Analyzing Anti-Rheumatic Properties of Novel Medicinal Flora (*Dodonaea viscosa)* through Expression Analysis

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A thesis submitted in partial fulfilment of the requirement for the degree of Master of Science in Healthcare Biotechnology

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I certify that this research work titled "**Analyzing anti-rheumatic properties of novel medicinal flora (*Dodonaea viscosa*) through expression analysis**" is my work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/referred to.

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DEDICATION

This thesis is dedicated to my parents, **Munawar Islam and Azra Munawar**, whose encouragement and guidance has been unmatched. I would like to thank them for always believing in me and helping me relentlessly to achieve my dreams.

Moreover, I would also like to dedicate this dissertation to my **sisters** for being my ultimate support system throughout this journey.

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Huda Munawar

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LIST OF ACRONYMS

ACPA	Anti-citrullinated protein antibodies
BALB	Bagg Albino Mice Strain
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CRP	C- reactive protein
D. viscosa	*Dodonaea viscosa*
DMARDs	Disease-modifying anti-rheumatoid drugs
DPPH	1, 1-diphenyl- 2-picrylhydrazyl assay
EULAR	European League Against Rheumatism
FRAP	Ferric reducing antioxidant power
IL	Interleukin
MHC	Major histocompatibility antigens
MMPs	Matrix metalloproteinases
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate Buffer Saline
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid factors
STAT	Signal transducer and activator
T2DM	Type-2 Diabetes mellitus
TNF-a	Tumor necrosis factor-alpha

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder characterized by bone and cartilage damage. Several factors are involved in the onset and progression of the disease including environmental and genetic factors. Currently, there is no cure for rheumatoid arthritis, however, conventional treatments like disease-modifying anti-rheumatoid drugs, non-steroidal anti-inflammatory drugs, and glucocorticoids are available associated with various side effects. In ancient times, medicinal plants were used for prolonged treatments to combat various diseases. Medicinal herbs containing therapeutic phytochemicals are cost-effective and have fewer side effects can be a great alternative for RA treatment.

Dodonaea viscosa is traditionally used in various pathological conditions such as anti-inflammatory, analgesic, spasmolytic, antiviral, anti-microbial, laxative, rheumatism, hypotensive, gout, fractures, wound healing, hemorrhoids, and snake bites. This study focuses on analyzing *Dodonaea viscosa* for its anti-rheumatic potential.

In the present study the phytochemical analysis was performed, the ethanolic extract of the plant showed multiple bioactive compounds presence including phenols, cardiac glycosides, tannins, coumarins, steroids, sterols, terpenoids, saponins, flavonoids, deoxysugars, carbohydrates and resins. The extract contains antioxidant and anti-inflammatory properties assessed by percentage inhibition of albumin denaturation assay and DPPH (1, 1-diphenyl- 2-picrylhydrazyl) assay.

The anti-arthritic potential of the plant extract was further confirmed using collagen induced arthritis (CIA) BALB/c mouse models. During the in-vivo and ex-vivo studies, plant extract presented no toxic effects confirmed by acute toxicity (LD_{50}). The mice models were evaluated by different clinical parameters before and after RA induction and treatment by the plant. *D.viscosa* treated groups showed significant decrease in paw measurements compared to the arthritic groups. The x-rays of treated groups presented less inflammation of soft tissues and less bone destruction indicating the protective function of the plant. The group treated with plant showed serum levels for Rheumatoid factor score within the normal range. Similarly, the liver catalase test indicated less ROS species production in the plant extract treatment group proving its therapeutic potential.

Further studies were performed to assess the Interleukin-6 expression through Sandwich ELISA and Real-time PCR. During both qualitative, and quantitative studies, significantly lower concentrations of of interleukin-6 were observed in treatment groups (*D.viscosa*) compared to arthritic group proving the drugs to be effective for RA. The study confirmed the anti-arthritic potential of *Dodonaea viscosa* plant through in-vitro and in-vivo analysis. Further in-silico studies can be performed to elucidate the underlying mechanism.

INTRODUCTION

1.1 Rheumatoid Arthritis (RA)

Rheumatoid Arthritis (RA) is an autoimmune disease that causes severe inflammatory arthropathy. It is a syndrome associated with bone and cartilage damage leading to disability, pulmonary vasculitis, systemic complications along with increasing socioeconomic decline and mortalities (Murphy et al., 2008). The treatment for RA has been revolutionized with the discovery of novel therapeutics transforming the articular and systemic outcomes (Aletaha et al., 2010).

The pathogenesis of this disease involves an interplay of genetic, epigenetic, and environmental factors. Twin studies indicate substantial contribution of genetic factors in RA, with 5% concordance rate among dizygotic twins and 15% among monozygotic twins (Aho et al., 1986; Macgregor et al., 2000). RA has prevalence of 1% worldwide making it the most common inflammatory arthritis. The RA prevalence is observed to be predominant in developed countries in comparison with developing countries (Goulielmos et al., 2016).

Many studies indicate gender as a predictor of RA incidence that is observed to be thrice in women than men. Moreover, it is reported that RA symptoms are worse in females in comparison to the males because of association with hormonal factors and the disease. Thus, females are less likely to attain remission of RA. The difference indicates that the female body is more susceptible to autoimmune diseases due to more reactive and strong immune system than the males. However, the link between gender influence on the occurrence of disease is still unclear (Sokka et al., 2009).

The susceptibility of onset of RA is not dependent on age (Crowson et al., 2011). Most epidemiological studies have shown onset of the disease in the fifth decade of life. Whereas some studies indicate a later onset of the disease (T Riise et al., 2000).

Autoimmune diseases have no cure, and the treatments should be taken for lifetime to help reduce progression of the disease. The current available treatment includes DMARDs, NSAIDs, and corticosteroids. These conventional drug treatments have many adverse side effects, limited efficacy, and high costs (Majithia & Geraci et al., 2007). The ineffectiveness of all available biologics in RA patients attests to the heterogeneity of the disease. Thus, new approaches should be developed to minimize the side effects and provide quality of life to the RA patients.

1.1.1. Rheumatoid Arthritis Types

RA is associated with immune dysregulation. Evidence suggests that RA has different serotypes based on the role of circulating autoantibodies. Despite the clinical overlap of seropositive and seronegative RA serotypes, both reflect different entities of the disease with respect to genetic features, cellular pathology, and response to the therapy (Aletaha et al., 2010b).

1.1.1.1 Seropositive Rheumatoid Arthritis

The term RA seropositivity refers to the presence of immunoglobulin M (IgM) rheumatoid factor (RF) and of circulating anti-citrullinated protein antibodies (ACPAs) that are independently involved in joint destruction (Schellekens et al., 2000). These peptides play the role of autoantigens to activate the T cells leading to production of a pro-inflammatory environment. This further activates the B cells to generate autoantibodies causing joint and bone damage (Meyer et al., 2006). Considering ACPA along with RF status in a patient can improve the early detection (a few years before the disease onset (Aletaha et al., 2010; De Stefano et al., 2021).

1.1.1.2 Seronegative Rheumatoid Arthritis

Seronegative RA patients are tested negative for RF and/or ACPA. However, many studies suggest the resemblance of seronegative patients with seropositive patients regarding clinical features and risk factors after different screenings for IgA/IgG RF and ACPA fine-specificities (Reed et al., 2020).

Yet both serotypes are independently identified based on genetic and environmental factors along with presence of cytokines in synovial fluid. The primary phase of the disease in seronegative patients is more vigorous and shows weak therapeutic responsiveness (Trouw et al., 2013). Almost one-third of rheumatoid patients are seronegative for RA markers (Somers et al., 2011). A new potential biomarker other than RF and ACPAs is being identified in both seropositive and seronegative population but not in healthy controls. It can be recognized as a new serum biomarker for seronegative RA as they lack the presence of RF and ACPAs (Bason et al., 2021).

1.2 Epidemiology

The worldwide prevalence of RA is around 0.24%. However, it is estimated to be higher in the United States and other northern European countries of around 0.5-1%. Most of the data on RA comes from these countries due to more studies being conducted. The estimated annual incidence rate in United States and other European countries is 40 per 10,0000 individuals (McInnes & Schett et al., 2017)

From years 1980 to 2019, estimated RA global prevalence was 460 individuals per 100,000 people. Thevariation in the reported data is observed because of differences in research methodology and geographical location (Almutairi et al., 2021). Throughout the world, RA is reported to be developed three times more in female than the males. The reason behind this is still unknown. However, accordingto some studies, low testosterone and increased prolactin levels during breastfeeding increasing the chances of the development and progression of RA (Colquhoun et al., 2022a; Minichiello et al., 2016). The mortality rate of RA patients is expected to be decreased by 10-14 years.Although, the early diagnosis of RA in patients reduces the risks and increases the life expectancy.

In America, the RA prevalence is reported to vary from 0.9 % in the Canada and 0.6 % in the US (Cross,Smith, Hoy, Nolte, et al., 2014). In Africa, the RA prevalencedata is very scarce, however, the reported studies show very heterogenous data because of geographical and regional variations (Slimani & Ladjouze-Rezig et al., 2014). Disease prevalence in Asia measured to be 0.75 % in India, 0.142 % in Karachi (Pakistan), 0.6 %-1 % in Japan and 0.26 in South Korea (Malaviya et al., 1993; Abbas Naqvi et al., 2017; Dougados et al., 2014).

In Pakistan, a study reported RA prevalence to be 26.9 % in Karachi with an average 44 years of age. Another study showed a significant correlation between clinical depression and disability in the patients (S. M. Alam et al., 2011).

1.3 Etiology of RA

Autoimmune diseases, like RA, are of unknown origin. In the early times, the onset of RA was only linked with the role of genetic and epigenetic factors. However, current studies confirmed the correlation of environmental factors with the inception of the disease (Karami et al., 2019a).

1.3.1 Genetic Risk Factors

Studies have estimated the heritability of RA to be approximately 60 % affecting both male and female (Alamanos & Drosos et al., 2005). The major genetic factors implicated in the pathogenesis of the disease are human leukocyte antigens (HLA) and non-HLA genes. Both HLA and non-HLA genes are associated with autoimmunity to anti-citrullinated protein antibody (ACPA) (Szodoray et al., 2010).

1.3.1.1 HLA Genes

Human Leukocyte Antigen (HLAgenes) have major role in body's defense against immune system anddiseases. Human Leukocyte Antigen – antigen D Related is a cell surface receptor of MHC ClassII encoded by human leukocyte antigen complex on chromosome 6 region 6p21.31. Some HLA alleles,such as HLA- DRB1 (DR4 and DR1) are highly linked with RA (Karami et al., 2019b).

The haplotypes (HLA-DRB1*04, DRB1*10, and DRB1*01) produce a "shared epitope (SE)" made upof 5 amino acid motifs: QKRAA, QRRAA or RRRAA in DRB1 chain resulting in increased susceptibility to RA. The SE codes for the positivity charged P4 peptide bonding pocket (Gregersen etal., 1987). The shared epitope bearing HLA-DRB1 haplotype acts as identification markers in seropositive or ACPA- positive RA patients. However, the non-shared epitope bearing haplotypes suchas HLA-DR3 act as markers for seronegative or ACPA negative RA (Scherer et al., 2020). The SE HLA-DRB1 alleles contribute 11 % to the genetic variance of the disease (Van Der Woude et al., 2009).

1.3.1.2 Non-HLA Genes

Other than HLA-DR alleles, many studies suggested the strong link between the non-HLAgenes with the susceptibility of the disease. Several studies identified the contribution of 31 non-HLA loci to RA risk. The second strongest association after HLA-DRB1 is shown by the gene encoding the intracellular phosphatase protein tyrosine phosphatase non-receptor type 22 (PTPN22). The associationof this allele is found with many other autoimmune diseases such as type I diabetes, lupus, systemic sclerosis, Addison's disease, and others (Goëb et al., 2008). PTPN22 is implicated in the signalling of T and B cells (Qamar & Mukherjee et al., 2021; Rieck et al., 2007). Other than PTPN22, strong linkage of IL23R genes is confirmed with RA predisposition. (Faragó et al., 2008). PADI4 haplotype association with RA was observed in Asian population. *PADI4* gene encodes for the peptidylarginine-deiminase 4 (PADI4) enzyme that plays role in protein citrullination, an important factor contributing to the development of the disease (Cha et al., 2007).

1.3.2.1 Environmental Factors

Various studies confirmed the involvement of lifestyle-related factors and environmental factors in the development and progression of RA.

1.3.2.1.1 Smoking

Tobacco exposure is one of the strongest environmental features that shows a consistent association with RA. In various geographical regions, smoking is associated with the developmentof extraarticular manifestations, such as cardiovascular complications and nodulosis (Lee et al., 2007). The relative risk of RF-seropositive RA in individuals with SE genes was estimated to be 2.8% in non-smokers, 7.5% in smokers. Moreover, smokers with two SE genes showed 15.7 relative risk of RF-seropositive RA (Padyukov et al., 2004). Tobacco smoke promotes synovial proteins citrullination leading to ACPA formation. In Western and Northern European cohorts, smokers carrying SE alleles showed increased ACPA positive RA chances (Pedersen et al., 2006).

1.3.2.1.2 Gut microbiota

The composition of the microbiota in RA patients is altered. This leads to the activation of intestinal TH17 cells that further promotes arthritis development in mice (Giannini et al., 2020).

Numerous infectious agents such as E coli, Proteus species EBV, heat-shock proteins, cytomegalovirus and many others are associated with RA. This association is linked due to similarities in the sequence between foreign and self-pathogen derived peptides causing production of autoreactive T and B cells. This phenomenon is known as molecular mimicry. Immune complexes formed during infection result in production of RF factor that is a strong affinity autoantibody against IgE and IgG (Kharlamova et al., 2016). Other gut microbiota such as Proteus mirabilis and Klebsiella pneumonia are linked withseropositive RA (Taneja et al., 2014).

1.3.2.1.3 Hormones

RA incidence in females is more common than in males. The actual mechanism is still unknown, however, there must a female hormonal variable than is impacting the risk of RA. The majorfactors that appear to be contributing to RA are early menopause, polycystic ovary syndrome, use of oral contraceptives and others. Moreover, estrogen also plays a role in RA development as it is involvedin bone formation and maturation. It is reported that pregnancy and breastfeeding reduce the risk of RAdevelopment. However, single women are less likely to develop RA (Alpízar-Rodríguez & Finckh et al., 2017; Deane et al., 2017).

