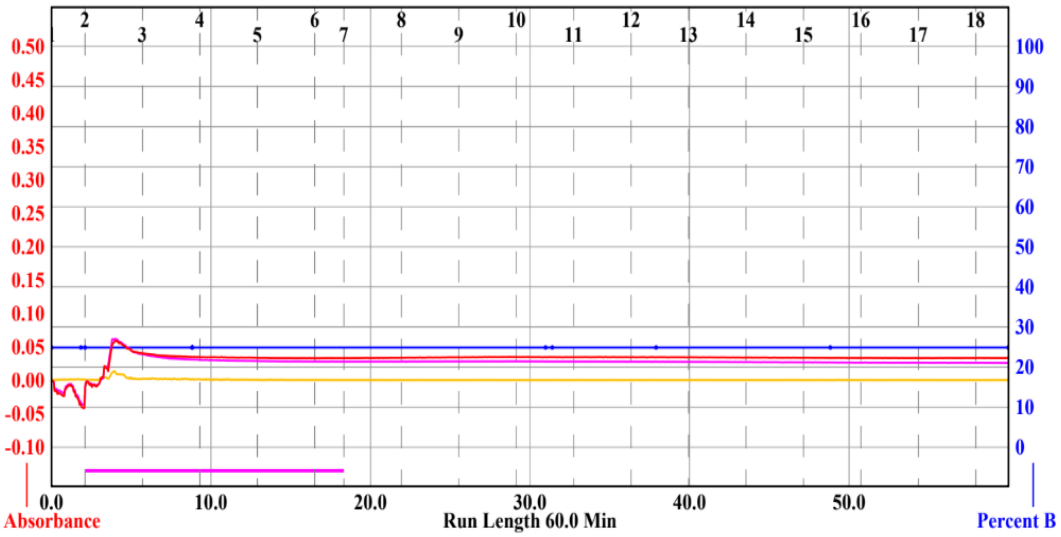


Figure No 5.9.2 Flash chromatogram of extracted quercetin

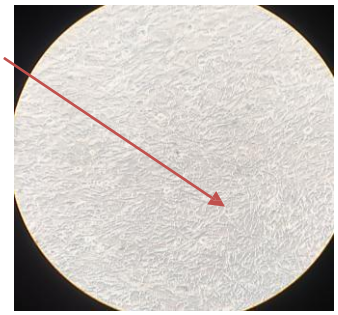
5.10 In vitro result analysis

To investigate the effect of isolated bioactive molecule from *Pithecellobium dulce* on NF-kB ligand RANKL mRNA expression in rheumatoid arthritis cell line.

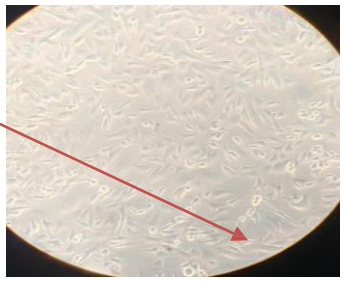
The bioactive compounds like gallic acid and quercetin were purified by flash chromatography and further subjected to investigation to explore their potential biological effects. The expression of NFkB ligand RANKL mRNA was evaluated in the SW982 rheumatoid arthritis.

a. Microscopic Observation of SW982 Cells under Different Conditions (Figure 5.10.1). Represents the microscopic images of SW982 rheumatoid arthritis under different conditions.

(I) Cell Confluency: This image shows SW982 cells with full confluency, densely packed and uniform indicating optimal growth.



(II) Cell Revival: The cells were spread out, regaining their morphology and growth pattern.



(III) Lipopolysaccharide induced Cells (LPS): after induction morphological changes were observed. The cells appeared more rounded and detached, indicating an inflammatory response characteristic of LPS stimulation

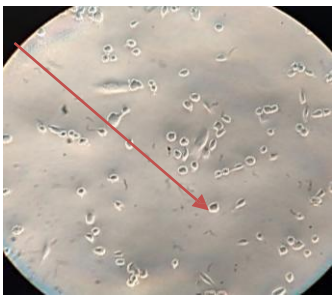


Figure No 5.10.1 Microscopic Observation of SW982 cell

5.11 MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay for Cell Viability.

The MTT assay was performed to investigate the cell viability effects of Pithecellobium dulce fruit crude extract, synthesized nano particles from *Pithecellobium dulce* fruit, extracted quercetin and extracted gallic acid on SW982 cells. Following graph and tables shown cell viability analysis.

***Pithecellobium dulce* fruits crude extract cell viability analysis**

Cell viability of *Pithecellobium dulce* fruits crude extract was assessed at concentrations from 200 µg/mL to 6.25 µg/mL (Table No 5.11.1). At highest concentration 200 µg/mL cell viability was 69.70% and lowest concentration 6.25 µg/mL cell viability increased 86.64%. The IC_{50} value 586.96 µg/mL, indicating low cytotoxicity shown in (Figure No 5.11.1a) cell viability improved as the concentration decreased.

Table No 5.11.1 *Pithecellobium dulce* Crude Extract Cell viability

Concentrations	Cell viability (%)	
	Mean	**SD**
200 ug/ml	69.70	0.11
100 ug/ml	77.95	2.82
50 ug/ml	74.66	5.3
25 ug/ml	78.77	1.98
12.5 ug/ml	76.25	5.71
6.25 ug/ml	86.64	0.69

IC50 value	**SD**
586.96	145.03

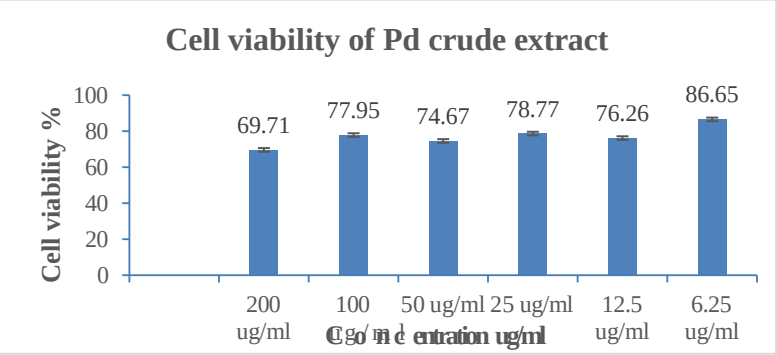


Figure No 5.11.1a Cell viability of Pd crude Extract

(I) *Pithecellobium dulce* fruits *nanoparticle* cell viability analysis

Cell viability assay of *Pithecellobium dulce* copper nanoparticle performed cell viability ranging from 6.25 µg/mL to 200 µg/mL (Table No. 5.11.2) Highest concentration 200 µg/mL shown cell viability 31.64%, lowest concentration 6.25 µg/mL increased cell viability 82.41 % (Figure No 5.11.2a.) with IC50 value 103.78 µg/mL.

Table 5.11.2 *Pithecellobium dulce* nanoparticle cell viability assay

Concentrations	Cell Viability (%)	
	Mean	**SD**
200 ug/ml	31.64	2.23
100 ug/ml	36.97	2.56
50 ug/ml	64.22	0.68
25 ug/ml	69.68	5.18
12.5 ug/ml	75.05	5.75
6.25 ug/ml	82.41	0.31

IC50 value	SD
103.78	5.52

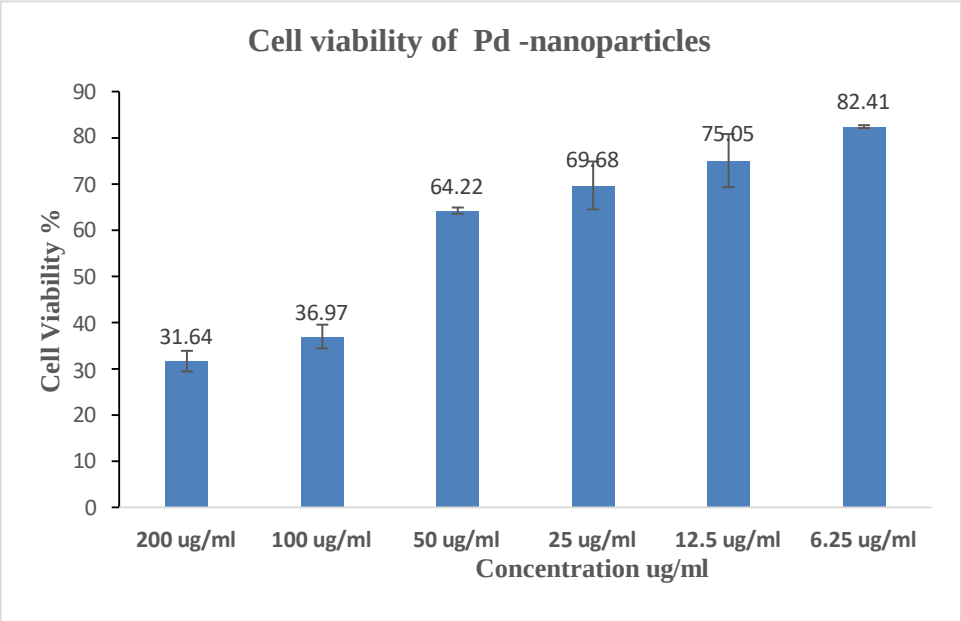


Figure No 5.11.2a Cell viability of *Pithecellobium dulce* nano particles

(II) *Pithecellobium dulce* extracted quercetin cell viability analysis

The extracted quercetin cell viability assay was performed using concentration from 20µM to 0.625 µm (Table No 5.11.3). At the highest concentration 20µM a significant decreased cell viability 16.70%, lowest concentration 0.625 µm showed increased cell viability 60.67% with SD ± 3.69 and IC50 value for quercetin was 1.3 µm, shown cytotoxic potential (Figure No 5.11.3a).

Table No 5.11.3 Extracted Quercetin cell viability assay

Concentrations	Cell Viability (%)	
	Mean	**SD**
20 µm	16.70	0.03
10 µm	22.77	0.07
5 µm	34.63	1.38
2.5 µm	41.30	6.87
1.25 µm	39.73	1.43
0.625 µm	60.67	3.69

IC50 value	**SD**
1.3	0.3

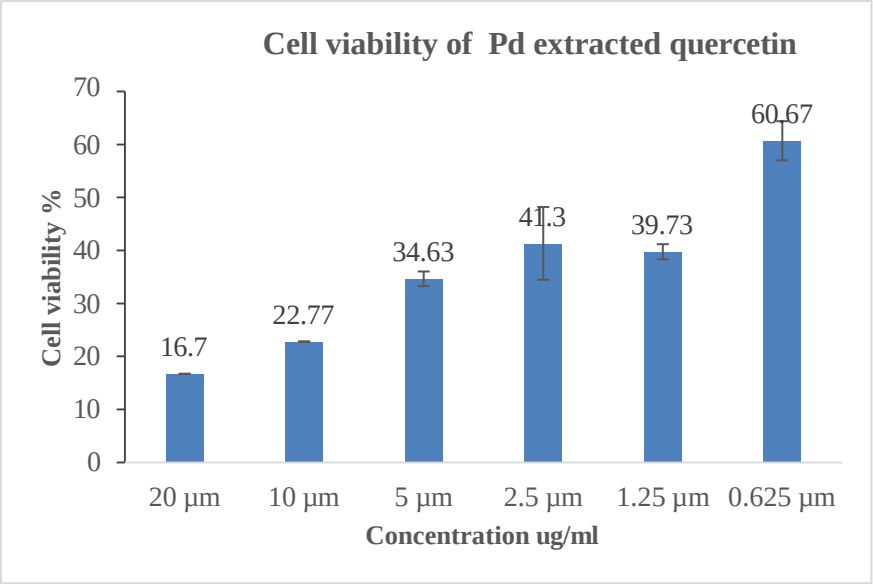


Figure No. 5.11.3a Cell viability of extracted quercetin

(III) Gallic acid Cell viability assay

The cell viability assay was performed for the gallic acid concentration taken from 100 µm to 3.125 µm shown in the (Table No 5.11.4). At the highest concentration cell viability decreased with 9.55% with SD ± 2.67, lowest concentration of sample around 3.125 µm shown increased cell viability up to 50.60% with SD ±0.92. IC50 value 34.35 µm, SD ± 4.4. indicating cytotoxic potential for lowest concentration dose graphically shown in the (figure 5.11.4a) and images showed in the (Figure No 5.11.5).

Table No 5.11.4 Extracted Gallic acid cell viability assay

Concentrations	Cell Viability (%)	
	Mean	SD
100 µm	9.55	2.67
50 µm	10.22	0.06
25 µm	10.45	0.17
12.5 µm	32.83	5.71
6.25 µm	39.98	2.97
3.125 µm	50.60	0.92

IC50 value	SD
34.35	4.4

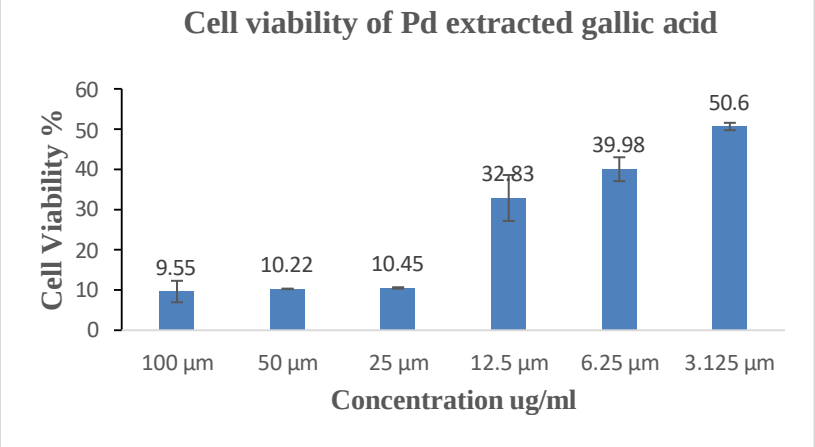


Figure No. 5.11.4a Cell viability of extracted gallic acid

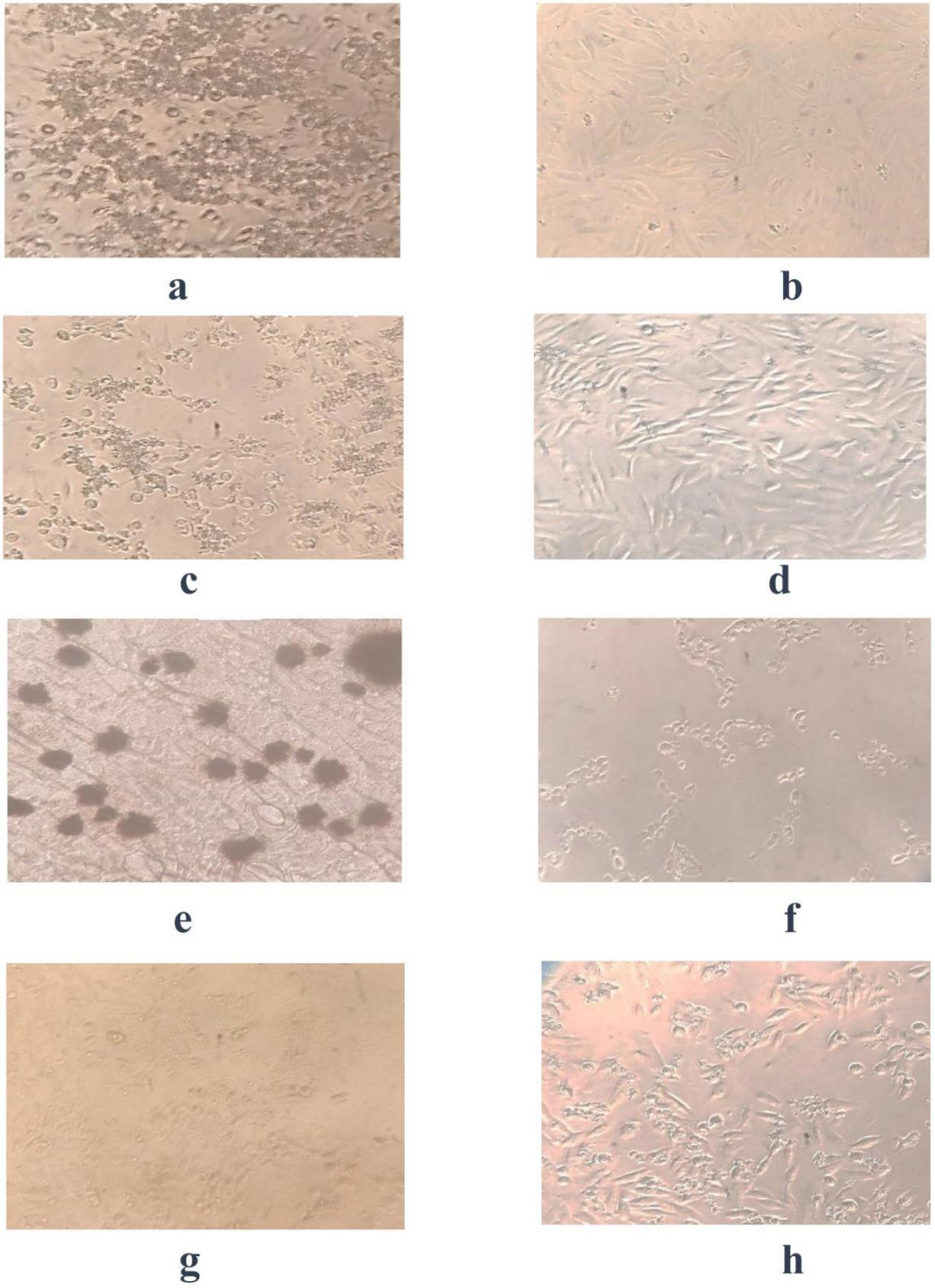


Figure No 5.11.5 Cell viability of Crude extract a. 200 ug/ml, b. 6.25 ug/ml. PdNPs c. 200 ug/ml, d. 12.5 ug/ml. quercetin e. 20 µm, f. 0.625 µm. gallic acid g. 100 µm, h. 12.5 µm.

5.12 Gene Expression results

MMP1 mRNA expression with or without Lipopolysaccharide induction (LPS)

To assess the effect of lipopolysaccharide (LPS) induction on MMP1 mRNA expression, quantitative mRNA gene expression analysis was performed and normalized 2log fold change calculated. (Figure No 5.12 a). The cells treated with LPS showed a significant upregulation in MMP-1gene compared to control group.

The normalized expression level of MMP1 in the LPS treated group increased to 2.54 ± 1.00, while without LPS treated control group decreased MMP1 expression it was 1.00 ± 0.04.

The statistical analysis was done using Mann-Whitney U test and P = 0.05 shown statistically significant increase mRNA expression after treated with LPS induction in the cell line. showed (Table No 5.12.1).

Table No 5.12.1 MMP1 mRNA expression with and without LPS induction

Normalized MMP1 mRNA expression				
Groups	**Mean**	**Std. Deviation**	**Mann-Whitney U**	**Significant P value**
Control without LPS	1.00	0.04	0.00	P=0.05*
With LPS	2.54	1.00		
P=0.05* Statistically significant				

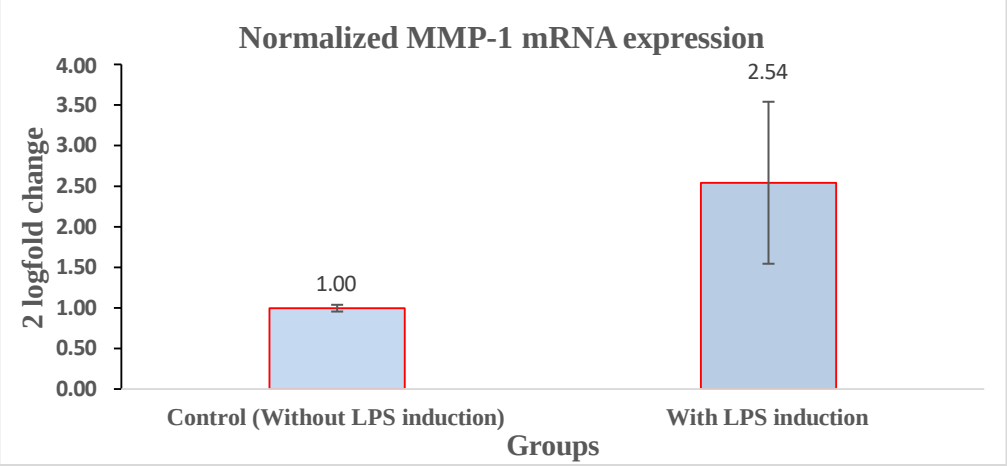


Figure No 5.12.a Normalized MMP1 mRNA expression

5.13 Effect of Bioactive compounds on MMP1 mRNA expression after lipopolysaccharide induction (LPS)

The analysis was performed to investigate effects of bioactive compounds extracted from *Pithecellobium ducle* and nanoparticle synthesized on MMP1 mRNA expression with LPS induction. Treated cells were assessed for quantitative real time RTPCR. the results were represented in (Table No 5.13.1).

Table No 5.13. 1 MMP1 mRNA expression treated with Bioactive compounds (after LPS induction)

Group1 Control without LPS treatment	Group 2 Crude extract 587 µg/ml	Group 3 Pd-CuNPs 103.78 µg/ml	Group 4 Quercetin 1.3 µg/ml	Group 5 Gallic acid 34.35 µg/ml
1.053 ± 0.41	0.633 ±0.03	0.432±0.22	0.519±0.01	0.299±0.25
P=0.05* Statistically significant.				

- In the control group 1 without LPS treatment MMP1 expression was 1.053± 0.41.
- In the group2 crude extract at 587 µg/mL significantly reduced MMP1 expression to 0.633± 0.03.
- In the group3 PdNPs 103.78 µg/mL which showed mean expression of 0.432± 0.22.
- In the group4 quercetin 1.3 µg/mL expression of mean 0.519 ±0.01.
- In the group5 gallic acid 34.35 µg/mL mean expression 0.299 ±0.25 indicating strong inhibitory effect.

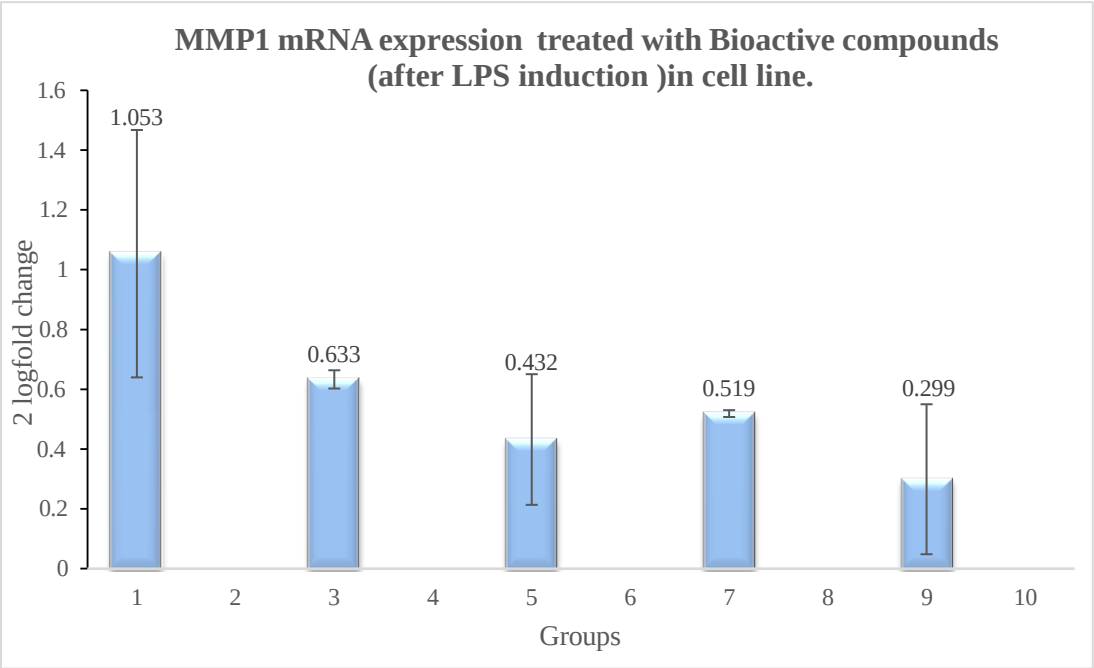


Figure No 5.13.1a Normalized MMP1 mRNA expression after treated with bioactive compounds (after LPS induction)

Among the five groups, the control group without LPS treatment showed higher MMP1 expression. Based on the calculated 2 log fold change, extracted Pd gallic acid demonstrated the most significant downregulation of MMP1 expression, followed by Pd-CuNPs, extracted quercetin, and the crude extract of *Pithecellobium dulce*.

These changes were found to be statistically significant (P = 0.05), indicating the potential anti-inflammatory effect of these extracted and nanoparticle synthesized compounds from *Pithecellobium dulce* fruit extract indicating its potential anti-inflammatory shown (Figure No 5.13.1a).

5.14 Synthesis of nanoparticles using *Pithecellobium dulce* fruit extract and its impact on rheumatoid arthritis cells.

5.14 a. Characterization of nanoparticles of *Pithecellobium dulce* fruit extract

In the present study, nanoparticles were synthesized using the aqueous extract of *Pithecellobium dulce* fruits. The synthesized nanoparticles were characterized by various techniques. Morphological analysis was done using high resolution transmission electron microscopy (HRTEM), and dynamic light scattering (DLS). Structural analysis was performed by (X-ray diffraction) XRD. Optical properties were evaluated by UV-visible spectroscopy and Fourier transform infrared (FTIR) spectroscopy.

5.15 High-resolution transmission electron microscopy (HRTEM)

HRTEM analysis was performed by HRTEM: Jeol/JEM 2100, instrument showed that the particle shape, size and surface features. The Particles were spherical and some were irregular. Size range form 10-200 nm. And particles were well dispersed. HRTEM images (Figure No. 5.15.1a and Figure No. 5.15.1b) confirmed the clear lattice fringes, with interplanar spacing between 2 nm and 5 nm, indicated a crystalline structure. The Crystallinity of nanoparticles were observed with Selective area electron diffraction (SAED) pattern (Figure No. 5.15.1c), which displayed bright concentric rings characteristic of crystalline material.

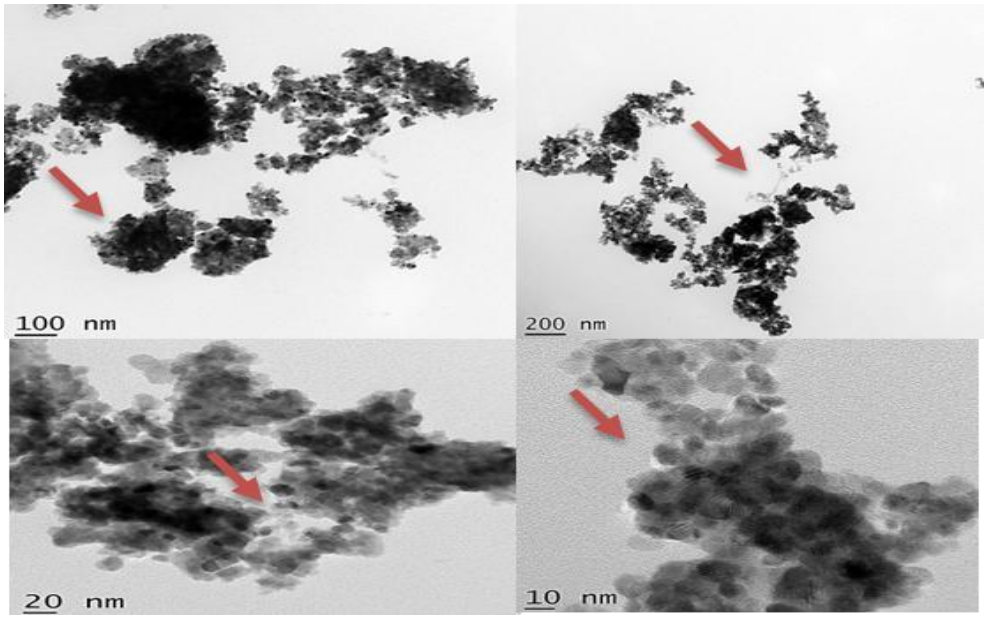


Figure No. 5.15.1 a General Morphology TEM of synthesized Pd-CuNPs

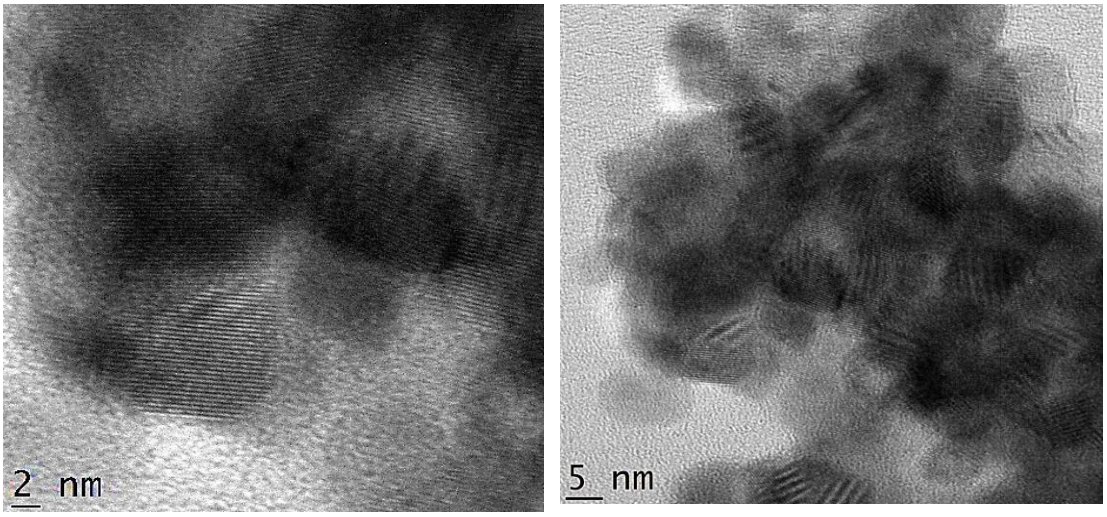


Figure No. 5.15.1b High-Resolution Structural Data of synthesized Pd-CuNPs

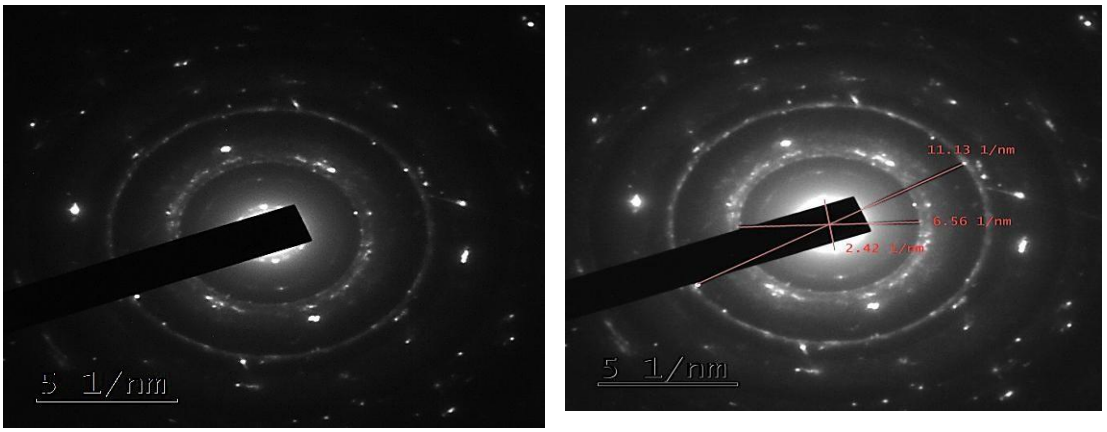


Figure No. 5.15.1c Selective area electron diffraction (SAED) pattern of synthesized Pd-CuNPs

5.16 Dynamic Light Scattering (DLS) (Particle size analyser)

Dynamic Light Scattering (DLS) was performed using the HORIBA SZ-100 instrument at 25.1 °C with a scattering angle 90°. The dispersion medium had a viscosity of 0.893 mPa·s. The Z-average particle size was 219.8 nm, representing the intensity-weighted mean size of the nanoparticles.

The polydispersity index (PDI) was 2.014, suggesting a highly polydisperse sample with significant variability in particle size (Table No. 5.16.1a). The size distribution graph showed a dominant peak with a mean particle size of 280.2 nm and a mode of 280.3 nm, representing the most frequent particle size present in the sample (Figure No. 5.16.1). These results indicate that the nanoparticles were broadly distributed, with a significant proportion in the higher size range.

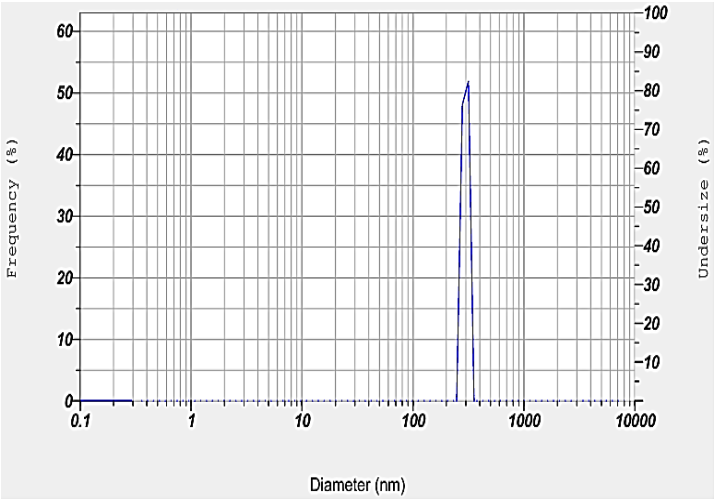


Figure No. 5.16.1 Dynamic Light Scattering (DLS) (Particle size analyser) of synthesized Pd-CuNPs

Table No. 5.16 1a. Dynamic light scattering (DLS) analysis

Peak NO	Mean	S. D	Mode
1	280.2 nm	17.0	280.3nm

Z- Average	219.8nm
PI	2.014

5.17 X-Ray Diffraction (XRD) Structural analysis

X-ray diffraction analysis was performed using the green synthesized nano particles to determine the crystalline structure. The crystalline nature of the sample was recorded in the 2θ range from 20° to 80° with sharp peaks. The major peaks observed at 2θ values were 29.71°, 36.56°, 42.41°, 43.10°, 61.55°, and 73.60°, corresponding to interplanar spacings (d- values) were 3.004 Å, 2.456 Å, 2.129 Å, 2.096 Å, 1.506 Å, and 1.286 Å. The sharp crystalline peaks were observed at 36.56°, with a peak height 33,743 counts per second (cps). The Full width at Half Maximum (FWHM) values from 0.65° to 1.74°, this value indicates the presence of small crystalline sizes and characteristic of nanocrystalline materials represented (Table No. 5.17.1a). The asymmetry factor values started from 1.2 and 1.2 indicated that the peaks were symmetric, and uniform crystal structure shown in the (Figure No. 5.17.1).