1.3.2.1.4 Role of Diet

Multiple dietary factors are variably associated with RA such as lack of vitamin D in diet, higher intakeof red meats, sugar, sodium, iron, and protein enhances the risk for susceptibility of RA (Merlino et al., 2004; Benito-Garcia et al., 2007). The consistent finding across different studies identified increased.

Intake of fish and omega-4 fatty acid reduces the risk for RA. Studies identified that by adding supplements like omega-3 fatty acid and other fatty acids biomarkers are found to reduce the progression of the disease (Linos et al., 2009).

Vitamin deficiency is highly observed in RA patients. Vitamin D plays a powerful anti-inflammatory role that leads to the reduction of IL-17 and IFN+T cells that are linked to RA. Moreover, in RA patientsthe synovial fluid containing T cells show less sensitivity towards vitamin D. Hence vitamin D restoration can act as a promising therapeutic approach for the disease (Badsha et al., 2018).

1.4 Pathogenesis of Rheumatoid Arthritis

Various genetic, epigenetic, and environmental factors influence the susceptibility, progression, and severity of the disease. Numerous immune modulators like cytokines and various effector cells are attributed to the pathogenesis of RA. These immune modulators have complex interactions with each other resulting injoint damage that starts at synovial membrane (Smolen & Steiner et al., 2003). Other than the joint complications, various RA patients deal with extra-articular or systemic problems. In some cases, the patients experience both extra-articular and systemic features at the same time resultingin more severity of the disease (Hochberg et al., 2008).

The first event in pathogenesis of RA is the activation of innate immune system. This happens by exogenous and autologous antigens activating dendritic cells that further provokes adaptive immune responses. The antigen-presenting cells (APCs) further activate the T cells by presenting arthritis-associated antigens to the class II MHCmolecules (Choy et al., 2012a). This results in activation and expansion of T cells in the joints. In the synovialmembrane, the T cells are further upregulated through various lymphokines including IL-2 and IFN- γ lymphokines. The activated T cells stimulate the production of B cells, macrophages, osteoclasts, and fibroblasts (Ehrenstein et al., 2004). The stimulated B lymphocytes not only contribute to the pathogenesis of RA through presenting antigens, but also through production of antibodies, cytokines, and autoantibodies. The B cells express differentiation antigens such as CD20 and CD22 molecules. The differentiation of B lymphocytes into plasma cells results in secretion of antibodies and autoantibodies to citrullinated peptides, RA antigens, IgG, fibrinogens, and others. Autoantibodies produce large immune complexes that further stimulate pro-inflammatory cytokines. Other than antigen presentation, APCs also contribute to osteoclastogenesis that is the prominent source of cytokines like TNF-α, IL-1 and IL-6. Several inflammatory mediators such as prostaglandins(PGs) and matrix metalloproteinases (MMPs) are produced within the synovial membrane due to the overproduction of fibroblast-like synoviocytes resulting in bone and cartilage destruction. The most dominant pro-inflammatory cytokines are TNF-α and IL-6, which are involved in the pathobiology of the disease. (Nakahara et al., 2003; Kudo et al., 2003).

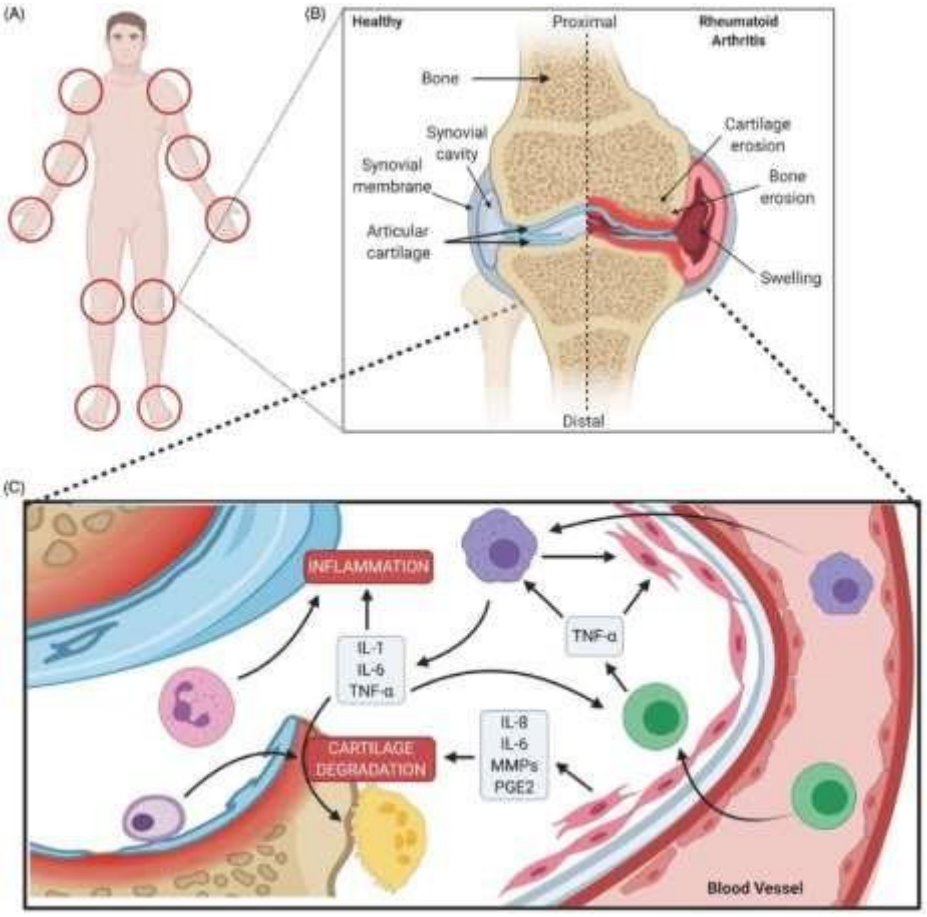

Figure 1 Overview of RA pathogenesis (Akram et al., 2021)

1.5. Diagnosis

RA is an inflammatory disease mainly affecting hands and feet joints. Many complicationsare linked with RA such as oligo-or polyarthritis in certain cases and sometimes monoarthritis as well (Colquhoun et al., 2022b). In 90 % of the patients, joint damage can be prevented through early diagnosis. Some common symptoms of RA include joint stiffness especially in hands and feet, edema and pain in the metacarpophalangeal joints, metatarsophalangeal joints and autoantibodies presence inthe body are all signs of RA (Aletaha & Smolen et al., 2018a).

Diagnosis of RA is based on clinical features of the patient like X ray images and presence/absence ofserological markers (autoantibodies). The most common serological marker is rheumatoid factor (RF) whose presence or absence determines RA in the body. Another more targeted and sensitive marker for RA is the anti-cyclic citrullinated peptide antibody (ACPA) that helps in prognosis and diagnosis of thedisease (Choi & Lee et al., 2018).

Moreover, a single blood test is not enough hence multiple tests are recommended for timely diagnosisand therapy of RA. These blood tests include identification of inflammatory markers, antibodies test like Rheumatoid factor, and anti-cyclic citrullinated peptide (ACP) and imaging tests (Colquhoun et al., 2022b).

The European League against Rheumatism (EULAR) established a criterion to help clinicians identify RA patients during clinical investigations (Aggarwal et al., 2015; Radner et al., 2014). The guidelines include cumulative points of at least 6 (out of a potential 10) from four domains that are as following:

- Location and number of joints involved.
- Presence of serological markers (RF and ACPA)
- Elevated inflammatory markers
- Time period for presence of symptoms

Criteria		Points
Joint involvement:	1 large 2 – 10 large 1 – 3 small 4 – 10 small > 10 (at least 1 small)	**0** **1** **2** **3** **5**
Serology:	Negative RF and ACPA Low positive RF/ACPA High positive RF/ACPA	**0** **2** **3**
Acute phase reactants:	Normal CRP/ESR Abnormal CRP/ESR	**0** **1**
Symptom duration:	< 6 weeks > 6 weeks	**0** **1**
		∑ = overall score

Figure 2 Frequently used clinical parameters for RA diagnosis (Lin et al., 2020a)

LITERATURE REVIEW

2.1 Autoimmunity

The immune system detects and destroys foreign antigens such as infectious agents or toxins. Lymphocytes are the most important immune cells involved in fighting external entities. However, due to the presence of diverse and large immune cell population, it becomes inevitable to avoid reactivity with the self-antigens. These self-antigens are the body's own proteins thatare detected as foreign particles leading to complicated diseases known as autoimmune diseases (Wahren Dorner et al., 2013).

Multiple epidemiological studies provide evidence of steady rise in autoimmune diseases worldwide. Autoimmunity is the same response as directed towards an infection by adaptive immune system. The self-antigens are recognized by T and B cell receptors leading to chronic inflammatory responses. The autoimmune responses can be localized or systemic. However, the trigger for the onset of an autoimmune response is unknown as many factors are involved. Many studies have reported that an infection can also trigger autoimmunity by releasing antigens like the body's own cells. Studies show evidence of multiple environmental and genetic factors influencing the susceptibility of autoimmune diseases such as in case of RA (Goodnow et al., 2005).

2.2 Role of B Cells

Both the innate and adaptive immune responses play a relative immunopathogenic part in various phases of RA. The overall course of the disease is categorized in three different stages i.e, initiation of the disease known as antigen independent phase involving the constituents of joints, perpetuation that involves inflammatory responses to specific antigens, terminal destruction that involves synovium degradation. The role of B lymphocytes is still unclear, but their contribution is quite significant in associating innate and adaptive immune system to promote inflammation by cellular components. Theoveractivation of B-cells causes the immune cells to migrate through chemokine receptors intosynovium. This step leads to further activation of T and B-cells to agglomerate and make tertiary follicular structures. These complex structures express CD40 ligand and produce cytokines such as IL-6 and IL-10 triggering the stimulation of B-cells, hence the increased mutagenesis in synovium. The over activated T-cells and B-cells aggravates the production of autoantibodies resulting in severe RA symptoms (Schröder et al., 1996; Dörner Burmester et al., 2003).

Moreover, B cells are also involved in producing rheumatoid factor (RF) and synthesizing B cells-mediated anti-CCP. The presence of B cells- mediated anti-CCP improved RA diagnosis by 90-98 %. Such patients are more prone to bone erosion, persistent joints, loss of function and other comorbidities (Koga et al., 2017).

2.3 Role of T Cells

In RA, T cells play the most pivotal role by activating the fibroblasts and macrophagesto act as tissue destructive cells. The activation of macrophages produces a range of chemokines and cytokines to

aggravate inflammation in joints. T cells are further activated by B cells, dendritic cells or macrophages causing CD4+ T-cells to interact with MHC-II and HLA molecules. The stimulation of CD4+ T-cells co-activates the CD28 molecules present on APCs to provoke the initiation of P13K signaling downstream signaling. This signaling is involved in antigenic activation of naïve CD8+ T- cells, CD4+ cells maturation, thus promoting inflammatory responses. CD4+ T-cells are also associatedwith the HLA-DR4 allele along with increased secretion of cytokines and chemokines causing more aggressive symptoms of RA. The type 1 T-helper cells (Th1) are involved in secretion of pro- inflammatory cytokines such as IFN-gamma (IFN-γ), TNF- α and IL-2 secretions. However, another type of CD4+ type 2 T-helper cells (Th2) are associated with B-cells activation by releasing anti- inflammatory cytokines such as IL-5 & IL-4. Moreover, IL-17 serum levels are reported to be higher inRA patients whose secretion is triggered by Th17 cells. IL-17 triggers the secretion of chemokine, pro- inflammatory cytokines, and matrix metalloproteinases (MMPs) and production of IL-8, MMP-1, VEGF-A, IL-6, and MMP-3 in the synovial fibroblasts. Moreover, many studies reported the involvement of IL-17 in osteoclastogenesis, pannus growth and neoangiogenesis. These multiple typesof T-cells and associated pathways lead to severe inflammation resulting in RA(Yap et al., 2018; Luckheeram et al., 2012).

2.4 Role of Cytokines

Various cytokines play their role in crucial biological processes such as cell growth and repair, inflammation, differentiation, proliferation, and immune responses regulation. Cytokines also play a crucial role in chronic inflammation leading to joint destruction during the pathogenesis of RA. Some cytokines are the protective immune regulating molecules known as anti-inflammatory cytokines such as IL-4, IL-10, IL-13, IL-27, and IL-35. While others are pro-inflammatory cytokines such as TNF-alpha, IL-1, IL-33, and IL-17 etc. They are associated with inflammation, cartilage and joint destruction, synovitis, and bone erosion (Mateen et al., 2016). TNF-alpha plays a prominent role in stimulation of proinflammatory cytokines that severely affects the physiological balance of ‘ant-inflammatory’ andpro-inflammatory mediators (J. Alam et al., 2017). Moreover, IL-6 is particularly involved in triggering neutrophils that further secrete intermediates of reactive oxygen and proteolytic enzymes causing inflammation and joint damage (Choy et al., 2012b).

2.5 Role of IL-6 in RA

In RA patients, high IL-6 serum levels are identified in synovial fluid suggesting a correlation with laboratory and clinical indices of RA. IL-6 acts as a multifunctional cytokine that contributes to the most crucial role in localized and systemic inflammation by providing SOS signal fortriggering the host defense response (Kishimoto et al., 2005; Tanaka et al., 2014). In case of infections, monocytes and macrophages produce IL-6 to stimulate the immunogenic and hematological responses required for pathogen elimination and tissue healing. Although, IL-6 and its prolonged elevated expression triggers chronic inflammatory activities in the body (Tanaka et al., 2012).

2.5.1. IL-6 Role in Immune Responses

During responses by acquired immune system, IL-6 is key cytokine is activating B and T lymphocytes.It activates the production of B lymphocytes into Ig-producing plasma cells that causes hypergammaglobulinemia and B cell stimulating factor 2 leading to chronic inflammation (Chihara et al., 2011). It is also involved in mechanisms that ensure the survival of precursor of plasma cells i.e., B-cell subpopulation plasmablasts. In synovium, circulating plasma cells and plasmoblasts are widely distributed acting as a source of ACPA in RA patients (Kerkman et al., 2016).