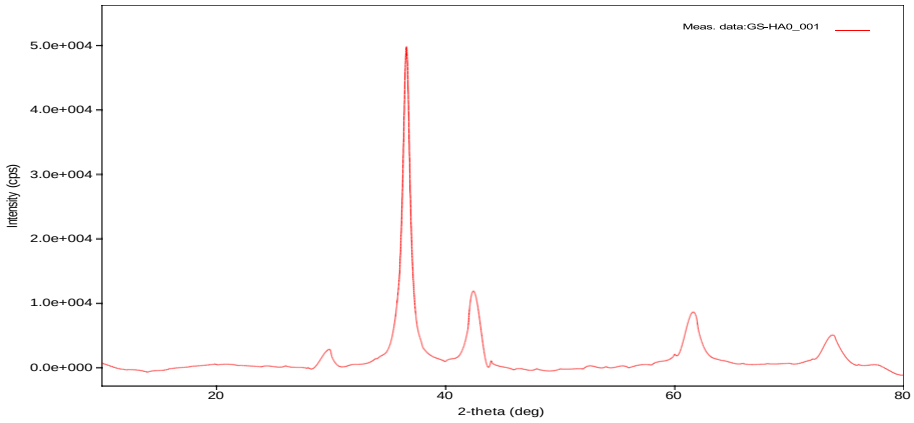


Figure No. 5.17.1. X-ray diffraction (XRD) structural analysis of synthesized Pd-CuNPs

Table No. 5.17.1a X-ray diffraction analysis

SI No	2-theta(2θ)	D(ang)	Height(cps)	FWMH (deg)	Asym. factor
1	29.71°	3.004 Å	2321	0.65°	1.2
2	36.56°	2.456 Å	33734	0.733°	1.05
3	42.41°	2.129 Å	8931	0.748°	0.98
4	43.10°	2.096 Å	1387	1.29°	0.98
5	61.55°	1.506 Å	6398	1.22°	1.06
6	73.60°	1.286 Å	3338	1.74°	0.88

5.18 UV- Vis Spectroscopy Optical property

UV-visible spectroscopy was performed to analyze the optical properties of $CuSO_4.5H_2O$ *Pithecellobium dulce* nanoparticles (pd-CuNPs). The absorbance was recorded by Shimadzu UV-1900i spectrophotometer with LabSolutions UV-Vis software (Version 1.14) with a wavelength range 200-800 nm. Samples with different concentrations were analysed 0.5 mL, 1mL and 2mL. Confirmed the presence of phytochemicals such as phenolics and flavonoids. Among the three samples the 2 mL sample showed the highest absorbance (4.71 a.u.), indicating the highest concentration of active compounds (Figure No. 5.18.1). The absorbance intensity increased with sample concentration. A sharper decline beyond 250 nm was observed, stabilizing near the baseline in the visible range (above 400 nm).

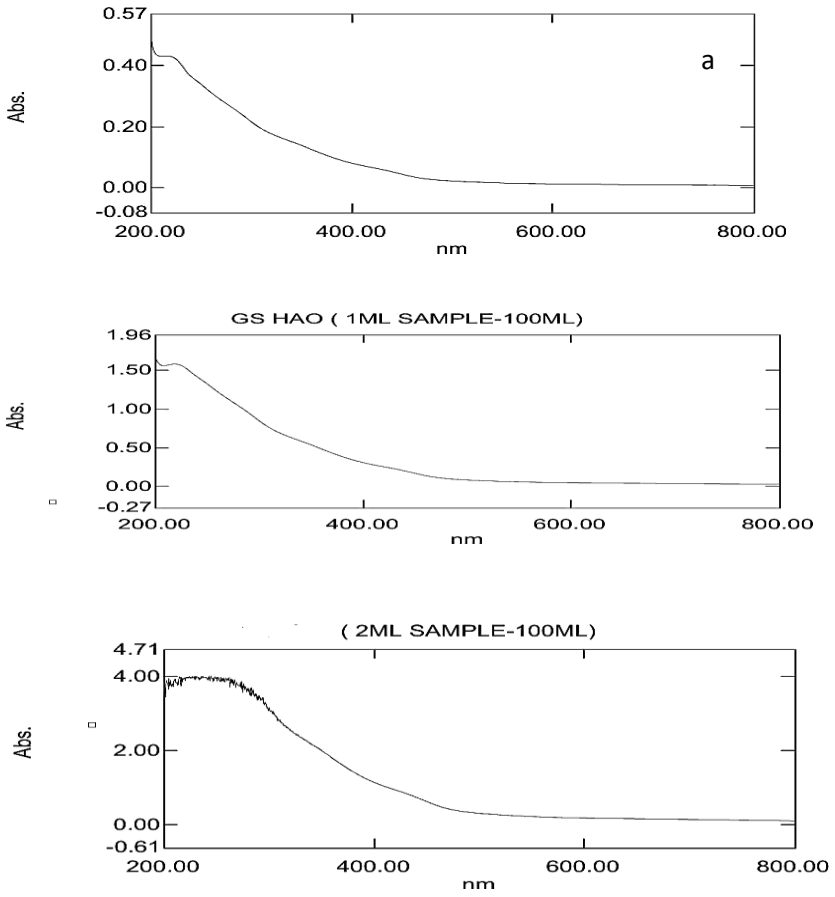


Figure No. 5.18 .1 UV-visible Spectroscopy a) 0.5 mL, b) 1mL, C) 2mL. of synthesized Pd-CuNPs

5.19. FTIR (Fourier Transform Infrared Spectroscopy

To identify the functional groups involved in the synthesis of *Pithecellobium dulce*-mediated copper nanoparticles (Pd-CuNPs), copper (II) sulphate pentahydrate (CuSO4·5H2O) was used as a trans metal precursor.

Fourier Transform Infrared (FTIR) spectroscopy was performed using a Bruker Alpha II ATR-FTIR spectrometer.

The sample was analysed in solid form, and the transmittance spectrum was recorded in the wavenumber range of 4000–600 cm^{-1}, with FTIR absorption showing peaks indicating the presence of phytochemical constituents.

Broad band around 3400 cm^{-1} was found with the hydroxy groups (O-H), confirming the phenolic compounds. Peaks near 2920 cm^{-1} were identified with the C-H groups aliphatic chains. Peak with 1630^{-1} indicates C=C aromatic rings confirmed the presence of flavonoids. Peaks between 1380-1410 cm^{-1} contain C-N groups, and identified amines.

Alcohol and esters were confirmed in regions between 1020-1080 cm^{-1}. Absorption in the 600- 800 cm^{-1} formation of Cu-O bonds indicated successful interaction between copper ions and plant metabolites shown in the (Figure No. 5.19.1). This investigation confirms the presence of *Pithecellobium dulce* phytochemicals in the biosynthesis of CuNPs and acts as both reducing and stabilizing agents.

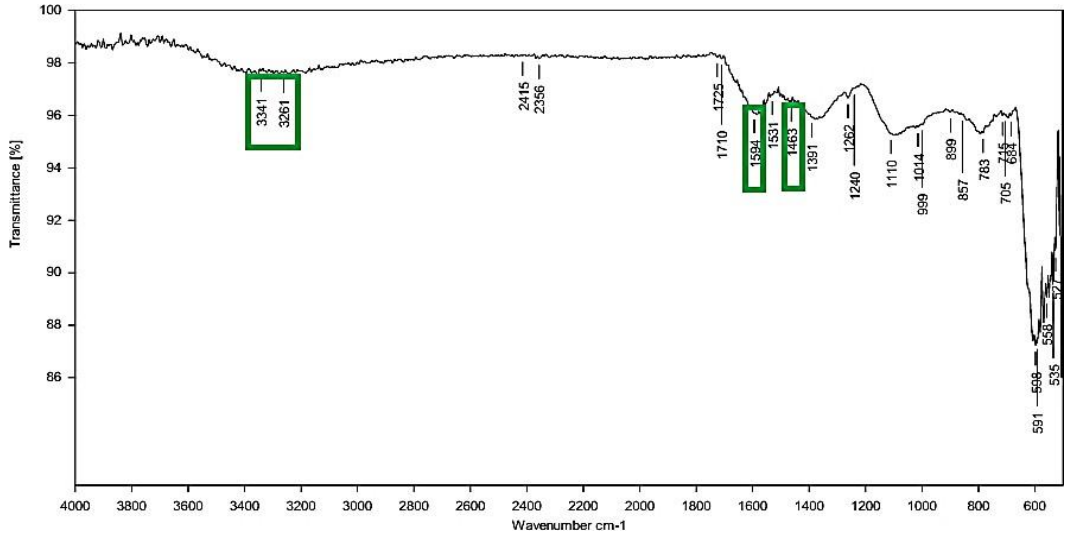


Figure No. 5.19.1 FTIR (Fourier Transform Infrared Spectroscopy of synthesized Pd-CuNPs

To Summarise:

- Green-synthesized nanoparticles (NPs) use plant extracts minimizing toxic chemical residues and making them safer for therapeutic applications.
- The synthesized nano particles from *Pithecellobium dulce* fruits using aqueous extract with trans metal copper sulphate Penta hydrate. The nano particle characterized, confirming their stability and bioactivity.
- TEM and DLS showed well dispersed nanoparticles (10-100nm)
- XRD- verified crystallinity. UV-Vis spectroscopy confirms the presence of phytochemicals like phenolics and flavonoids high concentration sample.
- FTIR confirmed functional groups responsible for capping and stability.
- These finding we considered to treat SW982 cell lines to see the effectiveness of Pd-Cu NP

5.20 Discussion

The *In Silico* study aimed to explore bioactive compounds from *Pithecellobium dulce* using various Computational databases. Such databases play crucial role in drug discovery for screening bioactive compounds and predicting pharmacokinetics. Screening of bioactive compounds from *Pithecellobium dulce* was performed using various computational databases. Toxicity analysis was conducted using the Swiss ADME/T database, and docking analysis was performed using the H-Dock server. The molecular dynamics confirmed stable, flexible protein-ligand interactions.

All bioactive compounds of *Pithecellobium dulce* exhibited high gastrointestinal absorption and blood-brain barrier permeability and complied with Lipinski's Rule of Five. Among them, gallic acid demonstrated a strong binding affinity with a docking score of -9.10 kcal/mol, a confidence score of 0.511, and a ligand RMSD of 53.11 Å. Quercetin also showed significant binding interactions, with a docking score of -7.52 kcal/mol, a confidence score of 0.313, and a ligand RMSD of 27.37 Å. These interactions are summarized in (Table No 5.5.1).

The target protein was MMP-1. Based on the docking interactions with MMP-1 amino acids and the corresponding molecular dynamics simulations, Pd-extracted gallic acid and Pd-extracted quercetin exhibited structural stability and flexibility. These findings suggest they are promising candidates for reducing inflammation in LPS-induced SW982 cell lines.

These finding was similar with recent study conducted on anti-inflammatory properties of gallic acid and quercetin Saraca asoca phytochemicals in vitro study on RAW 264.7 murine macrophage cells (Jain and Bhise et al. 2024), an *In Silico* analysis of quercetin and gallic acid from Saraca asoca, demonstrated their effective binding to VEGFR2 and COX-2, proteins involved in inflammation, wound healing. Quercetin exhibited a string binding affinity for VEGFR2 with good docking score of -9.3 Kcal/mol and similarly gallic acid showed binding with COX-2 with a docking energy of -6.5 Kcal/mol [1].

Koric et al. (2024) investigated a study on the anti-inflammatory potential of flavonoids and phenolic acids [2]. They showed that quercetin effectively inhibited adenosine deaminase (ADA), key biomarker in rheumatoid arthritis. Molecular

docking revealed strong binding and in vivo studies confirmed quercetin potential candidate to treat.

Hamdy, N. M. et al. (2024) found that anti-inflammatory potential of the polyphenolic compounds in Moringa oleifere, *In Silico* molecular docking. Investigated gallic acid and quercetin were found to be active constituents, docking studies revealed that quercetin exhibited docking score of -5.55 Kcal/mol and gallic acid -6.51 Kcal/mol. These finding supports our study findings that quercetin and gallic acid have a strong affinity to interact with inflammatory diseases [3].

Molecular docking conducted by Pandey et al. (2024) found that quercetin and niazirinin form Moringa oleifera showed good biding with HK-2 protein targeted in rheumatoid arthritis, with binding score -6.66 Kcal/mol, showed potential as an effective inhibitor for anti- arthritic therapy [4]. These findings are line with our findings.

These studies in accordance with our findings, highlight the important role of *In Silico* analysis for identifying anti-inflammatory potential of natural bioactive compounds from plant extract such as gallic acid and quercetin. Similar findings prove that bioactive compounds from plant extract from Pithecellobium dulce has promising approach.

This Phytochemical study aimed to extract, identify and isolate bioactive compounds from *Pithecellobium dulce* fruits using Soxhlet extraction, high-performance liquid chromatography (HPLC) and flash chromatography. Phytochemicals are essential natural compounds with potential applications in drug discovery. Phytochemical analysis of *Pithecellobium dulce* fruits confirmed the presence of alkaloids, phenolic acids, flavonoids, and terpenes. The extraction process was carried out using a Soxhlet apparatus, followed by isolation through HPLC, where extracted gallic acid and extracted quercetin were identified using standard references.

To further purify these bioactive compounds, flash chromatography was used, yielding 34.28 µg/mL of gallic acid and 8.64 µg/mL of quercetin. These purified compounds were used further studies to evaluate their therapeutic potential in the SW982 cell line, for efficacy.

A Similar study conducted by Vargas-Madriz et al. (2025) found that Pithecellobium dulce aril showed that oven-dried sample is rich in phenolics such as

gallic acid, quercetin, with methanol- water extraction gave the highest yields. Confirmed with HPLC and assessed anti- oxidant activity [5].

Another study by Yelugudari et al. (2023) investigated the study methanolic seed extract of *Pithecellobium dulce* is rich in fatty acids and flavonoids. The bioactive compound like 9-hexadecenoic acid identified by GC-MS and a purified fraction of HPLC showed anti-inflammatory activity, significantly reducing IL-8, IL-6, and PGE2 level in cell models. And the activity was found in the paw edema in rat, observed cytotoxicity suggesting its potential anti-inflammatory agent [6].

Sivakumar et al. (2019) studied on *Pithecellobium dulce's* water-soluble polysaccharide extraction form fruits. Hot water extraction followed by ethanol precipitation and purification with DEAE-cellulose column chromatography. Pithecellobium dulce water soluble polysaccharide showed potent antioxidant, antibacterial, and prebiotic activities its potential for food and pharmaceutical applications [7].

Selvakumar et al. (2021) extracted *Pithecellobium dulce* fruit peel using ethanol and confirmed the key phytochemicals. The extraction done by GC-MS analysis and purification by pinitol showed strong anti- oxidant activity and anti-cancer activity [8].

These findings highlight the therapeutic potential of *Pithecellobium dulce* fruit, due to its rich content of phenolics and flavonoids such as gallic acid and quercetin which have anti-inflammatory and anti-oxidant properties. We have also isolated and purified these compounds from Pd. Further these compounds were treated on LPS induced SW982 cell line.

Further in vitro study focused on the modulation of MMP1 expression in liposaccharide (LPS) induced SW982 cell line by *Pithecellobium dulce.* We evaluated *Pithecellobium dulce* extract, and its bioactive compounds like extracted gallic acid, extracted quercetin, crude extract and *Pithecellobium dulce* nanoparticles (Pd-CuNPs) which showed anti-inflammatory potential in LPS induced SW982 cell line. The LPS induction, increased the expression of MMP1 mRNA expression to (2.54 ± 1 2logfold change), confirmed inflammatory activation in the NF-kB ligand RANKL signaling pathway.

The treatment with *Pithecellobium dulce* compounds significantly downregulated MMP1 expression. Treated with extracted gallic acid (34.35 μg/mL) showed effective reduction in MMP1 expression (0.299 ± 0.25), followed by green

synthesized Pd-CuNPs (103.78 µg/mL) with (0.432 ± 0.22), extracted quercetin (1.3 µg/mL) with (0.519 ± 0.01) and crude extract (587 µg/mL) with (0.633 ±0.03).

These observations were statistically significant (P= 0.05), showing effective downregulation of the MMP1 expression and inflammatory response. The RT-PCR results normalized using GAPDH as the housekeeping gene, confirmed downregulation of MMP1 expression by extracted bioactive compounds, suggesting their potential role to modulate the NF-kB signaling pathway of inflammation.

Cell viability MTT assay showed that the concentration of compounds used were nontoxic and safe to treat. These findings show the anti-inflammatory effect of *Pithecellobium dulce* bioactive compounds, among them extracted gallic acid and quercetin are promising candidates to treat the rheumatoid arthritis (RA).

These results are in consistent with to Wiecek et al. (2022), who demonstrated that curcumin significantly reduced MMP1 expression and proinflammatory cytokine activity IL-1β, IL-6 and TNF-α in SW982 cells [9].

Another work conducted by Kim et al. (2018) supports our findings. They found that quercetin inhibited IL-17 and TNF-α expression in RANKL in synovial fibroblasts, reduced osteoclast formation intermediate pathway mTOR, ERK and NF-kB signaling [10].

In vitro, study conducted by Zamudio-Cuevas et al. (2022) found that poly-gallic acid (PGAL) significantly reduced expression of IL-1β, ROS, and MSU-induced cytotoxic in synovial cells. Their study found that gallic acid showed potential role in inhibiting NF-kB activation and MMP1 down regulation [11].

Bianet al. (2023) in their review on MMPs role in rheumatoid arthritis (RA), highlighted MMP-11 as key protein in extra cellular matrix (ECM) degradation. This review supports importance of MMP inhibition therapeutic strategy, and role of MMP-1 disease progression in inflammatory disease like RA progression [12].

In our study *Pithecellobium dulce* extracted bioactive compounds gallic acid and quercetin effectively downregulate MMP-1 expression in NF-kB ligand RANKL signaling (Table No. 5.13). Hence it can be stated that these compounds hold promising alternatives to treat rheumatoid arthritis in cell line.

Our study aimed to synthesize copper nanoparticles using *Pithecellobium dulce* fruit extract and copper sulphate pentahydrate. This is a green, eco-friendly, sustainable and biocompatible approach to traditional synthetic methods.

We have synthesized copper nanoparticles (CuNPs) using an aqueous fruit extract of *Pithecellobium dulce* the phytochemicals help the reduction of copper ions and stabilize the nanoparticles through capping mechanism. The Pd-Cu NPs showed characteristic surface plasmon resonance peak in UV-Visible spectroscopy, confirmed presence of phytochemicals like flavonoids and phenolic compounds (Figure No. 5.18).

Transmission Electron Microscopy (TEM) confirmed dispersed nanoparticle range from (10 to 100 nm) (Figure No. 5.15.1 a, b, and c). Dynamic Light Scattering (DLS) also showed well dispersed nanoparticles (Figure No. 5.16.1). X-Ray Diffraction (XRD) pattern confirmed the presence of crystalline nature of the nanoparticles. (Figure No.5.15.1). Fourier Transform Infrared Spectroscopy (FTIR) identified functional groups like hydroxyl (OH), carbonyl (C= O), and carboxyl group (COOH) (Figure No. 5.19.1)

These findings were confirmed with similar work conducted by Seku et al. (2025) they found that green synthesis of copper oxide nanoparticles using *Pithecellobium dulce* seed pods, reported these nanoparticles notably have antioxidant, and anticancer property [13].

Barani et al. (2021) found in their studies on synthesized copper nanoparticles using Withania somanifera extract [14]. The primary object of study was to develop eco-friendly and sustainable approach. Similar study was conducted by Shanmugasundaram et al. (2021) [15].

Singh and Sharma (2023) found significant anti-inflammatory properties by synthesized copper oxide nanoparticles using Azadirachata indica extract [16].

In line with these findings, we confirmed that *Pithecellobium dulce* is a potential candidate for green synthesis of biologically active nanoparticles. May have various applications in the anti-inflammatory, antimicrobial and anti-cancer, treatment in vitro.

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CHAPTER-VI
SUMMARY AND CONCLUSION

6.1 Summary

There is significant global impact of rheumatoid arthritis (RA) on health. There are limitations in current pharmacological treatments, with high costs, adverse side effects. This study explored plant-based alternatives, particularly bioactive compounds from *Pithecellobium dulce* fruit, which identified their nutritional and potential anti-inflammatory properties.

The present study investigated the *Pithecellobium dulce* fruit bioactive compounds' nutritional and therapeutic potential, focusing on their modulatory effects on the NF-kB ligand RANKL signaling pathway, with In Silico and in vitro approach.

In Silico analysis total twenty-one bioactive compounds were screened for the ADME/T analysis revealed toxicity, drug-likeness, Lipkin's rule five, pharmacokinetic profile of bioactive compounds.

Molecular docking analysis identified potentially active compounds like gallic acid and quercetin with their binding energy to matrix mettaloproteinase-1 (MMP-1). Molecular dynamic simulation confirmed the stability and flexibility of these protein-ligand complexes.

The preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, terpenoids, tannins, and saponins. Further confirmation of gallic acid and quercetin done through high-performance liquid chromatography (HPLC) and purified by flash chromatograph.

The in vitro molecular study conducted on LPS induced rheumatoid arthritis SW982 cell line, confirmed promising potential of Pithecellobium dulce fruit extracted bioactive compounds like gallic acid and quercetin with significant downregulation of MMP-1 expression in NF-kB ligand RANKL signaling pathway

Green synthesis is an eco-friendly sustainable approach of drug discovery and design. The green synthesis of Pd-CuNPs showed fast and effective absorbance in the cell line. Also, it showed significant inhibitory effect of MMP-1 expression in vitro.

The transmission electron microscope (TEM) showed particle size and shape (Figure No.5.15.1 a, b and c) UV-vis spectroscopy confirmed the crystallinity of particles (Figure No.5.18.1), dynamic light scattering (DLS) showed uniform spreading of particles (Figure No. 5.16.1), X-RD confirmed the presence of phytochemicals like flavonoids and phenolic compounds (Figure 5.17.1), Fourier

transmission infrared spectroscopy (FTIR) identified the functional groups like hydrogen, hydroxyl, carbon atoms (Figure No. 5.19.1).

These findings confirmed that *Pithecellobium dulce* fruit extracted bioactive compounds, namely gallic acid, and quercetin showed promising, alternative, in reducing inflammation in cell line.

6.2 Conclusion

Through the *In Silico* study, we found that the bioactive compounds of *Pithecellobium dulce* (Pd), namely quercetin and gallic acid, demonstrated strong binding interactions with the NF-κB ligand RANKL signaling MMP1 protein.

The presence of these compounds was confirmed through phytochemical analysis using various techniques.

Green synthesis of copper oxide nanoparticles using *Pithecellobium dulce* extract was demonstrated, with confirmed crystallinity, phytochemical composition, and functional group characterization.

In vitro, these bioactive compounds significantly downregulated MMP1 mRNA expression, indicating their potential in reducing RA-associated inflammation.

These findings highlight the therapeutic potential of *Pithecellobium dulce* bioactive compounds as natural supplements for reducing inflammation in RA management, offering a promising alternative to conventional treatments.

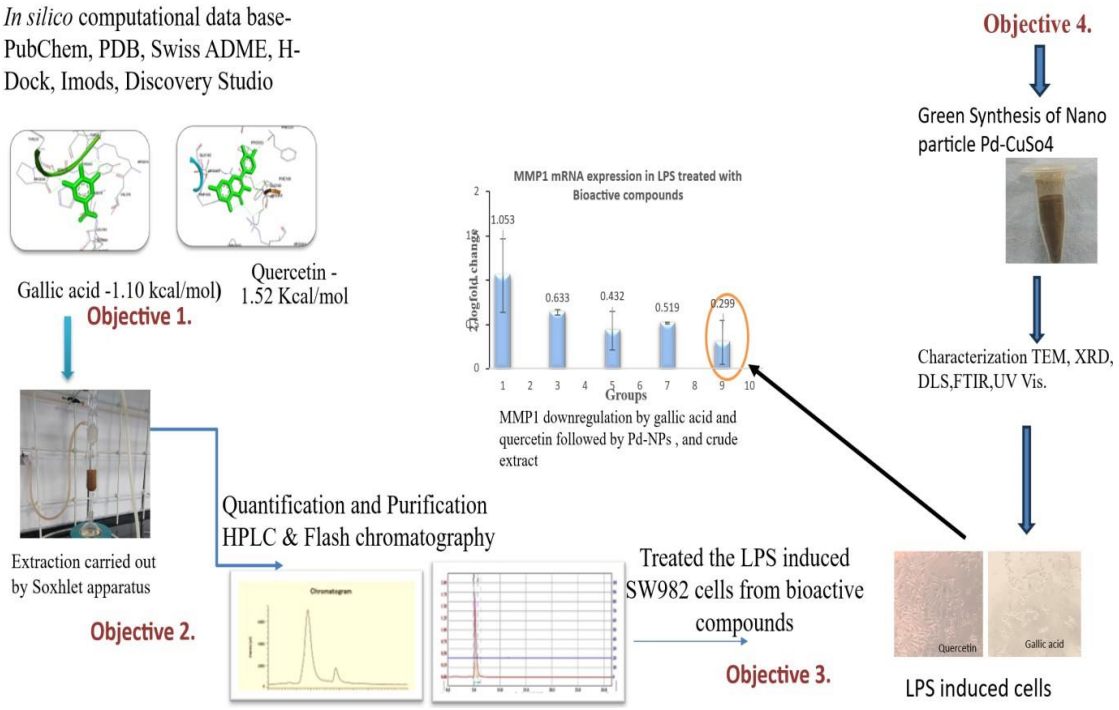
a) Limitations of this study

This study provides only *In vitro* evidence. Validation with advanced molecular techniques could not be incorporated due to duration and financial limitations.

b) Future Perspectives

Further In vivo studies using experimental animal models are required to confirm the effects of *Pithecellobium dulce* bioactive compounds, followed by clinical trials to validate their therapeutic potential for rheumatoid arthritis

GRAPHICAL REPRESENTATION



The graphical image represents the *In Silico* and in vitro approach. The bioactive compounds like gallic acid and quercetin were confirmed by *In Silico* analysis Swiss ADME/T and based on docking interaction, binding energy. further the compounds were extracted by Soxhlet and isolated by HPLC and Flash chromatography. The bioactive compounds anti-arthritic activity was evaluated by treating LPS-induced SW982 cells. Where a significantly downregulation of MMP-1 mRNA expression after treated with gallic acid followed by PdCuNPs and quercetin. Moreover, PdCuNPs were synthesized and characterized by using various techniques like TEM, X-RD, DLS, FTIR and UV-Vis spectroscopy. This integrated approach showed the therapeutic potential of gallic acid, quercetin and nanoparticles in targeting inflammatory pathways associated with rheumatoid arthritis.

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586103.

30/6/25

Name & Designation

Paper Publications

1. Dongre, N., Tungal, S., & Parvatikar, P. (2024, December). *In silico* analysis of bioactive compounds from *Pithecellobium dulce*: A promising approach for targeting proteins in rheumatoid arthritis. Research Journal of Biotechnology, 20(2), 164–172. https://doi.org/10.25303/202rjbt1640172.
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3. Accepted manuscript: Characterizing ADME/T and Physicochemical Properties of Bioactive Compounds from Manila Tamarind for Therapeutic Insights" for publication in the Research Journal of Biotechnology. Soumya Tungal, Nilima Dongre, Basavaraj B Devaranavadagi, Prachi Parvatikar.

Awards and Presentations

1. **Oral Presentation**

   Presented at the National Conference on Advances in Biotechnology and Nanotechnology, organized by the Department of Microbiology and Biotechnology, Karnataka University, Dharwad.

   Secured 2nd Place for Oral Presentation

   Held on 14th & 15th March, 2023.

2. **E-Poster Presentation**

   Presented at the XXXIVth *Annual National Conference of the Physiological Society of India (PSI)*, jointly organized by Shri B. M. Patil Medical College, Hospital and Research Center, BLDE (Deemed to be University), Vijayapura, Karnataka, and Vidyasagar University, Midnapore.

   Title: *"In Silico* Screening *of Pithecellobium dulce* Bioactive Compounds as MMP-1 Inhibitors for Rheumatoid Arthritis Treatment*"*

   Awarded Gold Medal for Best E-Poster Presentation (Young Scientist Category)

   Held on 2nd & 3rd November, 2023.

3. **Poster Presentation:**

   First National Symposium on Integrating Traditional Knowledge in Evidence-

Based Medicine ACTREC (Advance Centre for Treatment, Research and Education in Cancer), Tata Memorial Centre, Navi Mumbai, India. Held On:21-22 September.2023

Title: "Computational Screening of *Pithecellobium dulce* Bioactive Compounds as MMP-1 Inhibitors for Rheumatoid Arthritis Treatment"

Date: September 21–22, 2023

4. **Oral Presentation:**

   Scientific Academic and Research Society (SARS), BLDE (Deemed to be University), Vijayapura, Karnataka, India

   Title: "In Silico Approach and Quantification of Bioactive Compounds from *Pithecellobium dulce*: Targeting TNF-α in Rheumatoid Arthritis"

   Date: May 30–31, 2024

5. **Oral Presentation:**

   National Conference on "Current Trends in Biological & Health Sciences," Reva University, Bangalore, India

   Title: "Extraction, Quantification, and *In Silico* Pharmacokinetic Profiling of Bioactive Compounds from *Pithecellobium dulce*"

   Date: December 11–12, 2024

6. **Oral Presentation:**

   Scientific Academic and Research Society (SARS), BLDE (Deemed to be University), Vijayapura, Karnataka, India

   Title: "Isolation and Characterization of Bioactive Compounds from *Pithecellobium dulce* with In Silico ADME/T Analysis"

   Date: 8th May 2025

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ANNEXURE

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Extraction And Purification Of Pithecellobium Dulce Bioactive Compounds By HPLC And Flash Chromatography

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Abstract

Phytochemicals, being secondary metabolites made by plants, are well-known for their many uses in medicines and help a lot in finding and producing new drugs. Being widely known as jungle jalebi, Pithecellobium dulce (Pd) is a tropical fruit plant and is valued for its many medicinal properties. This work was focused on analyzing the phytochemical content of P. dulce fruit extract and improving the methods used to obtain its main bioactive components. Ethanol was used as a solvent in the Soxhlet extraction to get the crude plant extract. A preliminary screening of phytochemicals found that the plant contains alkaloids, flavonoids, phenols, tannins, terpenoids and saponins. HPLC confirmed the presence of gallic acid, known for its antioxidant and anti-inflammatory properties, after 2.8 minutes of analysis and showed a high degree of linearity when compared to the standard ($R^2 = 0.993278$, $p < 0.05$). The solution was then purified using flash chromatography to isolate gallic acid which appeared as well-separated peaks and was readily isolated according to its polarity and absorption profile at 280 nm. The results suggest that the purified gallic acid meets the required standards for pharmacological studies. The study suggests that P. dulce contains promising therapeutic compounds and using optimized methods like Soxhlet extraction, HPLC and flash chromatography plays an important role in separating and identifying them. The results point to the potential of P. dulce fruit in creating plant-based drugs and natural remedies, encouraging further study in laboratories as well as animal and human experiments.

Key words: *Phytochemicals; Pithecellobium dulce; Soxhlet; HPLC; flash chromatography, gallic acid.*

1. INTRODUCTION

Phytochemical analysis in chemical science is gaining popularity, with researchers focusing on the structure, synthesis and impact of plant compounds on the body [1,2]. Many industries such as pharmaceutical, medical, food and cosmetic make use of naturally occurring compounds due to their medical and health benefits [3]. Over the years, bioactive compounds have been valued in many traditional medical practices. Due to their phytochemicals and secondary metabolites such as alkaloids, flavonoids, phenolic acids, tannins, saponins, steroids and terpenoids, these plants are recognized for their therapeutic and nutraceutical potential. These bioactive compounds are found in edible and non-edible parts of the plants and trees including fruits, leaves, bark, stem, and root [5]. Their metabolites have several applications as antioxidants, anti-inflammatory, anti-cancer and anti-bacterial properties [6]. The increasing demand for natural remedies with health supplements and alternatives have acerated interest in less explored bioactive compounds.

One of the promising and alternative, Pithecellobium dulce (p. dulce), commonly known as jungle jalebi, manila tamarind, and vijayati Babula, with many other names in different regions, the tree belongs to the Fabaceae family. Originally native to tropical regions of the Americas, it was found in the plain of the India. The fruits of P. dulce contain wide variety of bioactive compounds with significant therapeutic properties [7,8]. The utilization of bioactive compounds from P. dulce is challenging, requiring the adoption of advanced techniques for the efficiently extracting, isolating, and purifying its phytochemicals. The process is inherently challenging and sensitive due to very minimal concentration of phytochemicals and bioactive compounds generally small amount and it's essential to extract without damaging critical and minor components in the raw material [9]. Furthermore, reasonable extraction procedures should ensure that molecules of interest are quickly separated

from the appropriate solvents [10]. Many conventional methods have been adopted for the extraction and purification of compounds from medicinal plants. Notably Soxhlet extraction, which remains a highly used and efficient technique for phytochemical analysis and its ability to extract broad range of constituent by continuous recycling [11,12]. The isolation and quantification of bioactive compounds is a demanding and increasingly popular area in analytical science, with High-Performance Liquid Chromatography (HPLC) and Flash Chromatography being among the most widely used techniques for quantification and purification. isolation and quantification of bioactive compounds is another one demanding and advanced gaining popularity in the field of analytical techniques among them high-performance liquid chromatography (HPLC) and Flash chromatography, which are consideration the most popular techniques for quantifications and purification [13]. HPLC provides high sensitivity and precision in identifying and quantifying phytochemicals, and flash chromatography is particularly advantageous forth rapid purification of targeted fractions [14]. The selection of appropriate extraction protocol focuses on many more factors, like nature of plant material, solvent purity, pH, temperature, and the solvent to sample ratio, all which influence the efficiency and quality of the extracted compounds [15].