In the case of T cells, IL-6 is involved in naïve CD4 + T cells differentiation. Moreover, IL-6 causes Th17 differentiation in association with transforming growth factor (TGF)-β that further resultsin secretion of IL-17, TNFα and IL-6 and plays the role of primary effector cells in autoimmune responses. However, IL-6 is also associated with TGF-β-induced regulatory T (Treg) cell differentiation inhibition that is involved in maintaining homeostasis of immune system and prevention of autoimmunediseases (Bettelli et al., 2006; Kimura & Kishimoto et al., 2010). Thus, IL-6 involvement results in Th17/Treg balance upregulation that is a pathological indication of multiple inflammatory autoimmune diseases including RA (Ma et al., 2012)

In addition to this, IL-6 develops a link between T cells and B cells. The production of IL-21 and differentiation of T follicular helper (Tfh) cells is also regulated by IL-6 that stimulates T cell-dependentB cell response and helps in immunoglobin class switching of B lymphocytes. Th17 and Tfh cells are involved in the regulation of tertiary lymphoid organs activated by inflammatory signals in the damaged tissue (Jones & Jones et al., 2016). These tertiary lymphoid organs produce autoantibodies strongly associated with aggravation of RA (Humby et al., 2009). The transition from innate to adaptive immunity is also linked with IL-6 based on neutrophils to monocytes and T cells (Kaplanski et al., 2003a; Hurst et al., 2001).

2.5.2. IL-6 in RA Pathogenesis

Various studies have reported less than 4 pg/ml IL-6 serum levels in healthy individuals. However, during infection or inflammatory diseases such as RA, the IL-6 serum levels are indicated between 10-100 pg/ml. In RA, synovial cells are prominent producers of IL-6, thus the IL-6 levelsin synovial fluids are higher than IL-6 serum levels. In pathogen-induced lesions, the production of IL-6 is induced by stimulated intracellular or cell-surface toll-like receptors (TLRs) such as that present on the macrophages and monocytes containing TLR-recognizing microbial components called as pathogen-associated molecular patterns (PAMPs) (Kawai & Akira et al., 2010). Several in-vitro studies identified the response to autoantigens was increased secretion of IL-17A, IL-6 and IL-23 secreted by naïve T cells that were cocultured with RA-microbiota stimulated dendritic cells in comparison with Tcells cocultured with healthy control-microbiota stimulated dendritic cells.(Maeda et al., 2016) This suggests that the dysbiosis of intestinal microbiota leads to pathogenesis of RA. Necrotic cells and the damaged extracellular matrix

release special molecules with damage-associated molecular patterns (DAMPs) that further trigger the TLRs to release IL-6 and other inflammatory cytokines (Kawai & Akira et al., 2010). Various studies indicated the presence of DAMPs in the tissues and synovial fluid of RA patients suggesting the involvement of DAMPs-TLRs pathway in the release of elevated levels inflammatory cytokines in joints. (Pope & Huang et al., 2009). Moreover, IL-6 stimulation is also achieved by activation ofcoagulation cascade. Various studies identified the release of IL-6 by administration of coagulation factor VIIa (De Jonge et al., 2003). Other than that, thrombin activates endothelial cells to secrete IL-6 (Kaplanski et al., 2003b). Fibrin also contributes to elevated levels of IL-6 via TLR4 in synovial fibroblasts of RA patients. It is reported that the citrullinated fibrin (RA specific autoantigen) involvedin more expression of IL-6 than the non-citrullinated fibrin in synovial fibroblasts (Sanchez-Pernaute et al., 2013).

However, the triggering factor in expression of IL-6 in the joints of RA patients is still unclear. Synovium inflammation promotes infiltration of inflammatory cells and synovial hyperplasia, while persistent synovitis induces joint swelling, pannus formation, neovascularization, and destruction of joints. These damaged joints further elevate the inflammation and promote the production of tertiary lymphoid follicles that leads to release of autoantibodies and exacerbated synovitis.

2.5.2.1 Therapeutic Targeting of IL-6 in RA

Different studies confirmed IL-6 role in pathogenesis of RA in various RA animal models by administration of IL-6-neutralizing antibody or IL-6-deficient mice such as anti-mouse IL-6 receptor mAb or IL-6 deficiency inhibited collagen-induced arthritic mice (Banda et al., 2002; Kishimoto et al., 2005; Tanaka et al., 2012). Similarly, another study indicated mild symptoms of RA in IL-6 deficient mice for methylated-BSA antigen-induced arthritis. However, the control mice group showed severe arthritic symptoms including pannus formation, neovascularization, and erosion of articular cartilage (Ohshima et al., 1998). All these observations led to the notion that blocking IL-6 expression could be considered a strategy for RA patients.

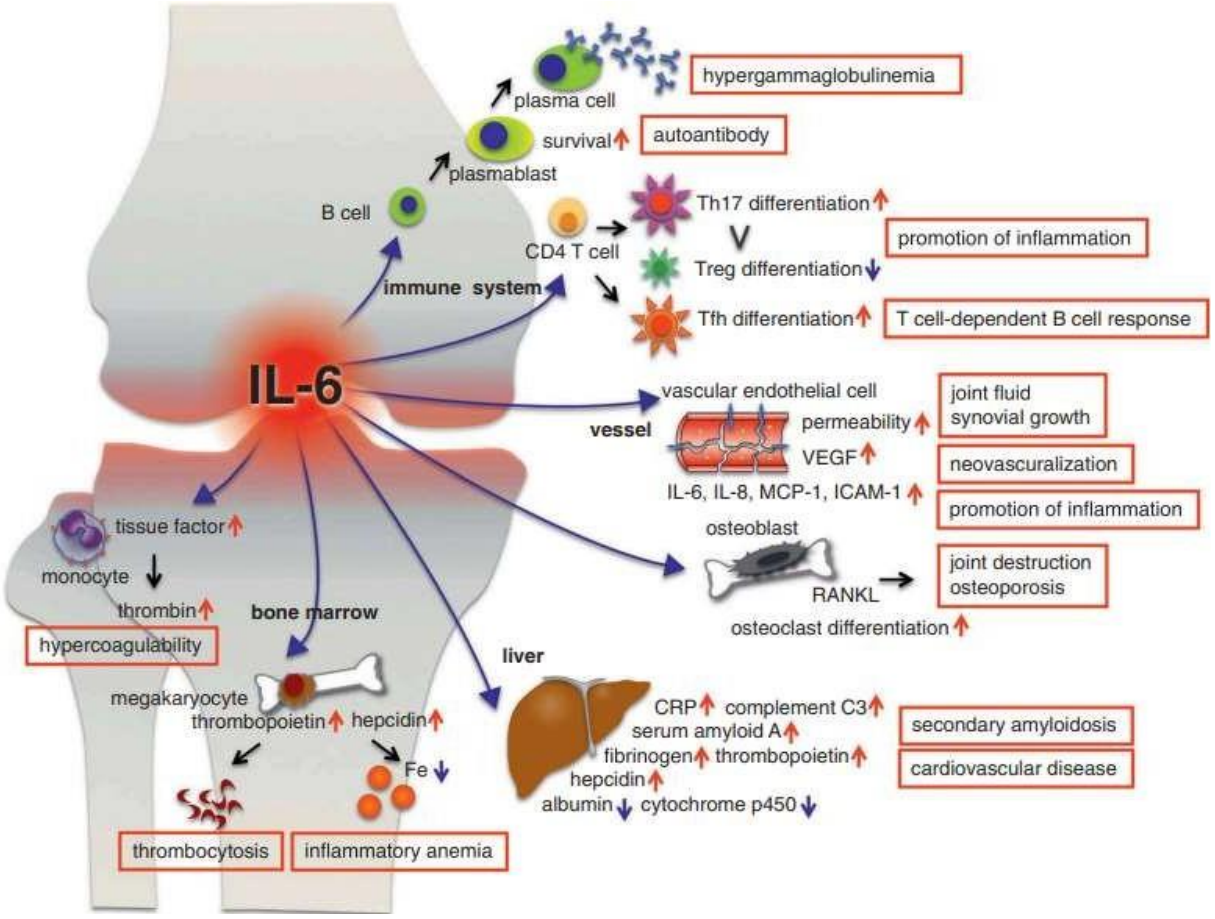

Figure 3 Role of IL-6 in RA (Narazaki et al., 2017)

2.6 Available Treatments for Rheumatoid Arthritis

Treatment is given to RA patients to reduce the symptoms of the disease within a period of 6 months. Generally, the treatment is useful to avert joint damage and reducethe long-term disabilities associated with RA. Personalized treatment is provided to the patients based on the health condition. Many factors contribute to making the treatment regime of that patient such asage, type of joint involved, disease progression and education etc. There are three main categories of available drugs for RA that are classified in the following categories: NSAIDs, glucocorticoids and DMARDs.

2.6.1 Analgesics (NSAIDs)

The first identified NSAID was salicylates, which was based on the salicylic acid extracted from the bark of willow trees. They provide the first line of therapy to reduce inflammation and pain, however, further progression of disease such as joint injury is not treated (Smolen et al., 2016). Nonsteroidal anti-inflammatory medicines (NSAIDs) are known for their fast action. They suppress the formation of prostacyclin (PGs) that is a product of arachidonic acid, and thromboxanes by cyclo-oxygenase inhibition. Arachidonic acid is a cell membrane phospholipid that acts as a chemical messenger duringtissue damage.

The conversion of arachidonic acid into PGs is accomplished by cyclooxygenase (COX)enzyme. NSAIDs suppress the production of PGs due to inflammation and tissue damage by inhibiting cyclooxygenase (COX). Moreover, cyclooxygenase-1 enzyme (COX-1) is inhibited by NSAIDs in different tissues like gastroduodenal mucosa and platelets causinggastrointestinal bleeding and ulceration that is indicated as a common side effect of persistent use of NSAIDs (Crofford et al., 2013).

One of the examples of NSAIDs is Aspirin (acetylsalicylate), the oldest anti-inflammatory drug used for joint discomfort, used in large dosages for RA patients. Some other drugs such as Ibuprofen (Advil, Motrin), naproxen (Naprosyn), and etodolac (Lodine) are also equally effective as aspirin. However, these medications are used in less dosages on daily basis (Bullock et al., 2018). Other than RA, NSAIDsare also used for reducing the symptoms associated with hepatic, renal, cardiovascular, and gastrointestinal effects (Crofford et al., 2013; Lin et al., 2020b).

2.6.2 Glucocorticoids

Glucocorticoids (GCs) are anti-inflammatory and immunomodulatory drugs that work by affecting primary and secondary immune cells. The most used GCs include prednisone, prednisolone, methylprednisolone, and dexamethasone. They function by inhibiting the activity of endothelial cells, fibroblasts, and leucocytes by limiting access to the site of inflammation. Moreover, GCs also suppress the production and activities of inflammatory humoral factors (Buttgereit et al., 2005).

GCs inhibit helper T cells (TH1) that further lowers the levels of proinflammatory cytokines. These drugs are proved to be effective in evading RA symptoms including morning stiffness, fatigue, pain and swelling of the joints.However, the side effects of long-term use of these medications include anxiety, depression, and manyothers (Ferreira et al., 2016). These drugs are used in low doses, due to more negative side effects thanNSAIDs, although the dose can be increased as per requirement of the RA patient.

2.6.3. Disease Modifying Anti-Rheumatic Drugs (DMARDs)

The objective of second-line therapy is to cause remission of the symptoms by reducing the progressionof joint deformity and degeneration. DMARDs are considered as slow-acting medications that lessen the risk of lymphoma, greatly associated with RA patients (Bullock et al., 2018; Smolen et al., 2010).
The available DMARDs are divide into three categories:

- Traditional synthetic DMARDs
- Targeted synthetic DMARDs
- Biologic agents (Littlejohn and Monrad et al., 2018).

2.6.3.1 Traditional Synthetic DMARDs

Conventional DMARDs are helpful to prevent damage to the tissues and joints. Moreover, these medications are non-specific immunomodulatory medications that help in reducing the course of the

disease (Littlejohn & Monrad et al., 2018).

The most significant traditional DMARD is methotrexate that is used for multiple reasons including for the treatment of RA for more than 50 years. Methotrexate can be used alone or in combination of glucocorticoids to reduce disease activity in almost 50% of the patients (Aletaha & Smolen et al., 2018b). In comparison to the DMARD monotherapy, triple therapy i.e., methotrexate & hydroxychloroquine is an effective regime for obtaining measurable results (Littlejohn & Monrad et al., 2018). Moreover, these medications are non-specific immunomodulatory medications that help in reducing the course of the disease (Littlejohn & Monrad et al., 2018).

2.6.3.2 Targeted Synthetic DMARDs

As the name suggests, the targeted DMARDs targets specific pathways involved in exhibiting inflammatory responses such as JAK-STAT pathway mediated by cytokines. The pathway is carried out when the pro-inflammatory cytokines attach to their receptors. In response to this, JAKs are recruited leading to the phosphorylation of receptor's intracytoplasmic components that further phosphorylates the STATs. The phosphorylated STATs homodimerize and translocate to the nucleus of the relevant cell.Thus, enhances the expression of pro-inflammatory genes to sustain tissue and joint damage (Damsky & King et al.,2017; Kotyla et al., 2018).

These medication work as inhibitors of the JAK family such as JAK1, JAK2, & JAK3, hence the downstream phosphorylation is also blocked resulting in low inflammatory cytokines expression. These drugs have made a substantial advance to treat RA in recent years such as Tofacitinib and Baricitinib (Damsky & King et al., 2017).

2.6.3.3 Biologic Agents

The biological agents work by targeting specific components of immune system (Rahimizadeh et al., 2021). There are four potential mechanisms by which biological DMARDs work; these are as follows:

- TNF-alpha or TNF(R) neutralization (Adalimumab, infliximab, Etanercept)
- IL-6 or its receptor neutralization (Tocilizumab)
- Scarcity of B cells (Rituximab)
- Suppression of T cell co-stimulation (Grijalva et al., 2007; Rein &Mueller et al., 2017; Kiely & Nikiphorou et al., 2018)

The efficacy of targeted DMARDs can be improved by using coupled therapy with conventional DMARDs(methotrexate) and biologic agents (Grijalva et al., 2007). These are mostly used in the later stage of the disease when the patients stop responding to the conventional DMARDs (Chaudhari et al., 2016).