This study uses appropriate solvents to evaluate standard and rapid, time-efficient extraction methods, such as Soxhlet extraction. Additionally, it involves phytochemical screening, quantifying targeted bioactive compounds using standard HPLC analytical techniques, purification via flash chromatography, and identifying bioactive compounds in Pithecellobium fruits.

2. MATERIAL AND METHODS

2.1 Collection of Pithecellobium dulce fruits

Fresh white aril fruits of Pithecellobium dulce were procured from the local market in Vijayapura, Karnataka, India, during the peak harvest season between March and April 2023. And authenticated by the Department of Dravya Guna, BLDE Association's AVS Ayurveda Mahavidyalaya, Vijayapura, Karnataka, India. The fruits were initially inspected to remove any spoiled or damaged ones to ensure sample integrity. To eliminate surface contaminants such as dust, microbes, or pesticide residues, the fruits were thoroughly washed under running tap water, followed by rinsing with distilled water. After cleaning, the fruits were spread out on clean trays and air-dried under shade for 3–5 days to retain maximum phytochemical content by avoiding direct sunlight exposure, which may degrade heat- and light-sensitive compounds.

Once adequately dried, the arils were separated, and the fruit pulp was subjected to coarse grinding using a mechanical grinder. The resulting material was then further pulverized using a laboratory-grade grinder to obtain a fine, homogenous powder. Approximately 100 grams of this powdered fruit material were stored in airtight, amber-colored containers at room temperature (25 ± 2°C) to prevent oxidation and moisture absorption until further use. Ethanol (500 mL) was selected as the extraction solvent due to its efficiency in extracting a broad range of polar phytochemicals, ensuring maximum recovery during Soxhlet extraction.

Fig. 1. Collection, Cleaning, Drying, and Powdering Process of Pithecellobium dulce Fruits.

Figure 1 illustrates the sequential steps involved in the preparation of Pithecellobium dulce fruit samples for phytochemical analysis. The process includes fruit collection, washing with tap and distilled water to remove impurities, shade drying to preserve bioactive compounds, and mechanical grinding to obtain a fine powder. This processed material served as the basis for solvent extraction in the subsequent experimental procedures.

2.2. Soxhlet Extraction Method

The extraction was conducted using a Soxhlet extractor, which is a standard and simple handling method for processing plant materials. The apparatus made up of a glass material with a spherical bottom flask, an extraction chamber connected by a siphon tube, and a condenser connected on top of the extractor. We have taken around 80grams of plant material and crushed by using a mortar and pestle, packed in filter paper, placed in a thimble within the extraction chamber. About 500ml of ethanol was poured into 500ml round bottom flask [16]. The apparatus was seated properly solvent was heated by providing 70-80°C temperature the ethanol was evaporated and pass through the condenser [17]. The condensed solvent dropped into the extraction chamber, where it come and contact with the plant material reached the siphon tube, the solvent along with extracted compounds flow back into the round bottom flask. This cycle was repeated continuously at the same temperature. The process was conducted over 48 hrs, approximately around 20 cycles have processed until the extraction was complete and all soluble compounds were extracted from the plant material [18].

2.3. Rotary Evaporator

Plant extract was processed using a rotary evaporator under vacuum conditions to separate ethanol from the extract. The vacuum system allowed ethanol to evaporate at a lower boiling point, which was then collected as the vapour condensed back into liquid form [19]. This sensitive method focused the concentration of the plant's importance without applying high temperatures [20]. The rotary evaporator was set to operate at 50°C and 80 revolutions per minute (rpm) to separate the ethanol from the plant sample.

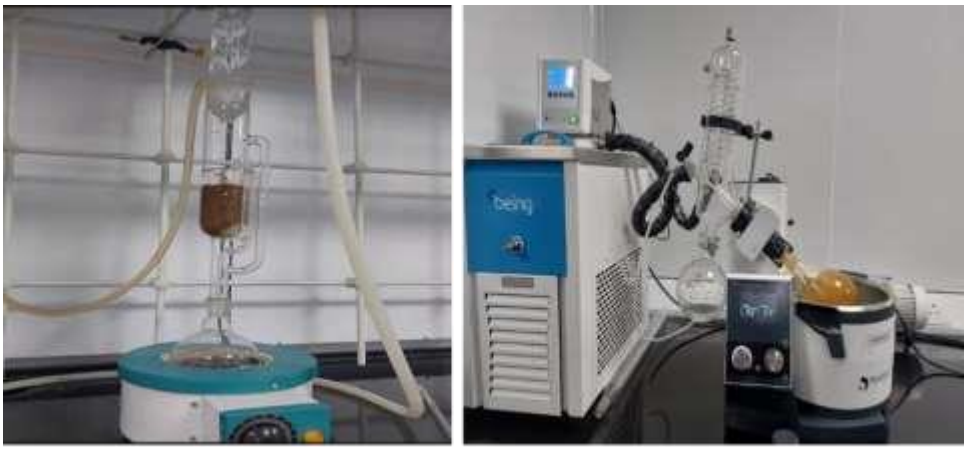

Fig. 2. Rotary Evaporation Setup for Concentration of Pithecellobium dulce Ethanolic Extract.

Figure 2 presents the equipment used for reducing the concentration of the ethanolic Pithecellobium dulce extract. The setup uses a flask that turns inside a vacuum, a water bath held at 50°C and a condenser for capturing the ethanol as it evaporates. With this technique, solvents can be removed under mild conditions, sparing sensitive active components from heat damage. Rotary evaporation helps maintain bioactive compounds and allows for a more concentrated extract to support phytochemical analysis and purification.

2.4. Phytochemical Screening

The preliminary phytochemical analysis of the extracts was performed using aqueous and ethanolic extracts utilising established techniques to identify the primary components [21]**.**

Table.1. Photochemical qualitative analysis.

SI NO	Phytochemical	Test	Reagents	Positive indication
1	Tannins	Dissolve extract in warm water and filter. Add 5% ferric chloride drops in 90% alcohol to the filtrate	5% ferric chloride in 90% alcohol	Bluish-black or greenish-black precipitate
2	Alkaloids	Stir 0.4 g of extract with 8ml of 1% HCL, warm and filter. To 2 ml of filter, add (a) Mayers reagent or (b) Dragendroffs reagent.	1% HCL Mayers reagent or (b) Dragendroffs reagent.	Yellow PPT
3	Flavonoids (Ferric Chloride Test)	Boil 0.5g of extract with ml of water then filter. Add 10% ferric Chloride solution to 2ml of filtrate	10% ferric Chloride solution	Yellow, orange, or red colour
4	Saponins	Boil 1g of extract with 5ml of water and filter. Add 3ml of water to the filtrate and shake vigorously for 5 minutes	water	Frothing (foam formation)
5	Terpenoids	Dissolve the extract in chloroform and add a few drops of Conc. Sulfuric acid	Chloroform, Conc. Sulfuric acid.	Red, pink, or purple colour
6	Phenols	Dissolve 500mg of extract in 5 ml of water. Add a few drops of neutral 5% ferric chloride solution	5% Ferric chloride (neutral ferric Chloride solution)	Dark green colour

Table 1 displays the preliminary qualitative testing results for the Pithecellobium dulce fruit extract. Several groups of phytochemicals were examined, including tannins, alkaloids, flavonoids, saponins, terpenoids and phenols. It describes the specific tests conducted which reagents are applied and how these can indicate the presence of each chemical group. The appearance of bluish black for tannins or yellow for alkaloids was used to determine if a reaction was positive. According to the table, P. dulce contains a range of phytochemicals, especially phenols, flavonoids and terpenoids, suggesting it may be helpful in treating health conditions.

2.5. Quantification of Pithecellobium dulce by High performance of liquid chromatography.

(HPLC)

Quantification of bioactive compounds using High-Performance Liquid Chromatography (HPLC) is increasingly recognized as one of the most important analytical techniques for herb quality control and fingerprinting research [22]. Plants are primarily analysed for non-volatile chemicals such as higher terpenoids, various phenolics, alkaloids, lipids, and sugars [23]. HPLC works best for substances detectable in the ultraviolet or visible ranges of the spectrum. Natural products are typically separated after evaluating a relatively crude extract in biological experiments to accurately characterize the active component [24,25]. HPLC results are interpreted by examining chromatograms that display the chemical separation in a sample. This approach involves comparing the chromatogram of a standard to that of a plant extract to quantify gallic acid. In our study, we have used the "JASCO AUTOSAMPLER" instrument with a reversed-phase C18 column (silica powder as the

stationary phase). Analytical procedures were performed at a wavelength of 280 nm with an injection volume of 20 µL. The mobile phase solvents consisted of HPLC-grade methanol and water. This setup allowed for precise measurement of flow rate, retention time, and wavelength. The retention time (RT) for both the standard and the extract was 2.8 minutes. This indicates that the chemicals were detected based on their travel time across the chromatographic column, which facilitated their identification by comparison with the standard.

2.6 Purification of Gallic acid by Flash chromatography

Flash chromatography combines medium and short-column chromatography with air pressure to achieve quick separation [26]. It is commonly used to separate molecular mixtures into distinct components, making it valuable for drug discovery and plant metabolite purification [27,28]. Flash chromatography differs from conventional methods in two significant ways: it uses slightly smaller silica gel particles (250–400 mesh), and to compensate for the reduced solvent flow caused by these smaller particles, pressurized gas (around 10–15 psi) is applied to push the solvent through the stationary phase column. This process results in fast and high-resolution chromatography, commonly referred to as "flash" chromatography [29,30,31].

Our study used the Combi Flash RF^{+} Lumen instrument to purify gallic acid. The ethanol extract was mixed with 4 grams of solid silica powder and loaded into the flash chromatography column. A solvent mixture of methanol and water was used, and the wavelength was set to 280 nm to detect gallic acid based on standard references. The sample was processed over 31.5 minutes.

Elution occurred when the mobile phase was pushed through the column under pressure, causing the compounds to move at different speeds through the stationary phase, depending on their interaction with both phases. Less polar compounds moved faster, while more polar compounds moved slower. Fractions were collected at predetermined intervals as the compounds eluted from the column.

Fig.3. Instrumentation used for phytochemical analysis: (A) JASCO HPLC Autosampler for quantification of gallic acid; (B) Combi Flash RF+ Lumen system for purification of bioactive compounds.

Figure 3 illustrates the advanced analytical instruments used in this study for the quantification and purification of bioactive compounds from Pithecellobium dulce fruit extract. Image (A) shows the JASCO HPLC Autosampler system equipped with a reversed-phase C18 column, which was employed for the precise detection and quantification of gallic acid based on retention time and peak area. Image (B) displays the Combi Flash RF+ Lumen chromatography system used for rapid purification of gallic acid from the crude extract. The use of these high-resolution instruments ensured accurate profiling and separation of targeted phytochemicals, enhancing the reliability and efficiency of the analytical process.

3. RESULTS AND DISCUSSION:

3.1. Soxhlet Extraction and Rotary Evaporator Yield

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The Soxhlet extraction method successfully separated plant material using ethanol as the solvent. This approach efficiently extracted compounds from plant materials partially soluble in ethanol and those containing insoluble contaminants. Around 31% of the sample was extracted from the Soxhlet setup using 100 grams of Pithecellobium dulce fruits. However, the method was noted to be unsuitable for thermolabile plant materials due to the heating involved in the process.

Rotary evaporator approximately 31% of a brown-coloured liquid extract was obtained from the plant material after processing. The collected sample was stored at 4°C for further use.

3.2 Phytochemical screening

Primary phytochemical screening analysis was performed by using various qualitative tests included tannins, alkaloids, Flavonoids, saponins, terpenoids and phenols in compare with the aqueous extract the ethanol extract sample has shown good presence phenols, flavonoids and terpenoids.

Table 2: Phytochemical qualitative analysis

SI No.	Test	Aqueous	Ethanol
1	Tannins	+	+
2	Alkaloids	++	+
3	Flavonoids	++	++
4	Saponins	-	+
5	Terpenoids	++	++
6	Phenols	+++	+++

Note: High +, Highest +++, Note present -

Table 2 summarizes the results of the qualitative phytochemical screening of Pithecellobium dulce fruit extracts prepared using both aqueous and ethanolic solvents. The presence of major phytochemical groups—such as tannins, alkaloids, flavonoids, saponins, terpenoids, and phenols—was evaluated based on colorimetric or precipitate-forming tests. The table compares the intensity of these compounds in both extracts using a relative scale, where "+" denotes low presence, "++" moderate, and "+++" high. The results reveal that the ethanolic extract showed a stronger presence of phenols, flavonoids, and terpenoids compared to the aqueous extract, indicating ethanol as a more effective solvent for extracting these bioactive compounds. This supports the selection of ethanol for further extraction and analytical procedures.

3.3 Quantification of Pithecellobium dulce by High performance of liquid chromatography.

(HPLC)

The HPLC method effectively separated and quantified the bioactive compound gallic acid from the plant extract. The standard peak height for 200 µg/ml was 114171 µV, with an area of 1714707 µV-sec. The retention time for both the standard and the extract was 2.8 minutes, indicating proper identification. A strong linear correlation was observed with an R-squared value of 0.993278, and the P-value was < 0.05, confirming statistical significance. The regression curve for absorbance showed a direct proportionality between concentration and peak response, ensuring accurate quantification

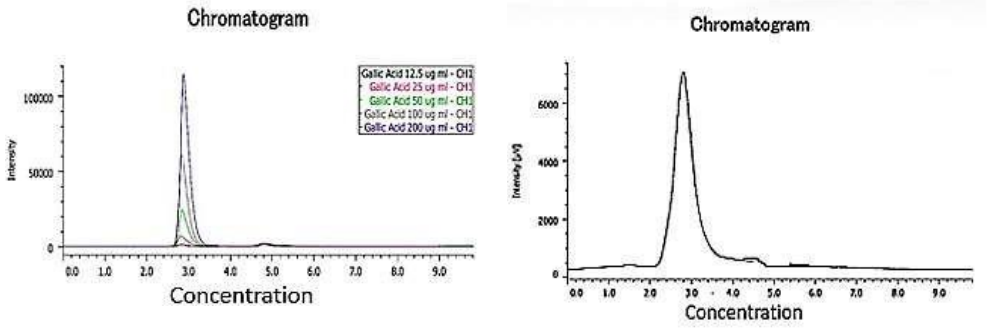

Fig. 4. HPLC Chromatograms of Standard Gallic Acid (Left) and Extracted Pithecellobium dulce Sample (Right).

Figure 4 displays the chromatographic profiles obtained through High-Performance Liquid Chromatography (HPLC) for the quantification of gallic acid. The left panel shows overlay chromatograms of standard gallic acid at varying concentrations (12.5–200 µg/mL), all exhibiting a sharp peak around the 2.8-minute retention time, indicating consistency and linearity of detection. The right panel presents the chromatogram of the ethanolic extract of Pithecellobium dulce, which also shows a prominent peak at approximately 2.8 minutes, corresponding to gallic acid. The similarity in retention times confirms the presence of gallic acid in the plant extract, validating the analytical method. The peak intensity further suggests a measurable concentration suitable for quantitative analysis, supporting the effectiveness of the extraction and detection protocol.

Figure 5 illustrates the standard calibration curve used for quantifying gallic acid concentration in Pithecellobium dulce fruit extract using High-Performance Liquid Chromatography (HPLC). The graph plots intensity (µV·sec) against known concentrations of standard gallic acid (µg/mL), showing a strong linear relationship. The linear regression equation (y = 1084.7x – 13734) and high coefficient of determination (R2 = 0.9817) confirm the accuracy and reliability of the method. This standard curve was used to interpolate the concentration of gallic acid present in the plant extract based on its chromatographic peak area, supporting precise quantification of this bioactive compound.

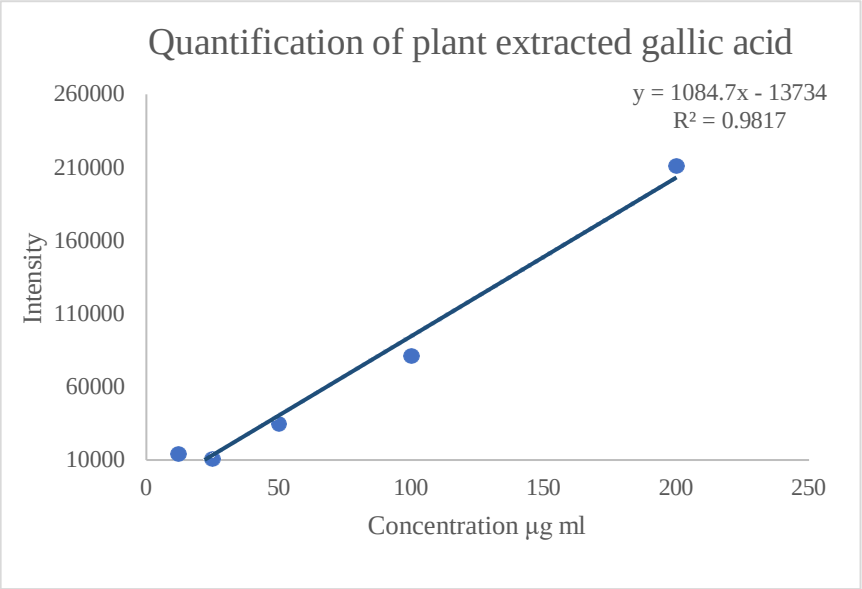

Fig. 5. Calibration Curve for Quantification of Gallic Acid by HPLC.