2.6.4. Side Effects Medications

Various studies reported the toxic side effects of available RA medications. Longer use of NSAIDs (more than 1 year) damages the gastroduodenal mucosa and causes upper GI bleeding, and stomach ulcers with other minor side-effects like dyspepsia and nausea. However, DMARDs show better results than NSAIDs, thus used as the first-line therapy according to the current clinical guidelines (Fries et al., 2000). On the other hand, corticosteroids possess excessive side effects but are more potent than NSAIDs. They are instructed to be taken in small dosages for a shorter period. The side effects cause bone thinning, diabetes, weight gain and immunosuppression (Liu et al., 2013).

2.7. Medicinal Plants

The current pharmacological treatments improve the quality of life, yet their side effects increase the chances of various systemic comorbidities such as cardiovascular and gastrointestinal diseases. As a result, the contemporary medical landscape favors traditional herbs and plants.

Since early times, plants have been used in ethnobotanical medicines and the information is passed on from generations to generations. The pharmacological industry gained extreme interest in medicinal plants because of their safe and effective use. Not all parts of the plant are used as there is a difference in the distribution of phytochemicals used against the disease in the different parts of plant. The herbal medications gained popularity among patients too due to fewer side effects and greater biocompatibility with term use when compared to allopathic pharmaceutics (Pandey & Tiwari et al., 2018; Singh et al., 2020a).According to the World Health Organization (WHO), there are 200,000 potential plant species and morewith medicinal properties.

2.7.1 Ethnobotanical Medicines as an Alternative Treatment for RA

Since ancient times, herbal medicines have been used to treat a variety of diseases. Researchers are increasingly interested in medical molecules produced from plants, as presently available medications either are expensive or have undesirable side effects (Sharmila et al., 2017). Various studies identified medicinal plants as an excellent alternative for RA treatment. There is a paradigm shift towards ethnomedicines proved to be safe and effective. Traditional medicinal plants with active ingredients such as terpenes, phenols, flavonoids, lactones, and carotenoids, as well as antioxidant and anti-inflammatory qualities, are successful therapy for RA (Singh et al., 2020b). However, very little

scientific evidence is available, hence scientific validation is required to further identify the medicinal properties on these plants.

In Nigeria, the pre-dominantly used plants against RA include ginger and garlic (Tongshuwar et al., 2020). *Vitex negudo* is used to treat rheumatism and many other diseases in India, China, and Unani along with rice, garlic, and gul. Similarly, *Piper longum, Aloe vera, Cassia fistula, Curcuma longa*, and *Lantana camara* are among the plants used Rheumatism treatment (Pandey et al., 2018).

In Pakistan, out of 6000 wild plant species 400-600 species are considered therapeutically important. Around 371 medicinal plant species belonging to 263 genera and 99 families are considered important for medicinal used against inflammatory disease in Pakistan. Most of these plants belong to the families of Asteraceae and Fabaceae are in Punjab and Khyber Pakhtunkhwa regions. The commonly used medicinal plants against various inflammatory diseases in Pakistan include *Berberis lycium Royle, Citrullus colocynthis(L.) Schrad, Calotropis procera (Wild.), Datura innoxia Mill, Ricinus communis L* and *Verbascum thapsus L* (Amjad et al., 2017; Rashid et al., 2015; Shah et al., 2021).

2.8 Subspecies of *Dodonaea viscosa*

Different species of *Dodonaea* are extensively used for medicinal purposes in various diseases. It is being used for a variety of pathological conditions including anti-inflammatory, analgesic, spasmolytic, antiviral, anti-microbial, laxative, rheumatism, hypotensive, gout, fractures, wound healing, hemorrhoids, and snake bites (Ghisalberti et al., 1998). Traditionally, the leaves, stems and fruits of this plantget utilized to make medicine. The most studied *Dodonaea* species include *D. angustifolia* and *D. Viscosa* (Shtein et al., 2011a). There are many subspecies available of *Dodonaea viscosa* such as:

- *D.viscosa subsp.angustafolia*
- *D.viscosa subsp. Angustissima*
- *D.viscosa subsp. Burmanniana*
- *D.viscosa subsp. Cuneata*
- *D.viscosa subsp. Mucronate*
- *D.viscosa subsp. Spatulate*
- *D.viscosa (L.) Jacq. subsp. Viscosa*

2.8.1 Taxonomic Hierarchy

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta

Division	Magnoliophyta
Class	Angiosperms
Order	Sapindales
Family	Sapindaceae
Genus	Dodonaea
Species	*Dodonaea viscosa*

Table 1 Taxonomic hierarchy of Dodonaea viscosa (Beshah et al., 2020)

2.8.2 Dodonaea viscosa

D.viscosa belongs to angiosperms (flowering plants) with almost 2000 species found in America, Africa, Australia, and southern Asia in temperate, tropical, and subtropical climates. It is named after a Flemish botanist, Rembert Dodoens. However, the term viscosa comes from the word viscous, which means 'sticky' (Ahmad Khaira et al., 2018). Different variations can be seen in different parts of the plant based on temperature and geographical location such as difference in the size and form of leaves (Shtein et al., 2011b).

The plant can thrive in coastal areas and is drought resistant. Although, it is frost-sensitive and does not thrive in the shade. *Dodonaea viscosa* has different names in different countries such as:

- In Australia: hop bush
- In Guam: lampuaye
- In New Zealand: akeake
- In Palau: mesechelangel
- In Argentina: chirca
- In Mexico: romerillo
- In Colombia: hayuelo
- In Pakistan: sanatha
- In Afghanistan: ghoraskai

Figure 4 Dodonaea viscosa (Beshah et al., 2020)

2.8.3 Geographical Location

Dodonaea viscosa is located worldwide across the southern hemisphere, including New Zealand, Australia, Southeast Asia, Latin America, and Arica as well as the southern United States. In Pakistan, 'Sanatha' may be found in Kala Chitta. Malakand, Parachinar, salt ranges in Pabbi Hills, Khyber Pass, Fort Sundyman, Nizampur, and Haripur's sub-mountain regions.

2.8.4 Ethnomedicinal uses of *Dodonaea viscosa*

D.viscosa is utilized for treating various ailments. The plant leaves are used for treating gastrointestinal diseases, wounds, skin problems and rheumatism, swelling, rash, and bone disorders (Rojas et al., 1992). Moreover, plant roots are used to cure headaches and ulcers. The plant is also used by making a mixture of roots and leaves (paste) as a remedy for indigestion, dental pain, constipation, and diarrhea (Rojas et al., 1996).

2.8.5 Studies on Plant Phytochemical Analysis

Phytochemicals are produced in the plant body by various primary and secondary metabolisms. Many factors contribute to the variation in the synthesis of metabolites including environmental factors, geographical locations, age, parts of the plant, and extraction procedures (Beshah et al., 2020; Sampaio et al., 2016).

The ethanolic and aqueous extracts of *Dodonaea viscosa* leaves showed the presence of saponins, flavonoids, steroids, terpenoids, triterpenoids, alkaloids, anthraquinone, polyphenols, glycosides, coumarins, while tannins were present only in aqueous extract. The most common compounds isolated from *Dodonaea viscosa* are different derivatives of flavonoids, isoprenylated flavanol, diterpenoids, saponins, volatile oils, and sterols (Hossain et al., 2019).

2.8.6 Pharmacological Properties of *Dodonaea viscosa*

Antioxidants strengthen the immune responses by controlling various underlying mechanisms. The acetonic and aqueous plant extract showed good antioxidant activity and a total reducing power (Malik et al., 2022). A research was conducted to identify the antioxidant effects of *D.viscosa*, through techniques through GCMS, HPLC, and FT-IR, shows remarkable results against damage produced by CCL4 in the liver, spleen, and kidney (Tong et al., 2021).

Many studies proved the anti-inflammatory role of *D.visosa* during inflammatory responses. Different inflammatory compounds are extracted from various plants. A study compared the plant extract efficacy with the standard drug indomethacin showed increased anti-inflammatory activity in (TPA) induced mice with different doses of Hautriwaic Acid (a diterpene, isolated from *Dodonaea viscosa*) (Salinas-Sánchez et al., 2012). Similarly, a compound Nebrodenside A isolated from *Dodonaea viscosa* showed significantly reduced inflammation in carrageenan-induced mice as compared to standard drug diclofenac (Khan et al., 2019).

The wound healing potential was confirmed using chloroform and methanol extract of *Dodonaea viscosa* that further predicted one active constituent or synergistic effect of more than one bioactive molecule (Nayeem et al., 2021). Moreover, the anti-diarrheal, and antimotility activity was also assessed in Swiss Albino mice using methanol extract (80%) of *Dodonaea viscosa.* Furthermore, this study confirmed the non-toxicity of the plant extract up to a dose of 2000mg/ml (Abdela et al., 2019).

METHODOLOGY

3.1. Plant Extract Preparation

Dodonaea viscosa plant leaves were collected from Margalla Hills, Islamabad, Pakistan. The accession number of plant was 00046453 that was retrieved from the Pakistan Museum of Natural History (PMNH). *Dodonaea viscosa* leaves were dried and converted into fine powder through mechanical grinder. The preparation of plant extract was done using Soxhlet apparatus. To make the extract, 20 g ofthe powdered leaves were dissolved in 200 ml of ethanol with a ratio of 1:10. The powdered leaves wereplaced in the thimble chamber of the Soxhlet apparatus and Whatman filter paper was used for filtration.The temperature was kept lower than the boiling point of ethanol at around 5˚C – 60˚C. The apparatuswas kept running for 48 hours until transparent solvent is seen through the siphon arm. The liquid wasfiltered and some of it was stored in the refrigerator at 20 degrees while the rest was air dried to achievethe dry plant extract (Mahire & Patel et al., 2020).

Figure 5 Ethanolic extract of Dodonaea viscosa prepared using Soxhlet apparatus

3.2. Phytochemical Analysis of *Dodonaea viscosa* Extract

The standard protocols were used to screen the phytochemical components in the plant extract.

3.2.1. Qualitative Phytochemical Analysis

3.2.1.1. Saponin

0.5 ml of plant extract was added to 5 ml of hot distilled water. The solution was vigorously shaken because of which 1 cm layer of froth was formed over the surface.

3.2.1.2. Phenol

1 ml ferric chloride (1%) was added to 1ml of plant extract to give a blueish- greenish color.

3.2.1.3. Anthraquinones

2 ml of extract was added in a test tube, to which 2 ml of (2N) of hydrochloric acid and a few drops of ammonia were added. The turning of pinkish red to Bluish violet coloration is an indication of the presence of anthocyanins.

3.2.1.4. Flavonoids

1 ml of extract is added to 1 ml of (10%) lead acetate in a test tube. Yellow precipitates indicate the presence of flavonoids.

3.2.1.5. Anthocyanin

2 ml of extract was taken in a test tube, to which 2 ml of (2N) of hydrochloric acid and a few drops of ammonia were added. Pinkish red to bluish violet coloration indicates the presence of anthocyanins.

3.2.1.6. Leucoanthocyanin

5 ml of plant extract was added in the test tube followed by mixing with 5 ml of isoamyl alcohol. The organic layer turning red indicates the presence of leucoanthocyanin.

3.2.1.7. Tannis

To 2 ml of extract, 2 ml of 10% ferric chloride and 2 ml of water were added. The presence of tannis can be confirmed when color is changed from blue or green to black colour.

3.2.1.8. Coumarin

2 ml extract was taken in a test tube, to which 3 ml of (10%) sodium hydroxide was added. The indication of coumarin is the presence of yellow colour in the test tube.

3.2.1.9. Terpenoids

The addition of 2 ml of extract, 2 ml chloroform, and a few drops of concentrated sulphuric acid results in deep red colour confirming terpenoids' presence.

3.2.1.10. Triterpene

2ml of extract and a few drops of concentrated sulphuric acid were added into the reaction tube. It was then shaken and stand for few minutes. The yellow color lower layer formation of the solution confirmed the presence of diterpenes.

3.2.1.11. Steroids

The addition of 2 ml of extract, 2 ml chloroform and 2 ml concentrated concentrated sulphuric acid produces a reddish-brown colour at the interface indicating the steroid's presence.

3.2.1.12. Sterol

Add 1 ml of extract, and a few drops of concentrated sulphuric acid. The solution is shaken then let it stand. The indication of presence of sterols is red colour layer in the lower layer of the solution.

3.2.1.13. Emodin

2 ml of extract was taken in the tube followed by addition of 2 ml ammonium hydroxide and 3 ml of benzene. The red coloration indicates the presence of emodin.

3.2.1.14. Glycosides

In a test tube, 2 ml of extract, 2 ml of chloroform, and 2 ml of acetic acid were added. Violet/blue to green colouration in the solution proves the presence of glycosides.

3.2.1.15. Cardiac glycosides

To 5 ml of extract 2 ml glacial acetic acid was added followed by the mixing of 1 ml of and 1 ml concentrated sulphuric acid in it. The confirmation about presence of cardiac glycosides is the green blur colour or brown ring in the test tube.

3.2.1.16. Deoxysugar

In a test tube, 2 ml of extract, 2 ml of acetic acid, a few drops of ferric chloride and 1 ml concentrated sulphuric acid was added. The appearance of violet color below brown color confirms the presence of deoxysugar in the plant extract.

3.2.1.17. Alkaloids

Hager's test was performed to confirm the alkaloids presence. 2-3 drops of Hager's reagent were added in 2 ml of extract in a test tube. Yellow precipitates indicate the presence of alkaloids.

3.2.1.18. Protein

The Xanthoproteic test was performed to confirm presence of protein. 1 ml of extract and 1 ml concentrated sulphuric acid was added. When the solution was boiled the white precipitates turned yellow indicating the protein presence.

3.2.1.19. Resins

In a test tube, 2 ml of extract, 3 ml acetone, 3 ml hydrochloric acid was added. The test tube with the solution was heated for 30 minutes in a water bath. The pink color changes to red to indicate resins presence.

3.2.1.20. Reducing sugar

For reducing sugar, benedicts test was performed. A few drops of benedict's reagent were added to the 1 ml extract and the solution was heated using a water bath. The presence of reddish-brown precipitates shows positive results.

3.2.1.21. Amino acid

For alkaloids presence, Hager's test was performed. In a test tube, 2 ml of extract was taken followed by addition of 2-3 drops of Hager's reagent. The presence of alkaloids is confirmed by the appearance of yellow precipitates.