Table 3. A. Standard gallic acid concentration and intensity. B. Plant extracted gallic acid concentration.

Sl. No.	Sample Type	Concentration (µg/mL)	Retention Time (min)	Intensity (Area in µV·sec)
1	Standard Gallic Acid	12.5	2.8	13,997
2	Standard Gallic Acid	25.0	2.8	105,209
3	Standard Gallic Acid	50.0	2.8	346,113
4	Standard Gallic Acid	100.0	2.8	859,005

5	Standard Gallic Acid	200.0	2.8	1,714,707
6	P. dulce Extract	34.28 (Interpolated)	2.7	211,061

Table 3 consolidates both standard and plant extract HPLC data for gallic acid quantification. Rows 1–5 represent standard gallic acid solutions at known concentrations (12.5–200 µg/mL), all showing consistent retention times (2.8 min) and increasing peak intensities with concentration, confirming linearity. Row 6 presents the data for gallic acid isolated from the Pithecellobium dulce extract, which showed a similar retention time (2.7 min) and a peak area of 211,061 µV·sec. By comparing this intensity to the standard curve, the gallic acid content in the extract was interpolated to be approximately 34.28 µg/mL. This validates the successful quantification and confirms the presence of gallic acid in the plant extract.

3.4. Purification of Gallic acid by Flash chromatography

The process yielded three distinct peaks in the chromatogram, indicating different concentrations of the separated compounds. The fractions were collected into three tubes (labelled 1, 2, and 3), based on the peaks. The second tube contained the highest concentration of gallic acid, with 10 mL of the sample collected. The sample was then analysed using high-performance liquid chromatography (HPLC). A chromatogram showed the separation of components over time with a flow rate of 18 mL/min, a peak width of 30 seconds, and the absorbance of gallic acid at 280 nm measured at 0.20 AU. Flash chromatography proved effective in isolating the desired compound.

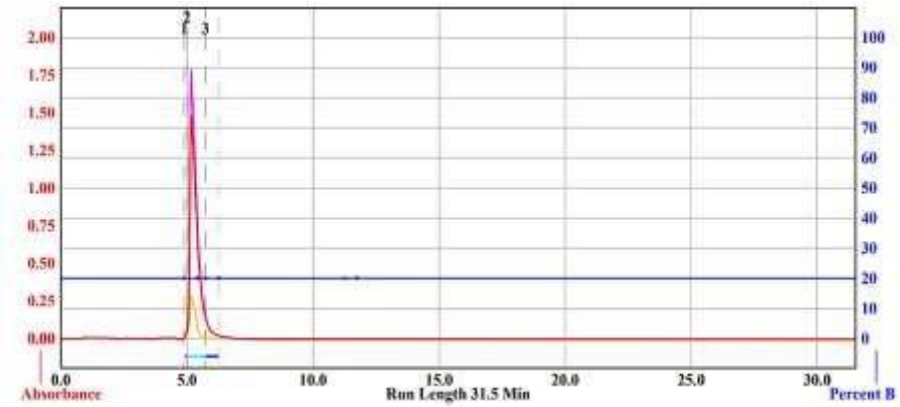

Figure 6. Flash chromatography gallic acid peaks.

Figure 6 illustrates the elution profile obtained during flash chromatography used for the purification of gallic acid from Pithecellobium dulce ethanolic extract. The x-axis represents the total run time (31.5 minutes), while the y-axes display absorbance (left, red line) and solvent composition (% Solvent B, right, blue line). Three distinct peaks were observed between approximately 4.5 and 6.0 minutes, corresponding to different compounds separated during the process. Among these, Peak 2 showed the highest absorbance, indicating the fraction with the highest concentration of gallic acid, as confirmed by further HPLC analysis. The gradient elution employed a mixture of methanol and water, with the solvent B composition gradually increasing over time (blue curve), aiding in the efficient separation of polar and semi-polar compounds. The narrow peak widths and clear resolution indicate successful and high-efficiency separation. This chromatographic profile validates the use of flash chromatography as a rapid and effective method for isolating bioactive phytochemicals.

Table 4 gathers the main points from the research done on Pithecellobium dulce. This process involved analyzing what was obtained from extraction, performing a qualitative screening, evaluating gallic acid levels quantitatively with HPLC and purifying it via flash chromatography. These methods were tested using specific observations

that proved their usefulness for isolating and analyzing gallic acid, a well-known therapeutic substance. Using this structure makes the main findings of the study more straightforward to understand.

Table 4. Summary of Results and Key Observations from Extraction, Phytochemical Analysis, and Compound Quantification of Pithecellobium dulce.

Sl. No.	Parameter	Method	Key Findings / Observations
1	Extraction Yield	Soxhlet Extraction (Ethanol)	Yielded approximately 31% extract from 100 g fruit powder; ethanol proved efficient solvent.
2	Crude Extract Concentration	Rotary Evaporator	Brown viscous extract concentrated at 50°C under vacuum; stored at 4°C for further analysis.
3	Preliminary Phytochemical Screening	Reagent-based qualitative tests	Positive for phenols (+++), flavonoids (++), terpenoids (++); ethanol extract showed higher yield.
4	Gallic Acid Quantification	HPLC	Retention time: 2.8 min; concentration in extract: 34.28 µg/mL; high correlation (R2 = 0.9817).
5	Standard Calibration Curve	HPLC (Standard Series)	Linearity observed from 12.5 to 200 µg/mL with regression: y = 1084.7x – 13734.
6	Gallic Acid Purification	Flash Chromatography	Three peaks observed; Peak 2 showed highest gallic acid purity at 280 nm with sharp elution.

CONCLUSION

This research points out that the use of various analytical methods helps efficiently pull out, measure and clean bioactive substances from the fruits of Pithecellobium dulce. When used together, Soxhlet extraction, HPLC and Flash Chromatography resulted in successful and efficient separation of our target phytochemicals, mainly gallic acid. Ethanol in the Soxhlet extraction recovered numerous polar compounds and HPLC was used to accurately measure the amount of gallic acid with consistent results. The gallic acid product was purified further through flash chromatography and the purity was confirmed by reviewing the chromatography results.

The tests revealed that P. dulce fruit has high levels of gallic acid, a phenolic substance known to protect against disease and diabetes. This research highlights the potential of using P. dulce as a source of nutraceuticals and plant-based bioactives in therapy. The approach used in this research could guide future studies on medicinal plants and their active chemicals.

However, while the in vitro quantification and purification results are promising, further research involving in vitro biological assays and in vivo preclinical studies is essential to validate the pharmacological efficacy, safety profile, and therapeutic relevance of gallic acid derived from P. dulce. Such comprehensive evaluations could pave the way for its application in drug development and functional food formulations.

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In silico Analysis of Bioactive Compounds from *Pithecellobium dulce:* A Promising Approach for Targeting Proteins in Rheumatoid Arthritis

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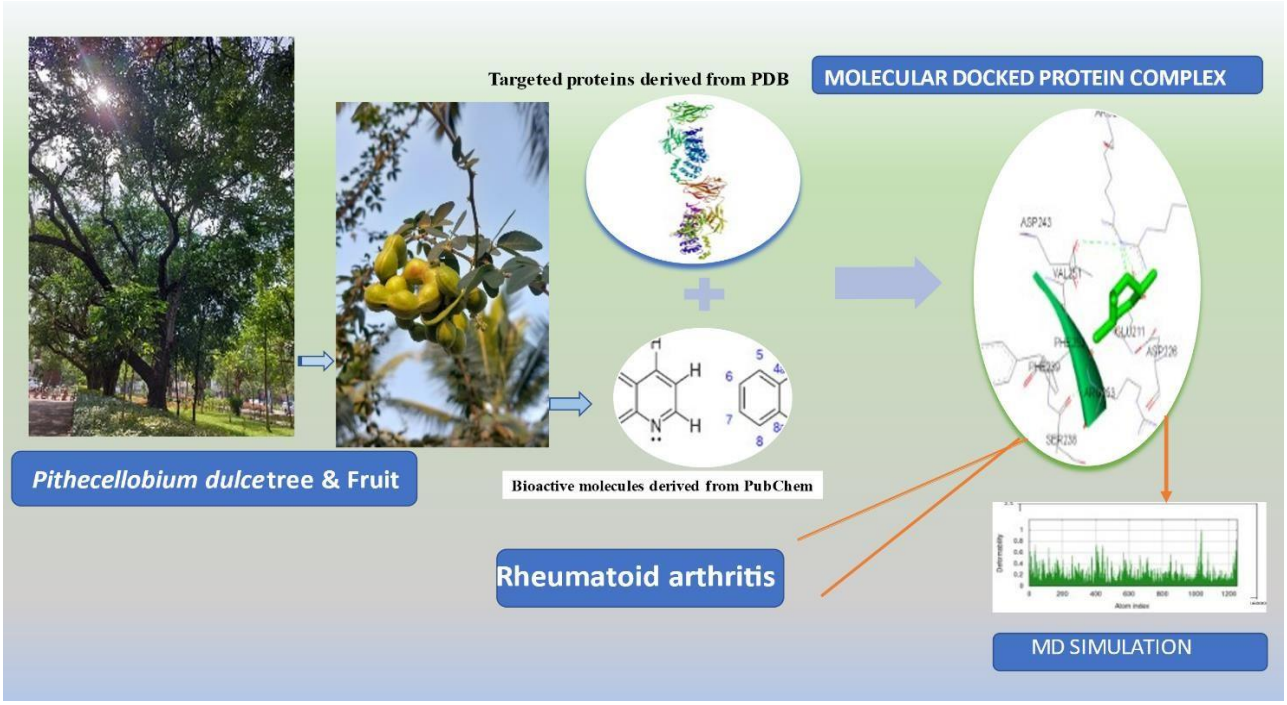
Abstract:
Rheumatoid Arthritis (RA) is a chronic autoimmune disorder that causes joint and bone destruction, severe pain, stiffness and disability, decreasing the quality of life for those affected. The study evaluated Pithecellobium dulce as a potential RA treatment using in silico analysis. Pithecellobium dulce's key bioactive components were tested for their ability to suppress rheumatoid arthritis (RA). Molecular binding experiments were carried out, evaluating their interactions with the new RA target proteins IkBα and MMP1, as well as their drug-likeness and toxicity. To investigate the efficacy and safety of bioactive compounds in the treatment of RA, molecular dynamics (MD) simulations were performed using IMOD software. The selected compounds, assessed through ADMET analysis, exhibited favourable drug-like properties like low toxicity. Additionally, molecular docking demonstrated strong binding energy with the target proteins. Further, MD simulations confirmed the stability and efficacy of the compound-protein complexes. These findings suggest that the bioactive compounds have potential therapeutic value. However, further in vivo and in vitro studies are necessary to determine its effectiveness and to validate its pharmacological activity conclusively.

Keywords: Rheumatoid arthritis, Bioactive compounds, *Pithecellobium dulce*, IkBα, MMP1, H- DOCK.



Graphical Abstract

Rheumatoid Arthritis (RA) is a chronic inflammatory condition that destroys joints and bones, lowering people's quality of life. As a result, there is a critical need for more effective and safer treatments. Natural therapy has the potential to treat RA more effectively, with less toxicity and fewer side effects. *In silico* analysis was used in a study to examine *Pithecellobium dulce* as a potential therapy for rheumatoid arthritis. Two of the eight bioactive compounds demonstrated high binding energy with target proteins indicating potential therapeutic efficacy. However, additional *in vivo* and *in vitro* investigations are required to demonstrate its efficacy and to validate its pharmacological action. Natural medicine may provide safer, more effective treatments for RA.

Introduction

Around 1% of the world's population suffers from rheumatoid arthritis (RA), a chronic inflammatory condition.[27] This autoimmune condition causes painful and uncomfortable symptoms such as joint swelling, stiffness and pain in the hands, wrists, knees and feet. These symptoms significantly impact the individual's quality of life. The pathogenesis of rheumatoid arthritis (RA) involves communication between immune cells and those in the joint.[9] Among these, fibroblast-like synoviocytes (FLS) play a crucial role. FLS are cells of mesenchymal origin that make up the intimal layer of the synovium and regulate local homeostasis while overseeing proper joint functioning. [14] The NF-kB signalling system, which includes RelA (p65), c-Rel, RelB, NF-kB1 (p50) and NF-kB2 (p52), is critical for inflammatory development.

Initially, inhibitory proteins like IkBα keep these factors in the cytoplasm and prevent NF-kB activation. Dysregulated IkBα function in RA causes continual NF-kB activation, leading to inflammation and joint degeneration. When stimulated by pro-inflammatory signals, IkBα degrades, allowing active NF-kB to reach the nucleus and drive gene transcription. In addition, MMP1, a matrix metalloproteinase, remodels the extracellular matrix in RA, contributing to tissue damage. Increased MMP1 activity accelerates joint cartilage breakdown and bone erosion, exacerbating the inflammatory response in RA.[3]

Understanding the functions of IKBα and MMP1 in RA offers an understanding of the dysregulated immune responses, ongoing inflammation and tissue destruction seen in RA.[19] By exploring new, innovative natural bioactive compounds, there is a potential to effectively reduce inflammation and facilitate remodelling processes associated with these proteins. Most commonly used modern medications like DMARDs for treating RA have side effects like nausea, vomiting and weakness, depending upon the drug type used. Thus, looking for alternative medicines with fewer or no side effects is necessary.

Natural bioactive phytochemicals have become popular treatment options for various inflammatory diseases.[1,19] *Pithecellobium dulce*, generally known as Manila tamarind, is a small to medium-sized, spiny, evergreen tree that grows over the Indian plains. *Pithecellobium dulce* fruit is edible and has many calories, protein, fat and carbohydrates.[23] The present study aims to investigate potential active compounds. It is necessary to explore the new drug design to target particular drugs with a 3-D design using different bioinformatics tools and techniques. The study predicts the drug potential and design of the structure of the drug with their atom's interaction and toxicity properties by *in silico* approach.

Material and Methods

Retrieval of the target protein: The 3-D crystal structure of the target proteins IkBα and MMP1 was obtained from the Protein Data Bank. The PDB IDs (https://www.rcsb.org/) were used respectively. The protein structures were then imported into Accelrys Discovery Studio for further analysis. Water molecules, ions, other non-receptor atoms and other substances were eliminated from the systems. The resulting protein structures were then prepared for docking studies.[8,12]

Physicochemical ADME/T properties: The selected active compounds were evaluated based on physicochemical parameters including molecular weight (<500), H-bond donor (HBD), H-bond acceptor (HBA), total number of rotatable bonds (<10), total polar surface area (<140) and atomic molar refractivity (42-130). These qualities are critical for understanding drug's interaction with biological targets, solubility and capacity to cross cell membranes. The SWISS ADMET tool was used to evaluate these characteristics. This tool uses computational methods to estimate a molecule's physicochemical qualities based on its structure. Furthermore, the technique predicts key ADMET characteristics such as human oral availability and brain/blood barrier permeability. BBB permeability is very significant since it impacts a drug's capacity to pass through the blood-brain barrier and reach its target in the brain.[8]

Molecular interaction Studies: Crystal structures of IkBα and MMP1 were obtained from the Protein Data Bank. Any undesirable ligands, linkages, or water molecules were eliminated from the 3D structures of the proteins using Discovery Studio. Ligands such as quinoline, nootkatone, 2,5,6 trimethyl 1,3 oxathiane, trans three methyl's 2N propyl thiophane, tetraneurin-f, ethyl two bromo four methyl's, six dimethyl silibenzophane and 2propyl tetrahydropyran 3-cool were retrieved from the PubChem Database in SDF format.

The selected ligands were docked against the IkBα protein using the HDOCK server (http://hdock.phys.hust.edu.cn).[12] This service provides a complete approach to macromolecular docking, template-based modelling, structure prediction and homology search. It takes both sequences and structures as input and uses intrinsic scoring systems to predict protein-ligand docking. HDOCK uses the following scoring functions:

Molecular Dynamic Simulation studies: This work used molecular dynamics (MD) simulations, a computational tool for evaluating atomic and molecule movement across time, to obtain insight into biological systems' dynamic properties. The iMod server (iMODS) aided these simulations by providing enhanced normal mode analysis (NMA) and a user-friendly interface for exploring potential paths engaging with created objects in 3D. Docking simulations used root mean square deviation (RMSD) to measure structural stability, ligand-protein interactions and binding energies.[22] Overall, MD simulations with iMod facilitated the analysis of dynamic behaviour and binding interactions in ligand-protein complexes, providing valuable insights into structural dynamics and functional consequences

Results

Screening of ligands from database and literature: *Pithecellobium dulce* ligands were screened from the literature and eight were further chosen. Due to their excellent nutritional value and medicinal potential, *P. dulce's* fruits have attracted much interest. Numerous pharmacological activities of *P. dulce's* fruit extract includes anti-diabetic, antioxidant, gastroprotective, anti-inflammatory, hepatoprotective, cardioprotective, anti-bacterial and H+/K+ ATPase inhibitory effects.

The human IKBα protein: IKBα, a protein having 317 amino acids and a molecular weight of 140.6 kDa, was structurally characterised using X-ray crystallography at 2.8 Å resolution. The crystallographic study revealed two chains within the asymmetric unit: chain A (IKB alpha) and chain B (p50 subunit of NF-kappa B). IKBα's ankyrin repeat domain inhibits NF-kappa B-mediated gene transcription via interacting with the p50 subunit's DNA-binding region.

Matrix metalloproteinase-1 (MMP1): MMP1, which has a molecular weight of 85.04 kDa, was structurally characterised using X-ray crystallography at a resolution of 2.15 Å. The asymmetric unit contains chains A (MMP1) and B (Zn^{2+} ion), with one zinc ion attached to MMP1's active site. MMP1 has a catalytic domain and a hemopexin-like domain. The catalytic domain is a tight globular fold with β-sheets and α-helices whereas the hemopexin-like domain has β-sheets and a short α-helix. MMP1's active site contains a zinc ion, three histidine residues and a water molecule. Furthermore, the catalytic domain has a conserved Met-Tyr-Gly-Cys-Thr-Pro-Cys sequence motif, essential for MMP1's enzymatic activity.

Table 1
PubChem details selected bioactive compounds from *P. dulce*

S.N.	Compound name	PubChem id	Molecular weight (g/mol)	Molecular formula
1	Quinoline	7047	129.16	C_9H_7N
2	Nootkatone	1268142	218.33	$C_{15}H_{22}O$
3	2,5,6-Trimethyl1,3 oxathiane	548225	146.25	$C_7H_{14}OS$
4	Trans three methyl, 2N-propylthiophane	6429953	140.25	$C_8H_{12}S$
5	Tetraneurin-f	01306773	366.4	$C_{19}H_{26}O_7$
6	Ethyl 2-bromo-4-methyl-6-dimethylsilylbenzothiophe 5-carboxylate	2824057	250.12	$C_7H_8BrNO_2S$
7	2-Propyltetrahydropyran-3-ol.	541755	144.21	$C_8H_{16}O_2$
8	Quercetin	5280343	302.23	$C_{15}H_{10}O_7$

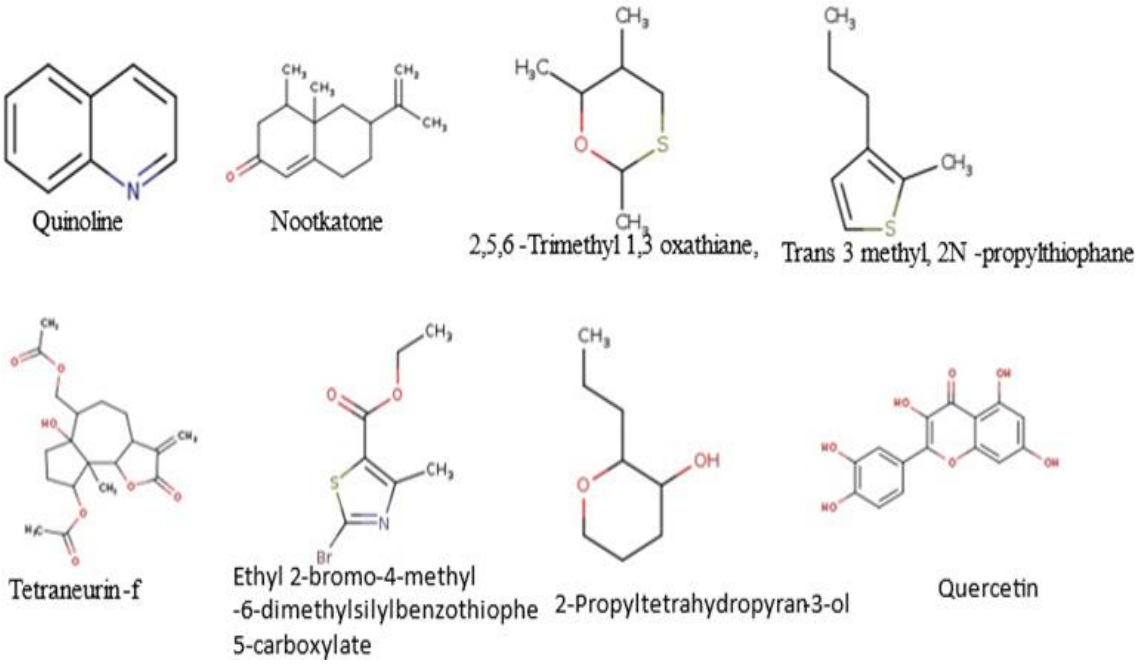


Figure 1: Chemical structure of selected 7 molecules by PubChem

Table 2
Selected targeted proteins

S.N.	Name of the Proteins	PDBID	Class	Molecular weight (g/mol)
1	IKBα	1NFI	Homo sapiens	140.6 kDa
2	MMP1	2CLT	Homo sapiens	85.04kDa

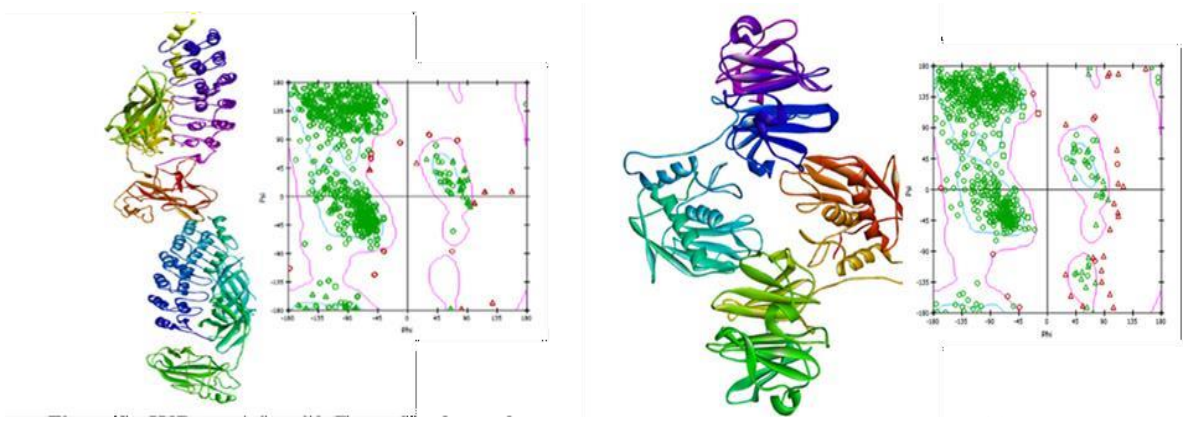


Figure 2: IkBα protein with Ramachandran plot and MMP1 protein with Ramachandran plot
The Ramachandran plot: Shows energetically allowed regions for backbone angles in protein structure, indicating favorable or unfavorable confirmations. It helps to evaluate protein structure quality and identify errors. Favored regions are energetically stable (green), while disallowed regions are unfavorable (red)

Table 3
DME/T analysis of the selected compounds

Name of the Compounds	No. H-bond acceptors	No. H-bond donor	TPSA <140	Class	GI absorption	BBB permeant	Lipinski rule	Violation
Quinoline	1	1	12.89 Å²	Soluble	High	Yes	Yes	0
Nootkatone	1	1	17.07 Å²	Soluble	High	Yes	Yes	0
2,5,6-Trimethyl1,3 oxathiane	1	1	34.53 Å²	Soluble	High	Yes	Yes	1
Trans three methyl, 2N-propylthiophane	0	0	28.24 Å²	Soluble	High	Yes	Yes	0
Tetraneurin-f	7	7	99.13 Å²	Soluble	High	No	Yes	0
Ethyl 2-Bromo -4-methyl-6-dimethylsilylbenzothiophe 5-carboxylate	3	3	67.43 Å²	Soluble	High	Yes	Yes	0
2-Propyltetrahydropyran-3-ol.	2	2	29.46 Å²	Very soluble	High	Yes	Yes	0
Quercetin	7	5	131.36 Å²	Soluble	High	No	Yes	0

Screening of ADME/T properties by SWISS ADMET online tool: The SWISS ADMET tool revealed that the drug candidates had favourable absorption and distribution qualities which complied with Lipinski's rule, a significant determinant in their efficacy. However, medications that did not follow this guideline were rejected due to possible solubility and permeability concerns. A selection of the remaining candidates underwent additional docking research to find favourable ligand-protein interactions. These data emphasise the need for further optimisation to improve the ADME/T characteristics of these medication candidates.

Molecular interaction studies: This work used molecular docking to analyse the binding and energy of chosen ligands with target proteins IKBα and MMP1. The top protein-ligand complexes were found based on intrinsic scoring functions. All selected ligands showed promising activity with IKBα and MMP1 having the highest affinity at the

active binding site. The binding energy of selected ligands was estimated using the Discovery Studio Visualizer tool and receptor-ligand interactions were further analysed to identify their 2D structure. The study identifies binding locations on IKBα and MMP1 surfaces that promote stable complex formation through Van der Waals interactions and conventional bonds. These data suggest that these ligands have potential for treating RA, but more research is needed.

Molecular dynamics simulation: The iMod server used molecular dynamics simulations and normal mode analysis to evaluate protein and ligand movements in docked complexes including quinoline, quercetin, IkBα and MMP1. Peaks on the main-chain deformability graph indicated extremely malleable regions, with atomic index 1000 having the highest peak at a deformability value of one.

iMod determined the B-factor values which indicate protein structural flexibility and temperature fluctuations. Higher B-factor values suggest more atomic mobility and conformational changes whereas lower values imply inflexible regions with minimal mobility which helps to identify functionally significant portions within the docked complex.

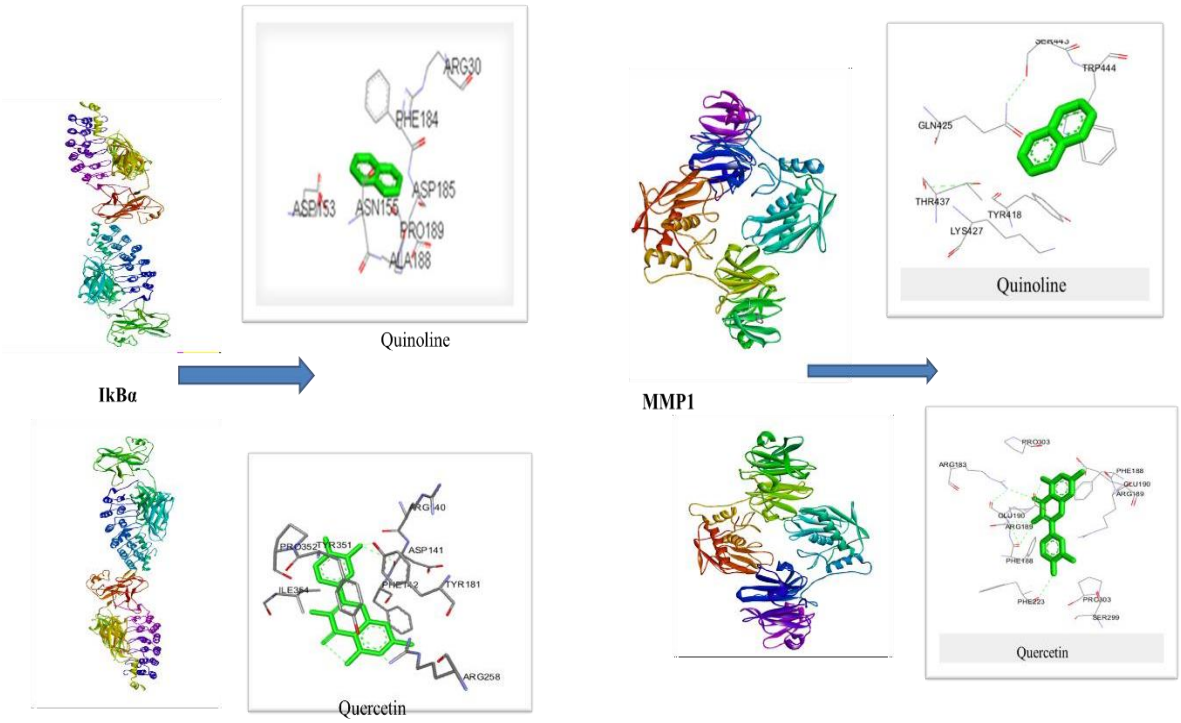


Figure 3: Structure in three dimensions with PBD ID (IkBα and MMP1), molecular interaction with quinoline and quercetin by H-dock server

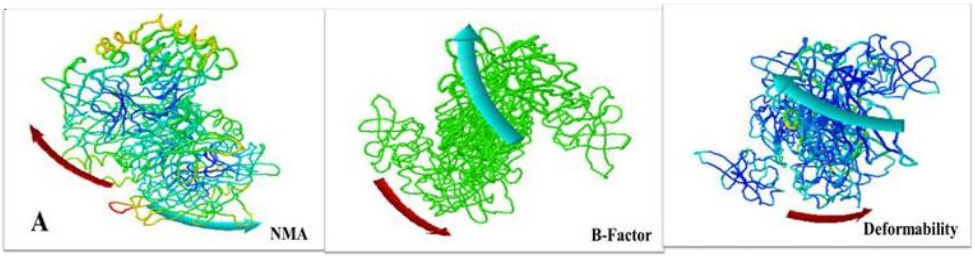
Table 4
Docking score of each of the compounds with IkBα and MMP1 protein

S.N.	Name of the compounds	IKBα			MMP1		
		Binding energy (kcal/mol)	Confidence Score	Ligand Rmsd A^0	Binding energy (kcal/mol)	Confidence Score	Ligand Rmsd A^0
1	Quinoline	-68.23	0.163	149	-84.38	0.212	82.68
2	Nootkatone	-79.97	0.197	100	-81.71	0.203	53.94
3	2,5,6-Trimethyl1,3 oxathiane	-57.33	0.135	118.74	-64.63	0.153	62.69
4	Trans 3 methyl, 2N-propylthiophane	-90.62	0.23	148.64	-108.42	0.303	67.59
5	Tetraneurin-f	-91.72	0.237	99.00	-95.3	0.251	54.76
6	Ethyl 2-bromo-4-methyl-6-dimethylsilylbenzothiophe 5-carboxylate	-64.45	0.153	65.76	-61.7	0.146	55.01
7	2-Propyltetrahydropyran-3-ol	-149.02	0.49	142.64	-136.4	0.43	54.84
8	Quercetin	-154.26	0.52	62.01	-152	0.511	53.11

Eigen values produced from iMod indicate vibrational frequencies associated with collective atom motions which provide information about system dynamics, flexibility and structural changes. Lower Eigen values indicate slower global motions, but higher Eigen values suggest faster localised vibrations. The covariance map in iMod displays a value matrix, with each element representing the covariance between pairs of atoms. The values are commonly represented by the covariance matrix specifies whether two residues are correlated (red), uncorrelated (white), or anti-correlated (blue).

iMod simulations using quinoline and quercetin docking indicate MMP1 protein flexibility, particularly at the hinge. B-factor values reflect adaptability: lower values imply rigidity while higher values indicate increased mobility and structural changes. The B-factor graph helps comprehend docked complexes by demonstrating atom flexibility. Eigen values from iMod show MMP1 dynamics and structure-function relationships with higher values indicating faster vibrations and lower values indicating slower motions.

The covariance map in iMod depicts atomic pair interactions in MMP1. Correlations are represented by red, no correlations by white and anticorrelations by blue. It indicates regions with correlated or anti-correlated movements, which are most likely relevant to protein activity. This map improves our understanding of MMP1 dynamics and structure-function relationships.