3.2.1.22. Carbohydrates

For screening the presence of carbohydrates, Fehling's test was used. Fehling's solution A and B were prepared ahead of performing the test. In 2 ml of plant extract, equal volumes of Fehling's solution A and B were added. The mixture was boiled for a few minutes and the presence of brick red precipitates indicates the presence of reducing sugars.

3.2.2. Quanitative Phytochemical Analysis

3.2.2.1 Total Phenolics Content (TPC) Determination

The determination of phenolic content was done using Folin Ciocalteu phenol (FC) reagent. 1% (1mg/ml) stock solution of gallic acid (Standard) in distilled water and 1 mg/ml of stock solution of plant extract was prepared. Multiple dilutions of gallic acid (standard) in different concentrations (10,30,50,70,90 µg/ml) were prepared and vortexed. 5 ml Fc reagent was added and vortexed again. After 5 minutes, 5 ml of 20% solution of sodium carbonate was added. The incubation was done at 25ºC for 25 minutes. The absorbance was measured at 760 nm against the blank. The calibration curve was plotted of gallic acid and TPC. The calculation was done by taking gallic acid equivalent (mg of gallic acid/ g of extract) (Fernando & Soysa et al., 2014)

3.2.2.2 Total Flavonoids Content Determination

A stock solution of 1mg/ml of Rutin (Standard) and plant extract in methanol was prepared. Different dilutions of standard in different concentrations (10,30,50,70,90 µg/ml) were prepared. Take 1 ml of sample, add 1 ml of aluminum chloride (10%), 1ml of 1M sodium acetate, and shake well. The mixture is incubated at RT for 45 minutes. The absorbance of the sample was measured at 415 nm. To identify the total flavonoid concentration, Rutin calibration curve was used. The obtained values were indicated as mg Rutin equivalents per gram of dry weight of the plant extract (mg/g) (Fattahi et al., 2014).

3.3 In-vitro Antioxidant Assays

3.3.1. DPPH (1, 1-diphenyl- 2-picrylhydrazyl) Assay

The scavenging capability of the plant extract is measured through this assay based on the free radical method. The decolorization of violet/purple ethanolic solution of DPPH to a yellow colour by spectrophotometric detection indicates the ability of the plant components to donate hydrogen. DPPH stock solution in ethanol 1:35 (1 mg in 35 ml ethanol) was prepared and kept on ice in the dark. Stock solutions of ascorbic acid as standard (1 mg in 1 ml of ethanol) in case of control and for test sample (1 mg of extract dissolved in 1ml of ethanol). Extract and ascorbic acid solutions were diluted in ethanolin multiple concentrations (30,90,150,210,270 µg/ml) with a gap of 20 µg/ml then 1 ml DPPH was added. This process was carried out in the dark as it is light sensitive. The solutions were incubated for1 hour at room temperature in the dark (Fattahi et al., 2014). Ethanol (3 ml) was taken as a blank and 3 ml ethanol & 1ml DPPH was taken as control. Absorbance was calculated at 517 nm. The percentageof inhibition was measured by using the following formula:

% inhibition = (control-sample/control) x 100

3.4 In-vitro Anti-Inflammatory Assays

3.4.1. Albumin Denaturation Inhibition Assay

This assay is used to evaluate the ability of an extract to prevent protein denaturation. Different dilutions of aspirin (in the case of standard) and ethanolic extract (0.1, 1,10, 100 µg/ml) was prepared and added to the mixture. To 0.2 ml of 1% BSA, 2.8 ml of (0.2M) Phosphate buffer saline (PBS) was added. The mixture was put at 37 degrees using the water bath for 15 min, followed by its transfer to an incubator at 70 degrees for 5 min. Take out samples and let them cool at room temperature. Absorbance of the samples is calculated at 620 nm in a spectrophotometer (Gunathilake et al., 2018). Distilled water was taken control. The calculation of percentage inhibition of albumin denaturation was done using the formula:

% Inhibition of albumin denaturation = ([sample/control]-1) x 100

3.5 In-Vivo Anti-Inflammatory Assay

3.5.1. In-Vivo Acute Oral Toxicity (LD_{50}) Study

Approval of experiments on mice model was acquired from Institutional Review Board (IRB) with Performa number 32-IRB-ASAB-2016 at Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Pakistan.

This study was performed on BALB/c mice (age 5-6 weeks) to observe the toxic effect of crude *D.viscosa.* The protocol is carried out in three phases and each stage has 3 major groups with an equal

number of mice in every group (n=6). The parameters used for observation throughout the experiment include behavioral changes, physical signs of toxicity and mortalities for 24 hours before the start of next phase. The result of each stage determined the next stage. The mice were acclimatizedfor 2-3 days before starting the experiment (Zakari et al., 2018).

In phase I, 20 ml of three doses of 30 mg/kg, 300 mg/kg and 500 mg/kg were used. In phase II,1000 mg/kg, 2000 mg/kg, and 3000 mg/kg of doses were administered. In phase III, 5000 mg/kg of doseof plant extract was used, which is the limit dose as provided by the EPA/OECD and considered as high enough dose to test for acute toxicity. The formula used to prepare the doses was:

Extract volume = Weight of mice x Dose Stock concentration (expected therapeutic level of the plant)

3.6. In-Vivo Evaluation of Therapeutic Efficacy of *Dodonaea viscosa*

3.6.1. Animal Procurement

Both genders of BALB/c mice of age 5-6 weeks were obtained from National Institute of Health (NIH) Islamabad, Pakistan. The Animal House of ASAB, NUST was used to keep the animals. The animals were subjected to acclimatization under normal conditions for a few days. The mice were provided with normal feed and tape water for a week. All experimental protocols to be followed on animals were under the criteria of the guidelines given by the National Institute of Health. The permission to carry out experimentation was permitted by Institutional Review Board, ASAB NUST.

3.6.2. Mice Grouping for Experimentation

Mice were divided into 4 groups with an equal number of mice in each group (n=6). The grouping of mice was as follows:

Group No.	Groups	Administration
1.	Normal	Standard feed
2.	Negative Control (Arthritis Induced)	Standard feed (no treatment)
3.	Positive Control (Arthritis Induced)	Commercial drug (leflunomide)
4.	Experimental/Treatment group (Arthritis Induced)	*Dodonaea viscosa* extract

Table 2 Mice experimental groups

3.6.3. Arthritic Mice Model Preparation

For preparation of Collagen-induced arthritic (CIA) models, Bovine Collagenase Type 2 and Freud's Complete adjuvant (FCA) were used. The immunization mixture is made up of Collagen type-ll mixed with glacial acetic acid (0.1 M) in a ratio of 2:1. To make glacial acetic acid (0.1 M) 0.89% Hartmann solution was mixed with it. The collagen type II solution was mixed with Freund's adjuvant (2 mg/ml) in the ratio of 1:1. The addition of FCA to the solution was done slowly then vortexed for 2 minutes until pure white in colour. The immunization mixture (200 µl-100 µl) was filled into the 1ml insulin syringes and was kept on ice. The first 2 injections were given intravenously in the tail while thelast injection was given in the sub-dermal region of paw of mice. The injections were given on day 0, day 7, and day 14. Moreover, on day 21, a booster injection was also given (0.1 ml/100 µl) in the left paw of mice. The mice paws were assessed using non-invasive parameters including clinical arthritic score, volume of paws, ankle thickness via vernier calliper measurement to determine the intensity of inflammation as an indication of arthritic induction. All the mice were tagged, weighed and measurements of left hind pawwere taken with the help of a vernier calliper. The timeline of arthritis induction is as follows:

3.6.4. CIA Scoring of Mice Models

Mice paws were assessed for swelling and inflammation. The scoring was done based on the criteria on a scale of 0-4. The mice with a score of 3-4 were used for treatment studies. (Lin et al., 2020)

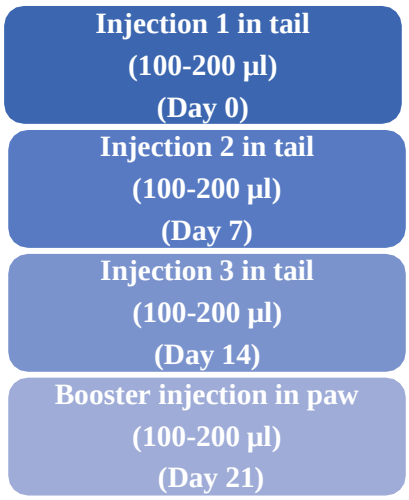

Figure 6 Schematic diagram of arthritic induction via CIA injection on day 0, 7, 14 and 21

CIA SCORE	OBSERVATIONS
0	Normal paw
1	One or two swollen/inflamed toes with normal paw and ankle

2	Three or more toes effected but no/mild paw swelling
3	Entire paw's swelling
4	Entire paw and toes swelling

Table 3 Clinical CIA impairment scoring table

3.6.5. Treatment with Plant Extract

The plant extract was provided to the experimental group; 900 mg/kg dissolved in tape water was given orally for 2 weeks. The concentration was selected based on the Acute Toxicity Test. While to the positive control group was given standard drug leflunomide (300 mg/kg) for 14 consecutive days.

3.6.6. Paw Size Measurements

The left hind paws were measured six times before injections for inductions, and before the beginning and after the completion of treatment. The size was calculated using vernier calliper of 0.05mm least count. The measurements were recorded in mm.

3.6.7. Collection of Blood, Liver, Spleen and Paw

After the completion of treatment, mice were sacrificed. Chloroform was given to anesthetize the mice before dissection. Blood was collected by cardiac puncture and was further utilized for clinical parameter like RF. Paw samples were collected for RNA extraction and X-rays. Spleen samples were collected for performing spleen indexing. Liver was collected for performing liver catalase test. The results of all four groups were compared to assess the therapeutic potential of the *Dodonaea viscosa*.

3.6.8. Spleen Indices

The spleen samples were collected from mice and weighed to calculate spleen indices. The following formula was used for measurement of spleen indices:

Spleen indices = weight of spleen in (mg)/ Total body weight of mice

3.6.9. Estimation of Rheumatoid Factor

Rheumatoid factor is the most popular diagnostic test of RA. After mice sacrifice, blood samples were centrifuged to obtain serum that was utilized for measuring RF levels. Normal range of RF is between 14-20 IU/ml.

3.6.10. Liver Catalase Assay

Catalase protects the cells from reactive oxygen species (ROS) produced due to oxidative stress. This oxidative enzyme present within the tissue produces water and oxygen because of degradation of hydrogen peroxide. The liver sample was converted into tissue homogenate using liquid nitrogen. The

crushed sample was added in 2.5 ml of cold PBS. The centrifugation of mixture was done, and the supernatant was collected. Further,the mixture was prepared by adding 1.0 ml of 0.01 M PBS and 0.4 ml of 2 M Hydrogen peroxide in tissue homogenate supernatant (0.1 ml). At the end, dichromate-acetic acid reagent (2 ml) was added into the mixture. The absorbance was measured three times (after a few seconds interval) at 620 nm. The blank was preparedin the same way except for the tissue homogenate (Kiziltunç et al., 2009). To calculate the catalase activity following formula was used:

$$\text{CAT U/g} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \times \frac{1}{\text{grams of tissue homogenate used}}$$

3.6.11. Radiological Evaluation

After dissection of mice, paw samples were collected for radiological evaluation. The examination was conducted based on the following factors i.e, soft tissue inflammation, joint structure destruction, spaces between joints and swollen joints.

3.6.12 Qualitative and Quantitative Analysis on CIA Model

3.6.12.1 Sandwich ELISA

For qualitative analysis, sandwich ELISA was performed to identify the IL-6 expression in different groups of CIA model. The procedure was performed on 96 well plates pre-coated with mouse IL-6 specific antibody. The protocol was followed as per manufacturer's instructions (Elabscience®)

After mice dissection, EDTA blood tubes were used for blood collection. The blood was allowed to settle in the tube for 60 minutes at room temperature. To obtain the serum, centrifugation was done at 1000 x g for 20 minutes while maintaining the temperature at 2-8 ˚C. The supernatant was collected to proceed the assay. All the samples and standards were assayed in duplicates.

First, different concentrations of 100 µl standard working solution were added in the first two columns. All the samples were added in duplicate. Incubationwas done for 90 minutes at 37 ˚C. After removing the liquid; 100 µl of biotinylated detectionAb/Ag was added followed by 1 hour incubation at 37 ˚C. Washing of each well was performed using wash buffer (350 µl). The washing step was repeated 3 times.100 µl of Avidin-Horseradish Peroxidase (HRP) conjugate working solution was added followed by incubation for 30 minutes at 37 ˚C. The blue color in the wells indicates the presence of mouse IL-6. The washing was performed again 5 times. 90 µl of substrate reagent was added to each well. Again, the plate incubation was performed for 15 minutes on the previous temperature. Lastly, the ongoing enzyme-substrate reaction was stopped using stop solution (50 µl). The OD was measured using pre-heated micro-plate reader at 450 nm. The OD value and Mouse IL-6 concentration has a direct proportional relation. The concentration of IL-6 can be calculated by comparing the sample OD values with the obtained standardcurve.

3.6.12.2. RNA Extraction from Tissue Samples

Mice paws samples were collected and stored at – 80 ˚C after submerging them in liquid nitrogen. RNA extraction was carried out using a mice tissue sampleof 0.2 mg. The sample was prepared using liquid nitrogen. 700 µl trizol was added to crushed tissue sample followed by 5 minutes incubation at room temperature (RT). 400 µl chloroform was added and 3 minutes incubation was done at RT. The centrifugation of homogenate was performed at 12,000 rpm at 4˚C for 10 minutes to allow phase separation. An aqueous upper layer was added in the tube;to maintain the temperature, placed on ice. Pre-chilled isopronol was added in equal ratio of aqueous layer. The mixture was incubated on ice for 10 minutes. Later, the samples centrifugation was performed at 4˚C for 10 minutes 12,000 rpm. The supernatant was discarded. The washing of the pellet was done 2 times using 1ml of 70 % ethanol at 7500 rpm for 5 minutes at 4˚C followed by air drying of the pellet. To keep the RNA free from RNases, 40 µl of RNase free water or DEPC was added. Until further experimentation, the extracted RNA was stored at 80 ˚C. Nanodrop plate was used for RNA quantification (Skanit RE 4.1, Thermoscientific). The absorbance was calculated at 260, 280 and 320 nm.

3.6.12.3.cDNA Synthesis

After RNA extraction, RNA was converted to cDNA, viva cDNA synthesis kit (Vivantis). The mixing and centrifugation of reagents was performed briefly. RNA-primer mixture was prepared as follows:

Reagents	Stock Conc.	Working Conc.	Vol/reaction	Vol/n(12)
Total RNA			7 µl*	-----
Primers oligodT	40 µM	4 µM	1 µl	12 µl
NF H_2O			2 µl	24 µl
Final Volume			10 µl	

Table 4 RNA-primer mixture for cDNA synthesis

Where “n” is the number of reactions. The incubation was performed for 5 minutes at 65˚C and chilled for 2 minutes on ice. Spinning of the mixture was done for a brief time. The cDNA Synthesis Mix was prepared as follows:

Reagents	Stock Conc.	Working Conc.	Vol/reaction	Vol/n(12)
M-MuLV Reverse Transcriptase	5000U	100 U	1 µl	12 µl

M-MuLV buffer	10X	2X	4 µl	48 µl
NF H_20			2 µl	24 µl
Ribolock			1 µl	12 µl
dNTPs			2 µ	24 µl
Final Volume			10 µl	

Table 5 cDNA synthesis mix

Where "n" is the number of reactions. 10 µl of cDNA Synthesis Mix was added to each RNA-primer mixture followed by centrifugation and incubation for 60 minutesat 42 ˚C. Lastly, the termination reaction was done by incubating the tubes at 85˚C for 5 minutes. After that,ice was used to keep the tubes chilled and centrifuged briefly. The storage of synthesized cDNA was done at -20˚C.

3.6.12.4. Real-Time PCR

For quantitative analysis, the synthesized cDNA was first used to optimize the PCR reaction. Firstly, four primer sets (IL6-F, IL6-R, GAPDH-F, GAPDH-R) were used for optimizing the annealing temperature (Ta). The primer sequences are as follows:

Primers	**Primer Melting Temperature (Tm)**	**Sequence**
IL6-F	57.2 ˚C	5'TACCACTTCACAAGTCGGAGGC 3'
IL6-R	55.5 ˚C	5' CTGCAAGTGCATCATCGTTGTTC 3'
GAPDH-F	55.7 ˚C	5'-CAACTCCCTCAAGATTGTCAGCAA-3'
GAPDH-R	54.4 ˚C	5-'GGCATGGACTGTGGTCATGA-3'

Table 6 Primer sequences for real-time PCR

The PCR reaction was performed on Galaxy XP Thermal Cycler (BIOER , PRC) to optimize PCR reaction conditions.

PCR Reagents	**Stock Conc.**	**Working Conc.**	**Vol/Rec**	**Vol. x (26)**
cDNA template	-	-	1 µl	
P_F	10 µM	0.2 µM	0.2 µl	5.2µl
P_R	10 µM	0.2 µM	0.2 µl	5.2µl
dNTPs	10 mM	0.2 mM	0.2 µl	5.2µl
Buffer	10X	1X	1 µl	26µl
$MgCl_2$	25 mM	2.5 mM	1 µl	26µl
Taq Polymerase	5U/ µl	1.5 U	0.3 µl	7.8µl
PCR H_2O			6.1 µl	158.6µl
Final Volume			10 µl	

Table 7 Optimised conditions for PCR reaction before performing real-time PCR

Where "n" is the number of reactions. In the case of GAPDH primer, after identifying melting temperature of primers, the gradient was run from 42 ˚C to 52 ˚C. The GAPDH primer with cDNA dilution of 1:10 was optimized at 48.1 ˚C. In the case of IL6, after identifying the Tm of primers, the first gradient was run from 50 ˚C to 60 ˚C. The IL-6 primer with cDNA dilution of 1:10 was optimized at 60 ˚C.

Mic PCR (Bio Molecular System) was used to perform Real-time polymerase chain reactions. The following table shows the optimized concentration used in the reaction:

PCR Reagents	Stock Conc.	Working Conc.	Vol/Rec	Vol. x (23)
cDNA template	-	1:10	1 µl	
P_F	10 µM	0.2 µM	0.2 µl	4.6 µl
P_R	10 µM	0.2 µM	0.2 µl	4.6 µl
Syber Green	2X	1X	5 µl	115 µl
PCR H_2O			3.6µl	82.8 µl
Final Volume			10 µl	

Table 8 Concentrations were optimzed before performing real-time PCR reaction

Steps	Sub-cycles	Conditions	PCR cycles
Initial Denaturation		95 ˚C, 12 minutes	1
PCR Cycles	Denaturation	95 ˚C, 15 seconds	40
	Primer annealing	-----, 20 seconds	
	Primer extension	72 ˚C, 20 seconds	
Hold		95 ˚C, 15 seconds	

Table 9 Conditions were optimzed before performing real-time PCR reaction

The relative IL-6 gene expression analysis in different groups was further done using 2 (-Delta Delta C (T)) Method. Further, the cycling graph and melt curve graph was obtained for better result interpretation.

RESULTS

4.1 Plant Extract Preparation

The plant extract was prepared using absolute ethanol. A dark greenish extract was obtained using Soxhlet apparatus. The extract was poured into the Petri dish and was solidified through air drying. The dried extract was stored at 4 ˚C in a covered tube for further use in experiments.

Figure 7 Ethanolic D.viscosa extract prepared using Soxhlet apparatus

4.2. Phytochemical Analysis of the Plant Extract

4.2.1. Qualitative Analysis

The qualitative analysis of ethanolic plant extract was performed. The results were characterized by physical changes as per standard. Following is the list of the obtained results:

Test	*Dodonaea viscosa*	Appearance
Phenol	+	Blue black colour
Anthraquinone	-	Pink/red/violet color
Flavonoids	+	Colourless solution/ yellow precipitates
Anthocyanin	-	Pinkish red to bluish violet coloration
Leucoanthycyanin	-	Organic layer turns into red
Alkaloid	-	Yellow precipitates
Tannins	+	Transient Greenish to black coloration
Coumarins	+	Yellow color

Terpenoids	+	Deep red colour
Steroids	+	Reddish brown color
Sterols	+	Red color at interface
Saponins	+	Formation of foam
Cardiac glycosides	+	Green/blue colour or brown ring
Glycosides	-	Violet to blue to green colouration
Deoxysugars	+	Formulation of brown ring
Proteins	-	White precipitate turned yellow on boiling
Resins	+	Pink color change to red
Reducing sugars	-	Reddish brown precipitation
Amino Acid	-	Violet color
Carbohydrates	+	Red precipitation
Emodin	-	Red coloration
Triterpene	-	Formation of yellow colored lower layer

Table 10 Phytochemical analysis of the plant extract in ethanol. + sign shows a positive

4.2.2. Quantitative Analysis

For qualitative analysis of phytochemicals, total phenolics and total flavonoids content was measured.

4.2.2.1 Total Phenolics Content

The Folin-Ciocalteu reagent was used for quantitative determination of the total phenolic content in terms of gallic acid equivalent. The total phenolic concentration is represented as mg gallic acid equivalent of per gram weight of dry extract. Linear regression was used to create the standard graph of gallic acid. The linear regression equation (y=0.0001x+0.0562) was used by putting the value of plant extract absorbance at "y" and calculating the value of "x". The formula used to calculate the concentration was:

$$C = x \,.\, V/m \text{---------- (equ. 1)}$$

Where "V" is the volume and "m" is the mass of plant extract. A significant quantity of phenols was found in the ethanolic extract (29.88 mg GE/g of dry weight extract)

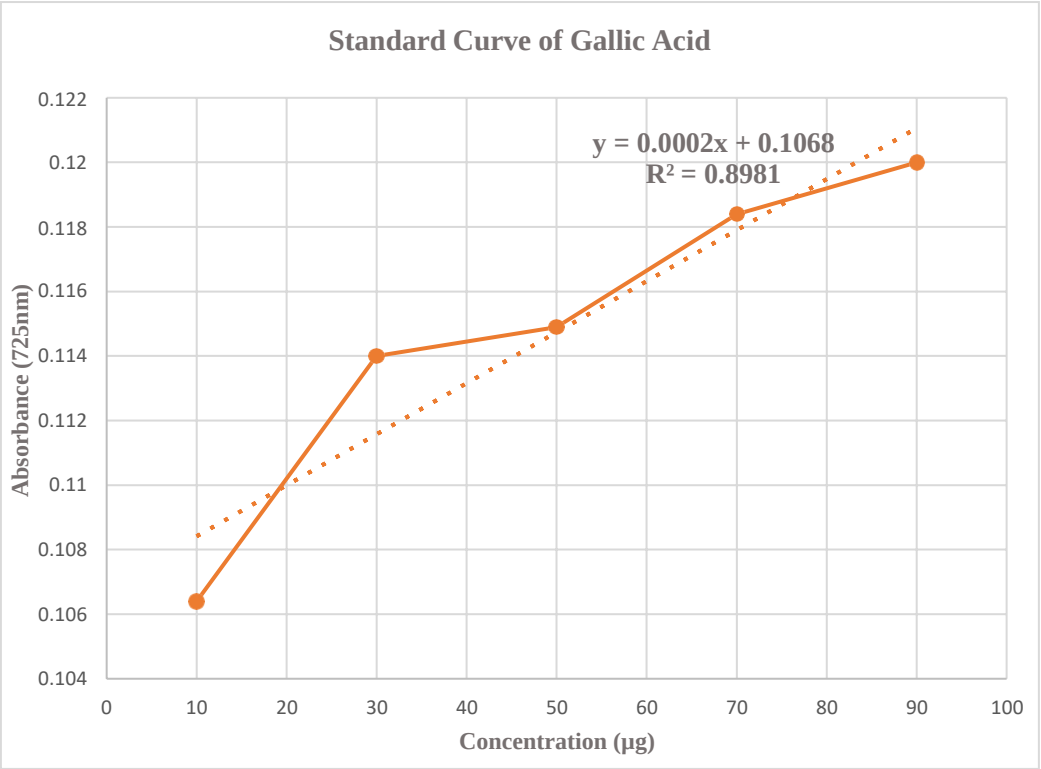

Figure 8 Graph for total phenolics content of Dodonea viscosa extract i.e. 29.88 mg GE/g of dry weight extract

4.2.2.2. Total Flavonoids Content

The aluminum chloride colorimetric assay was used for calculating total flavonoid content of ethanolic extract. The standard curve was obtained using Rutin. The results are expresses as mg Rutin equivalent per gram weight of dry extract. The Flavonoid Content was calculated from linear regression equation (y=0.0001x + 0.02). The formula used to calculate the concentration was:

$$C = x \,.\, V/m \text{----------} \quad \text{(equ. 1)}$$

Where "V" is the volume and "m" is the mass of plant extract. Significant quantity of flavonoid was found in ethanolic extract of *Dodonaea viscosa* (54.33 mg RE/g of dry weight extract).

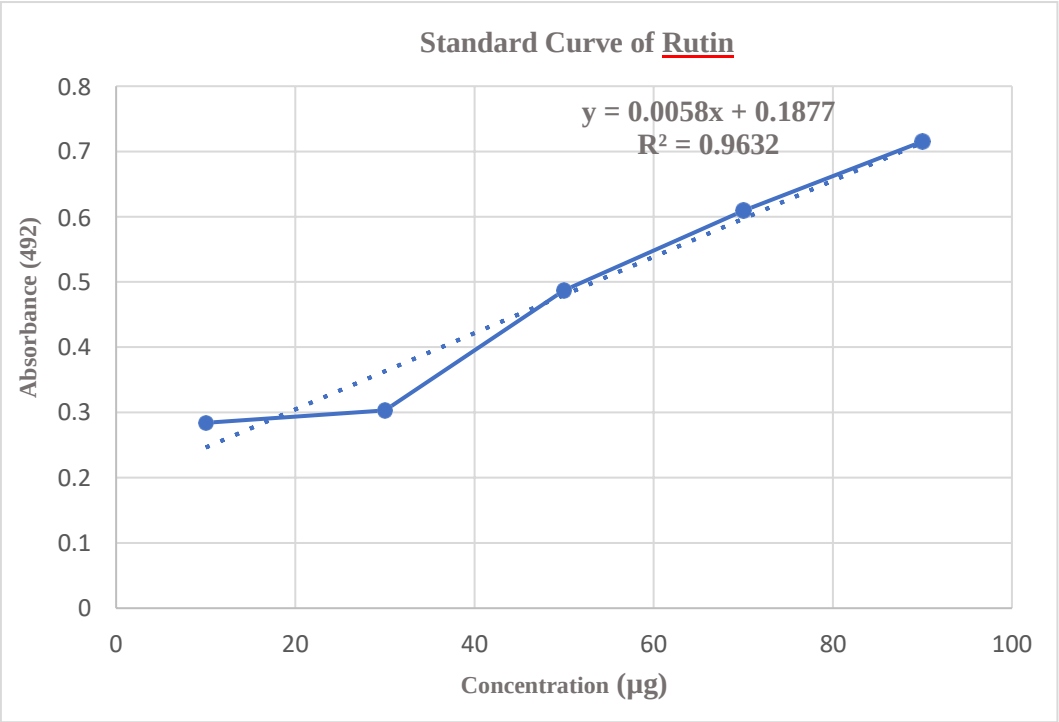

Figure 9 Graph for total flavonoid content of Dodonea viscosa extract i.e. 54.33 mg RE/g of dry weight extract

4.3 In-Vitro Antioxidant Assays

4.3.1 DPPH (1, 1-diphenyl- 2-picrylhydrazyl) assay

The results were obtained by determining the percentage inhibition and radical DPPH scavenging activity *D.viscosa* extract was expressed as mg Ascorbic Acid equivalent per gram dry weight of plant extract. Ascorbic acid was taken as a standard. Standard graph was calculated using the linear regression to measure the DPPH scavenging activity. The results showed an increase in radical scavenging potential with the increase of concentration and comparable to the standard that showed that our plant has more antioxidant potential.