Molecular mobility evaluated by NMA of the docked complexes: IkBα with A) Quinoline,

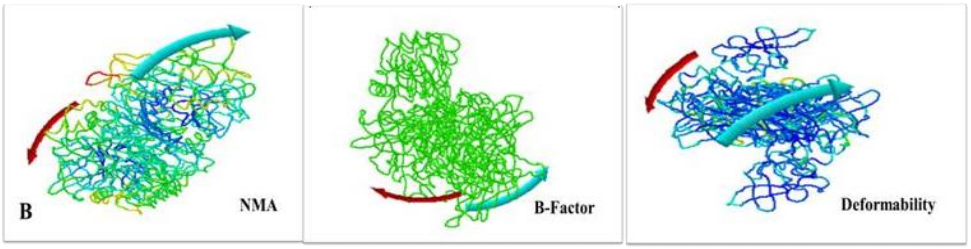


Figure 4: Molecular mobility evaluated by NMA of the docked complexes: IkBα with B) Quercetin

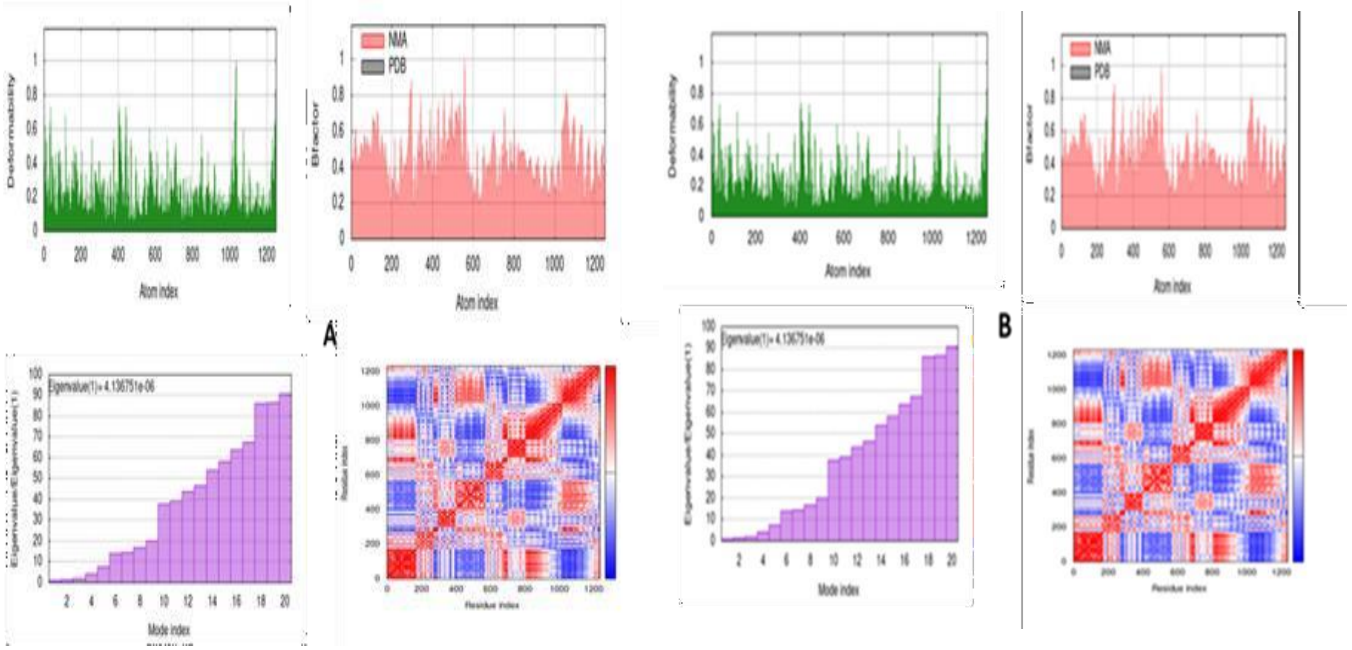


Figure 5: Outputs of Molecular dynamic simulation in iMODS for IkBα with (A) Quinoline and (B) Quercetin deformability factor plot, Eigen value, Variance plot and Covariance plot

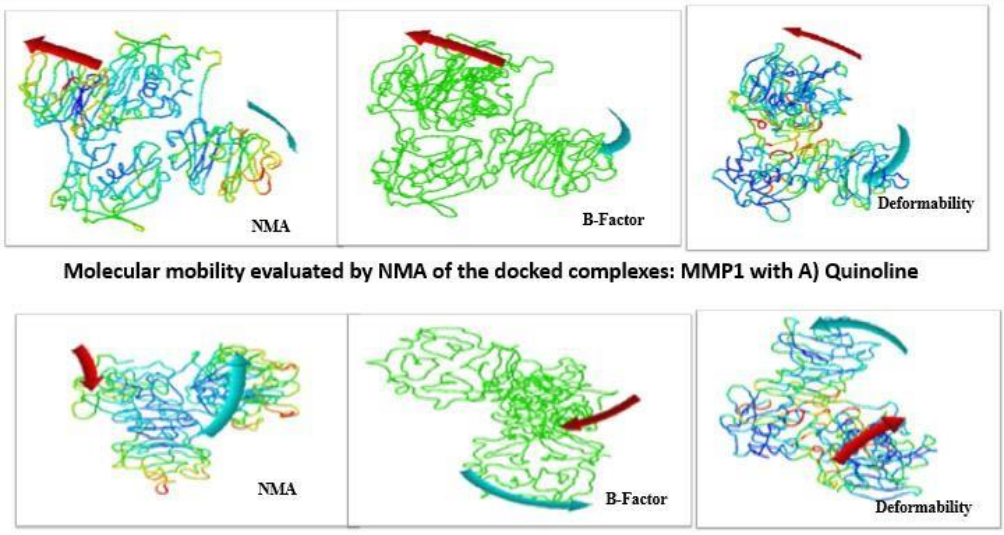


Figure 6: Molecular mobility evaluated by NMA of the docked compexes: MMP1 with Quercetin

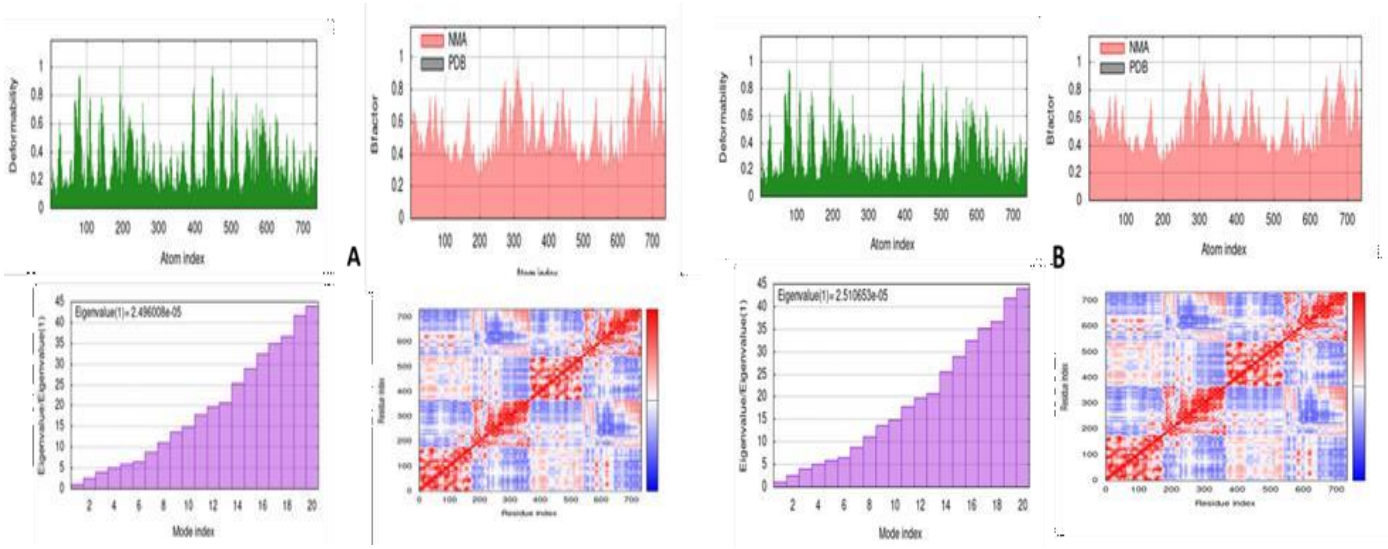


Figure 7: Outputs of molecular dynamics simulations in iMODS for MMP1 with (A) Quinoline and (B) Quercetin: deformability-factor plot, Eigen value and Variance plot and Covariance map

Discussion

Millions of people throughout the world suffer with rheumatoid arthritis (RA), a chronic autoimmune illness. Constant inflammation is one of its defining features and various symptoms that significantly diminish the quality of life for those affected.[27] The development of RA is believed to be influenced by various factors including genetic, epigenetic, environmental, metabolic, immunological and microbial factors.[13] The interaction between various immune cells also plays a role in the development of RA, with different behaviour depending on the specific disease context and microenvironment.

Biological DMARDs target extracellular factors to successfully treat RA inflammation whereas NSAIDs only provide brief pain relief. As a result, over 60% of RA patients seek alternative herbal therapies for long-term treatment.[15]The hunt for natural bioactive compounds with few side effects has escalated as a result of the health hazards associated with inflammation and the growing interest in natural alternatives to synthetic medications. These compounds should promote leukocyte recruitment, improve microvascular function, raise vascular permeability and decrease the release of pro-inflammatory cytokines.[23]

Inflammation sets off a series of biochemical reactions involving adjacent blood arteries, the immune system and numerous cells in the damaged tissue. Chronic inflammation occurs when inflammation persists, causing persistent changes in tissue composition characterised by a cycle of tissue damage and repair. Experimental procedures are employed.[16] Our research aimed to uncover useful nutraceuticals from the *Pithecellobium dulce* plant that could successfully cure arthritis while posing minimal biological hazards. We used *in silico* drug discovery, which makes use of existing drug databases, to find new medications for conditions such as arthritis. Molecular docking investigation found that IkBα and MMP1 proteins have a significant role

in lowering RA inflammation, highlighting the potential of natural medicines in controlling inflammation-related illnesses. More *in vivo* and *in vitro* researches are required to confirm the efficacy of these compounds.

Conclusion

Our findings suggest that quinoline and quercetin are attractive chemicals for producing anti-inflammatory supplements. Their nutritional and pharmacological qualities make them ideal candidates for therapeutic intervention in a variety of disorders. Molecular docking study indicates a considerable affinity for targeted proteins, specifically IkBα and MMP1, which are important in the pathogenesis of inflammatory disorders. Furthermore, ADME/T study showed low toxicity, ensuring safety for rheumatoid arthritis supplements. However, more in vivo and *in vitro* researches are required to completely evaluate their usefulness. Our findings help to better understand the therapeutic potential of quinoline and quercetin from *Pithocellobium dulce* and establish the framework for future research in this area.

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3.	कृति के प्रतिलिप्यधिकार में आवेदक के हित की प्रकृति Nature of the applicant's interest in the copyright of the work	:	OWNER
4.	कृति का वर्ग और वर्णन Class and description of the work	:	LITERARY/ DRAMATIC WORK
5.	कृति का शीर्षक Title of the work	:	EFFECT OF BIOACTIVE COMPOUND FROM PITHECELLOBIUM DULCE (PD) ON LIPOPOLYSACCHARIDE INDUCED RHEUMATOID ARTHRITIS CELL LINE
6.	कृति की भाषा Language of the work	:	ENGLISH
7.	रचयिता का नाम, पता और राष्ट्रीयता तथा यदि रचयिता की मृत्यु हो गई है, तो मृत्यु की तिथि Name, address and nationality of the author and if the author is deceased, date of his decease	:	DR. NILIMA DONGRE , DEPARTMENT OF BIOCHEMISTRY, SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE, BLDE - DEEMED TO BE UNIVERSITY, SMT. BANGARAMMA SAJJAN CAMPUS, B. M. PATIL ROAD, SOLAPUR ROAD, VIJAYAPURA, K-586103 INDIAN DR. PRACHI PARVATIKAR , DEPARTMENT OF BIOTECHNOLOGY, SCHOOL OF ALLIED HEALTH SCIENCE, BLDE - DEEMED TO BE UNIVERSITY, SMT. BANGARAMMA SAJJAN CAMPUS, B. M. PATIL ROAD, SOLAPUR ROAD, VIJAYAPURA, KARNATAKA-586103 INDIAN MRS. SOUMYA TUNGAL , DEPARTMENT OF BIOCHEMISTRY, SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE, BLDE - DEEMED TO BE UNIVERSITY, SMT. BANGARAMMA SAJJAN CAMPUS, B. M. PATIL ROAD, SOLAPUR ROAD, VIJAYAPURA, K-586103 INDIAN
8.	कृति प्रकाशित है या अप्रकाशित Whether the work is published or unpublished	:	UNPUBLISHED
9.	प्रथम प्रकाशन का वर्ष और देश तथा प्रकाशक का नाम, पता और राष्ट्रीयता Year and country of first publication and name, address and nationality of the publisher	:	N.A.
10.	बाद के प्रकाशनों के वर्ष और देश, यदि कोई हों, और प्रकाशकों के नाम, पते और राष्ट्रीयताएँ Years and countries of subsequent publications, if any, and names, addresses and nationalities of the publishers	:	N.A.
11.	कृति में प्रतिलिप्यधिकार सहित विभिन्न अधिकारों के स्वामियों के नाम, पते और राष्ट्रीयताएं और समनुदेशन और अनुज्ञप्तियों के विवरण के साथ प्रत्येक के अधिकार का विस्तार, यदि कोई हो। Names, addresses and nationalities of the owners of various rights comprising the copyright in the work and the extent of rights held by each, together with particulars of assignments and licences, if any	:	BLDE - DEEMED TO BE UNIVERSITY , SMT. BANGARAMMA SAJJAN CAMPUS, B. M. PATIL ROAD, SOLAPUR ROAD, VIJAYAPURA, KARNATAKA-586103 INDIAN
12.	अन्य व्यक्तियों के नाम, पते और राष्ट्रीयताएं, यदि कोई हों, जो प्रतिलिप्यधिकार वाले अधिकारों को समनुदेशित करने या अनुज्ञप्ति देने के लिए अधिकृत हों Names, addresses and nationalities of other persons, if any, authorised to assign or licence of rights comprising the copyright	:	BLDE - DEEMED TO BE UNIVERSITY , SMT. BANGARAMMA SAJJAN CAMPUS, B. M. PATIL ROAD, SOLAPUR ROAD, VIJAYAPURA, KARNATAKA-586103 INDIAN
13.	यदि कृति एक 'कलात्मक कृति' है, तो कृति पर अधिकार रखने वाले व्यक्ति का नाम, पता और राष्ट्रीयता सहित मूल कृति का स्थान। (एक वास्तुशिल्प कृति के मामले में कृति पूरी होने का वर्ष भी दिखाया जाना चाहिए) If the work is an 'Artistic work', the location of the original work, including name, address and nationality of the person in possession of the work. (In the case of an architectural work, the year of completion of the work should also be shown).	:	N.A.
14.	यदि कृति एक 'कलात्मक कृति' है जो किसी भी माल या सेवाओं के संबंध में उपयोग की जाती है या उपयोग किए जाने में सक्षम है, तो आवेदन में प्रतिलिप्यधिकार अधिनियम, 1957 की धारा 45 की उप–धारा (i) के प्रावधान के अनुसार व्यापार चिह्न रजिस्ट्रार से प्रमाणन शामिल होना चाहिए। If the work is an 'Artistic work' which is used or capable of being used in relation to any goods or services, the application should include a certification from the Registrar of Trade Marks in the provision to Sub-Section (i) of Section 45 of the Copyright Act, 1957.	:	N.A.
15.	यदि कृति एक 'कलात्मक कृति' है, तो क्या यह डिजाइन अधिनियम 2000 के अंतर्गत पंजीकृत है? यदि हां, तो विवरण दें। If the work is an 'Artistic work', whether it is registered under the Designs Act 2000, if yes give details.	:	N.A.

उमा पी पंडित
Registrar of Copyrights

16. यदि कृति एक 'कलात्मक कृति' है, जो डिजाइन अधिनियम 2000 के तहत एक डिजाइन के रूप में पंजीकृत होने में सक्षम है, तो क्या यह औद्योगिक प्रक्रिया के माध्यम से किसी वस्तु पर प्रयुक्त की गई है और यदि हाँ, तो इसे कितनी बार पुनरुत्पादित किया गया है?
If the work is an 'Artistic work', capable of being registered as a design under the Designs Act 2000.whether it has been applied to an article though an industrial process and ,if yes ,the number of times it is reproduced. : N.A.

17. टिप्पणी, यदि कोई हो/Remarks, if any :

डायरी संख्या/Diary Number: 32009/2024-CO/L

आवेदन की तिथि/Date of Application: 14/10/2024

प्राप्ति की तिथि/Date of Receipt: 14/10/2024

Registrar of Copyrights

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Department of Microbiology and Biotechnology

NATIONAL CONFERENCE ON
ADVANCES IN BIOTECHNOLOGY AND NANOTECHNOLOGY
14 & 15th March, 2023

Certificate of Merit

This is to certify that Dr./Mr./Ms. SOUMYA TUNGAL of Dept of Biochemistry, B.M.Patil Medical college has presented a Oral / Poster in the National Conference on Advances in Biotechnology and Nanotechnology held during 14 and 15th March, 2023 at Department of Microbiology and Biotechnology, Karnatak University, Dharwad.

He / She has been selected for First/Second/Third Place.

Prof. A. B. Vedamurthy
Organizing Chairman

Prof. V. Shyam Kumar
Organizing Secretary

Certificate of Appreciation

This certificate is awarded to

Soumya Tungal

for participating in Poster/Oral presentation in the

First National Symposium on Integrating Traditional Knowledge in Evidence Based Medicine

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XXXIVth Annual Conference at BLDE (DU), Vijayapura

Certified that Soumya Jungal
of Shri B.M. Patil Medical College B.L.D.E (Deemed to be University)
has been awarded VIII Organising Committee (1996), PSI, Vidyasagar University (Midnapore) Medal Award *for the best paper presentation entitled* In Silico Screening of Pithecellobium dulce Bioactive Compounds as MMP1 inhibitors for Rheumatoid Arthritis treatment.
in the year 2023 among the young scientists.

3rd November 2023

Prof. Amar K Chandra
President

Rajen Haldar
Hon. General Secretary

REVA UNIVERSITY
Bengaluru, India

CERTIFICATE OF PARTICIPATION

School of Allied Health Sciences

This is to certify that

Dr/Ms/Mr. Soumya Jangal has presented/ participated on Extraction, Quantification, and In-Silico pharmacokinetic Profiling of Bioactive compounds from Pithecellobium dulce. in the National Conference on **"Current Trends in Biological & Health Sciences"** organised by School of Allied Health Sciences, REVA University on December 11 & 12, 2024.

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