Standard: $R^2 = 0.9432$

Plant extract: $R^2 = 0.9738$

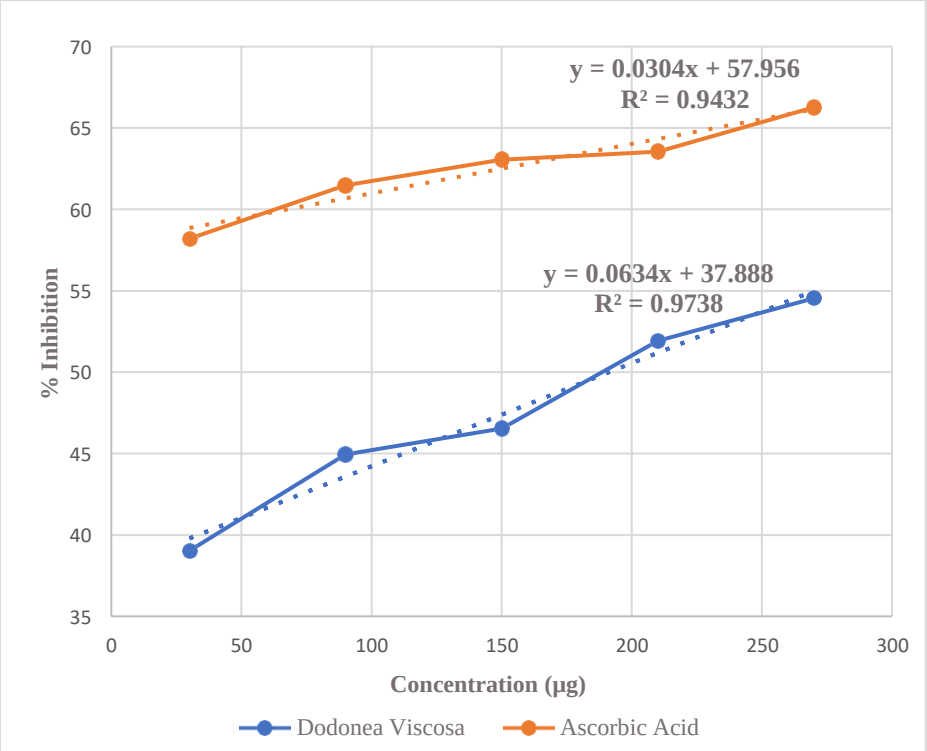

Figure 10 Graph shows increasing radical scavenging activity of Dodonaea viscosa with the increase in concentration

4.4. In-Vitro Anti-Inflammatory Assay

4.4.1 Albumin Denaturation Inhibition Assay

The results were obtained from this assay using the percentage inhibition formula. The Standard used in this assay was Aspirin. The results showed an increase in % inhibition with the increase in concentration. The values of plant extract were higher than that of standard showing the anti-inflammatory potential of the plant extract.

Standard: $R^2 = 0.9901$

Plant extract: $R^2 = 0.8716$

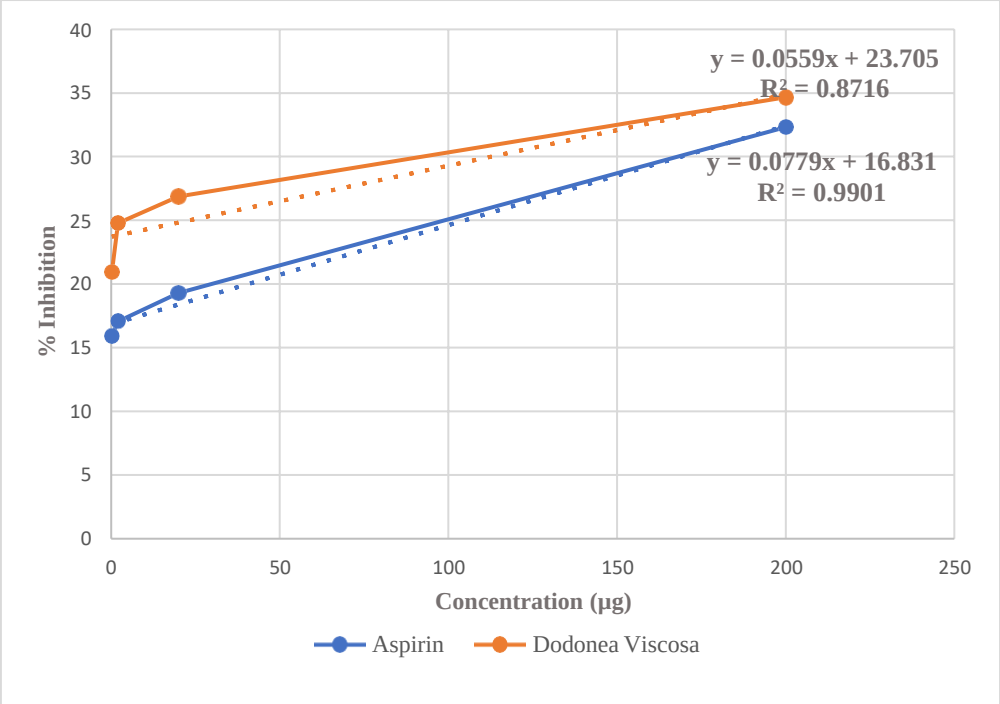

Figure 11 Graph shows increasing plant extract ability to prevent protein denaturation with the increase in its concentration

4.5. Toxicological Assay

4.5.1. Acute Toxicity Analysis (LD_{50})

The mice were calm throughout the experiment and showed an increase in weight with no mortality reported. The LD_{50} of the test substance has a high degree of safety as it is said to be greater than 5000 mg/kg.

PHASE NO.	DOSE CONCENTRATIONS	SIGNS OF TOXICITY	MORTALITIES
Phase 1	30, 300, 500 (mg/kg)	No	0
Phase 2	1000, 2000, 3000 (mg/kg)	No	0
Phase 3	5000 (mg/kg)	No	0

Table 11 Acute toxicity test suggested safe use of plant up to 5000 mg/kg

4.6. In-Vivo Evaluation of Therapeutic Efficacy of *Dodonaea viscosa*

4.6.1. Arthritis Induction

Collagen Induced Arthritic (CIA) models were prepared to check the anti-arthritic property of plant extract. Arthritic induction was measured based on an increase in the paw sizes of the mice from day 0 to day 14. The normal and arthritic groups were compared for paw measurements that were taken in mm^2. Moreover, CIA scoring was done. The mice with 3-4 CIA scoring were taken for further treatment. Increase in the paw sizes of arthritic and normal mice was observed with a P value of 0.0001 (significant). The graph shows an increase in Paw size in mm^2 over the course of arthritic induction compared to the healthy control group. Two-way ANOVA was applied, and the results are expressed as ±SD.

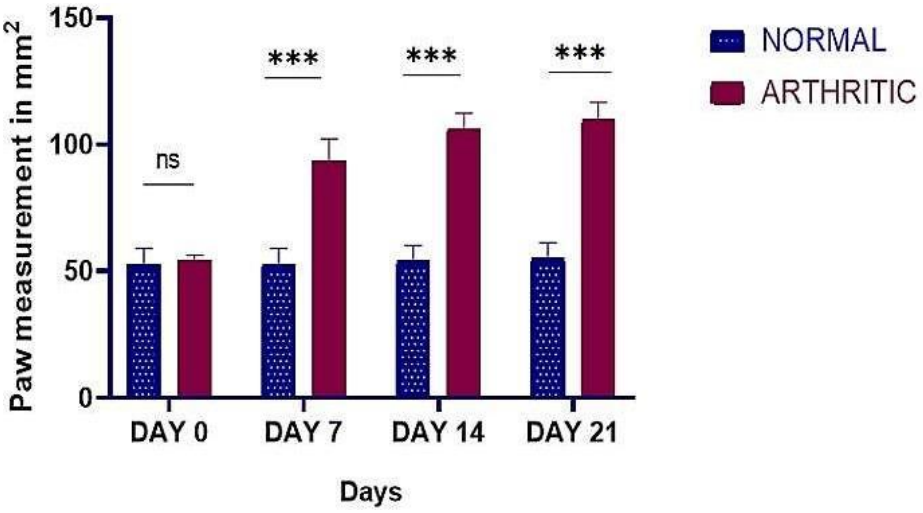

Figure 12 Paw measurement in mm^2 of normal and arthritic mice with each injection administration. The graph shows an increase in paw size with each dose of injection. Two-way ANOVA was applied, and the results are expressed as ± SD with P< 0.0001

4.6.2. Treatment with standard drug and plant extract

After the arthritic induction, mice were subjected to the treatment for 14 days. Paw measurements were taken before the treatment and during the treatment on day 0, day 7 and day 14. Negative control and normal groups showed no significant difference whereas there was a significant decrease in paw measurements in group 1, group 2, and positive control. The graph showed a decrease in Paw size in mm^2 over the course of treatment of Leflunomide/*D.visocsa* as compared to the arthritic group. The following are the mice groups subjected to different treatments. Two-way ANOVA was applied, and the results are expressed as ±SD.

Group No.	Groups	Administration
1.	Normal	Standard feed
2.	Negative Control (Arthritis Induced)	Standard feed (no treatment)
3.	Positive Control (Arthritis Induced)	Commercial drug(leflunomide)
4.	Experimental group (Arthritis Induced)	*Dodonaea viscosa* extract

Table 12 Mice were divided into groups for treatment phase

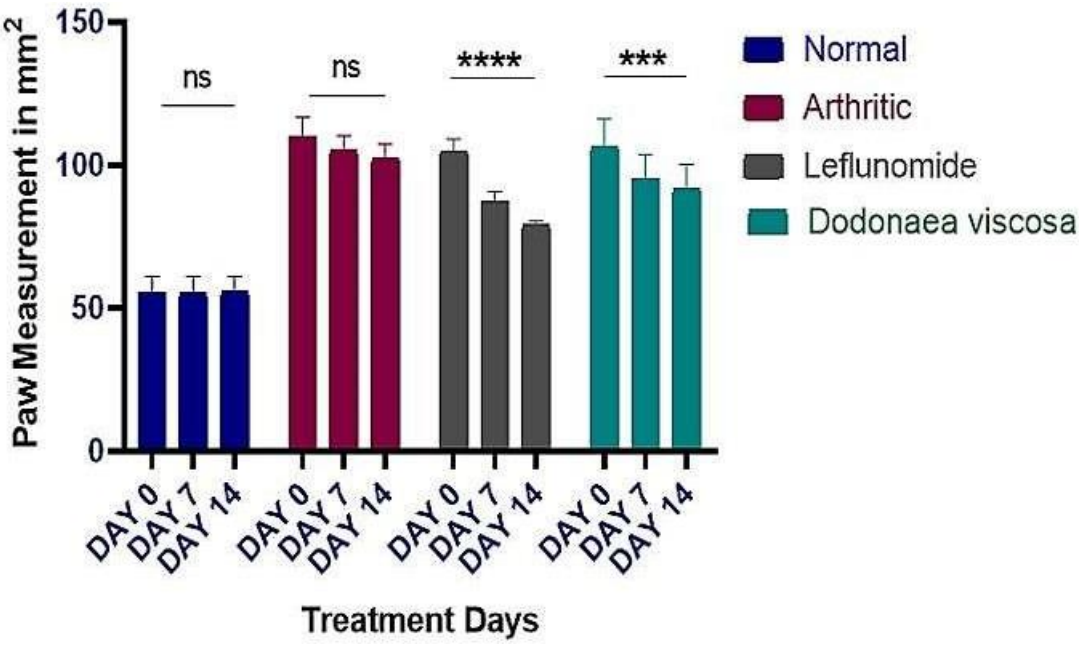

*Figure 13 Significant reduction in paw size over the course of treatment with the standard drug (leflunomide) and Dodonaea viscosa. Two-way ANOVA was applied. The results are expressed as ± SD with **** : P <0.001 & *** : P ≤ 0.0003*

4.6.3. Spleen Indices

In an autoimmune disease, the spleen size increases. Spleen Indices were measured and compared between the mice groups (normal, arthritic, leflunomide and *Dodonaea viscosa*-treated groups) to see the effect of different treatments. The values of arthritic and normal groups indicated significant spleen enlargement. However, the treated groups showed values closer to normal group indicating a protective function. One-way ANOVA was applied, and the results are expressed as ±SD with the P values as following *** $P \leq 0.001$, ** $P \leq 0.0048$, *$P \leq 0.0126$.

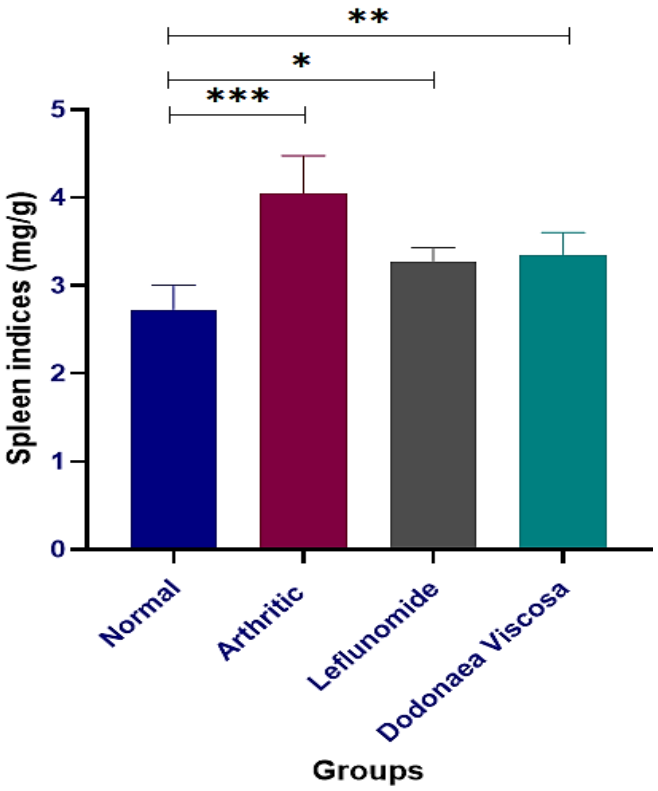

*Figure 14 Comparison of spleen indices of 6 samples from each group showed significant results. The maximum spleen enlargement was observed in arthritic group. One-way ANOVA was performed. Results are reported as mean ± SD with the P values as following *** P ≤ 0.001 ,** P ≤ 0.0048, *P ≤ 0.0126*

4.6.5. Rheumatoid Factor Score

In RA, the RF levels increase the normal levels causing the immune system further to cause further damage at the joint site. The normal levels of RF in human are around 0-14 IU/ml serum. A dramatic difference was revealed between the serum levels of normal and negative control groups. Moreover, the treated groups showed values are within the normal range suggesting the positive results of the plant extract and commercial drug (Leflunomide) in suppressing the release of RF in serum. One-way ANOVA was applied, and the results are expressed as ±SD with the P values as following ***P ≤ 0.001, **P ≤ 0.0009.

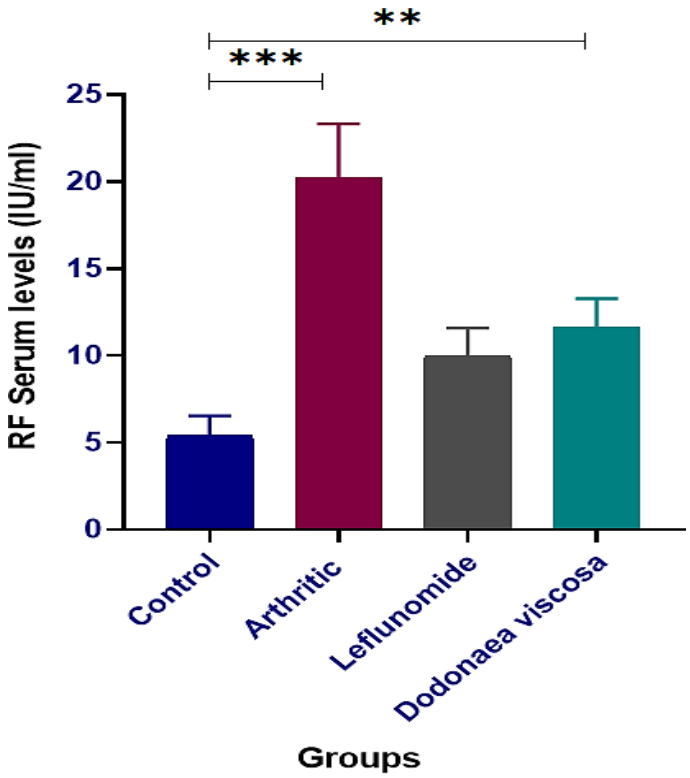

*Figure 15 Rheumatoid factor for control, arthritic & treatment groups. n=6 in each group. The most elevated levels of RF serum were observed in arthritic group. Statistical analysis was performed using one-way ANOVA. Results are reported as mean ± SD with the P values as following *** $P \leq 0.001$,** $P \leq 0.0009$*

4.6.6. Liver Catalase Test

The antioxidant enzyme, catalase, protects the organs from oxidative stress due to production of ROS species. Liver tissues of arthritic mice, normal and treated mice were compared to assess the catalase activity. A significant difference was observed. The graph showed higher levels of catalase enzyme in arthritic mice. Whereas less ROS species were produced in the treated group indicating the therapeutic potential of the plant extract. One-way ANOVA was applied, and the results are expressed as ±SD with the P values as following ***$P \leq 0.001$.

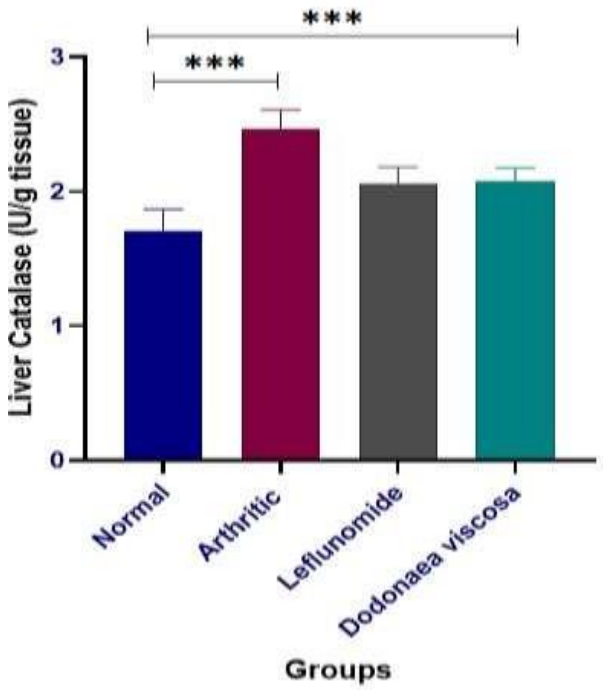

*Figure 16 Catalase activity for control, arthritic & treated groups was observed. n=6 in each group. The most increased liver catalase activity was seen in arthritic group showing maximum ROS presence. Statistical analysis was performed using one-way ANOVA. Results are reported as mean ± SD with the P values as following *** P ≤ 0.001*

4.6.7. X-rays of Hind Paws

After the sacrifice of the mice their hind paws were taken for radiological evaluation, to see the therapeutic efficacy of the treatment phase. X-rays of right hind paws showed significant distance between the joints, inflammation in toes, bone erosion, bone destruction, and soft tissue inflammation. However, there was no soft tissue inflammation in the normal paws. The treatment groups showed less bone destruction and less swelling indicating the therapeutic effect of plant extract. The red arow shows bone destruction in toes, while the yellow arrow indicates soft tissue inflammation.

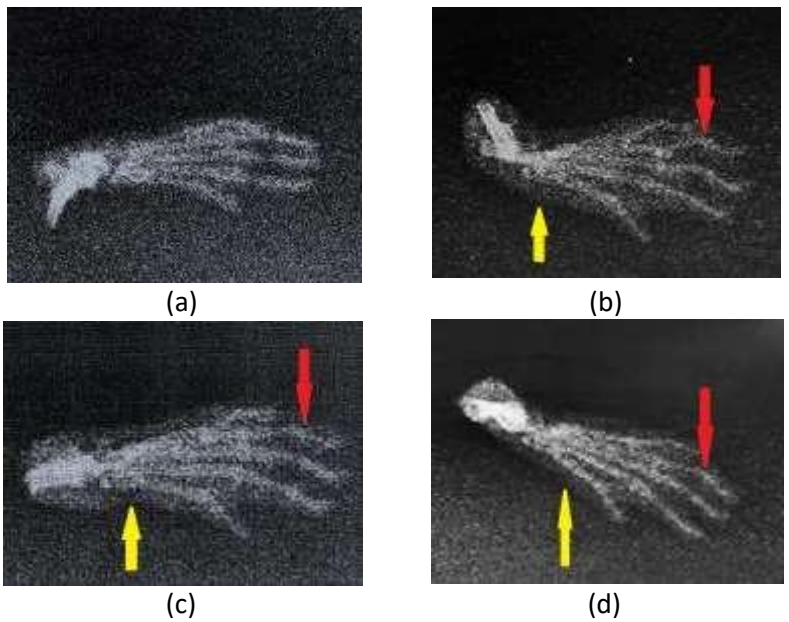

Figure 17 Images (a), (b), (c), (d) shows the radiological examination of normal, arthritic, leflunomide, and Dodonaea viscosa groups respectively. The red arow shows bone destruction in toes, while the yellow arrow indicates soft tissue inflammation.

4.6.8. Qualitative and Quantitative Analysis on CIA Model

4.6.8.1. Qualitative Analysis (Sandwich ELISA)

Serum Interleukin-6 concentrations were measured for control, arthritic and treated groups to establish possible correlation between IL-6 & the development and progression of arthritis. The results showed significantly lower levels of IL-6 in normal mice. However, enhanced production of IL-6 in serum was seen in arthritic mice. The treatment groups (leflunomide/*Dodonaea viscosa*) showed lower levels compared to arthritic group proving the drugs to be effective. The results were significant with P-value <0.001. Statistical analysis was performed using two-way ANOVA. The values were presented as mean ± SD.

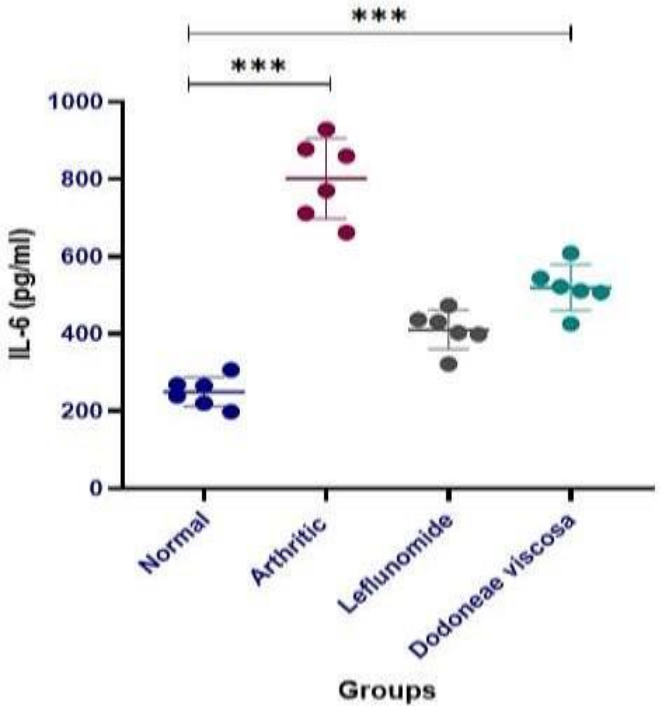

*Figure 18 Enzyme-linked immunosorbent assay was used to evaluate IL-6 serum levels where n=6 in each group. The maximum relative IL-6 expression was seen in arthritic group. Statistical analysis was performed using two-way ANOVA. The values were presented as mean ± SD with *** $P \leq 0.001$*

4.6.8.2. Quantitative Analysis (Real-Time PCR)

The mRNA levels of interleukin-6 in mice ankle joints were determined using real-time PCR to observe the therapeutic effect of *D.viscosa*. The expression of IL-6 gene relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; endogenous control) was quantified using 2 (-Delta Delta C (T)) Method. The results of the treatment groups showed significantly decreased IL-6 levels as compared to the arthritic group. Moreover, the arthritic group showed several folds higher expression of IL-6 mRNA as compared to the normal group. The values were presented as

mean ± standard error of the mean (n=6 samples per group) analyzed by ANOVA test with P-value <0.001.

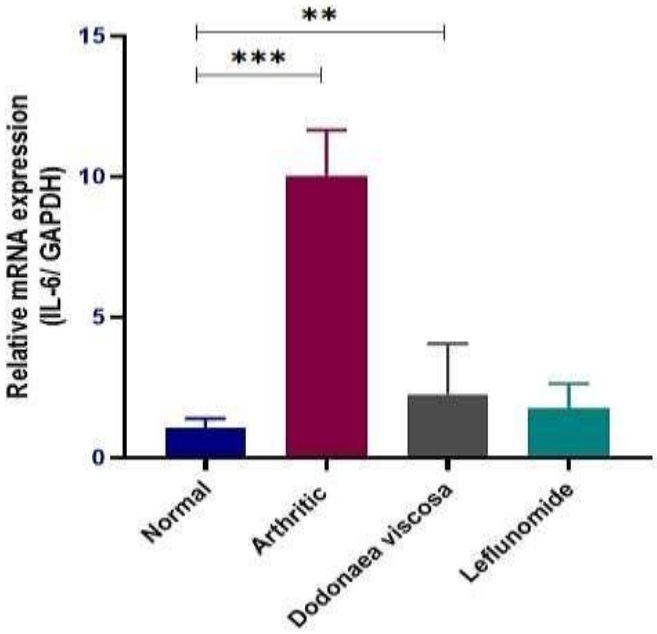

*Figure 19 IL-6 expression in ankle joints detected by Real-time PCR where n=6 in each group. The maximum relative IL-6 mRNA expression was seen in arthritic group indicating maximum IL-6 expression. Statistical analysis was performed using two-way ANOVA. The values are presented as mean ± SD with the P value of *** P ≤ 0.001, ** P ≤ 0.01*

DISCUSSION

Rheumatoid Arthritis (RA) is an autoimmune disease that causes chronic inflammatory arthropathy. It is a syndrome associated with bone and cartilage damage leading to disability, pulmonary vasculitis, systemic complications along with increasing socioeconomic decline and mortalities (Murphy et al., 2008). Autoimmune diseases have no cure, and treatments should be taken for a lifetime to help reduce the progression of the disease. All the currently available pharmacological treatments improve the quality of life yet increase the chances of various systemic comorbidities such as cardiovascular, gastrointestinal diseases and others. Various studies identified medicinal plants as an excellent alternative for RA treatment. There is a paradigm shift towards ethnomedicines proven to be cost-effective and safe with fewer side effects. *Dodonaea viscosa* is traditionally used in various pathological conditions such as anti-inflammatory, analgesic, spasmolytic, antiviral, anti-microbial, laxative, rheumatism, hypotensive, gout, fractures, wound healing, haemorrhoids, and snake bites.

The goal of the study was to investigate the anti-rheumatic potential of the *Dodonaea viscosa* plant through in-vitro and in-vivo analysis. Firstly, the plant was subjected to qualitative phytochemical analysis that resulted in the identification of various bioactive compounds as described in the results. Our data is consistent with the other studies where the ethanolic extract of plant leaves showed the presence of similar compounds (AL-Oraimi & Hossain et al., 2016). Further, the quantitative phytochemical analysis i.e. total phenolic content (TPC) and total flavonoid content (TFC) was performed. The TPC and TFC of *Dodonaea viscosa* were 29.88 mg GE/g of dry weight extract & 54.33 mg RE/g of dry weight extract, respectively. These results confirmed and extended the data shown by other researchers (Riaz et al., 2012; AL-Oraimi & Hossain et al., 2016). The presence of phytochemicals owes to the effective use of the plant for various pathological conditions traditionally. A study performed by Nayeem et al. determined the antioxidant activity of the methanolic of the plant. In another study, *Dodonaea viscosa* crude extracts in different solvents were subjected to identify the redox properties using DPPH assay. The maximum antioxidant activity was shown by hexane and ethyl acetate extract along with the isolation of 2 flavanol and sterol compounds confirming the antioxidant potential of the plant leaves (Habsi & Hossain et al., 2019). Consistent with these observations, the DPPH assay results of our study also showed an increase in free radical scavenging ability as the extract concentration increased. Based on these findings, we can suggest *Dodonaea viscosa* as a good source of antioxidant compounds. In this study, albumin denaturation inhibition assay was also performed to evaluate the anti-inflammatory potential of the plant. The results showed an increased anti-inflammatory activity of the plant, as compared to the standard i.e., aspirin, with the increase in concentration of the extract. Another study performed by Muhammad et al tested the anti-inflammatory potential of a compound (Nebrodenside A) isolated from *Dodonaea viscosa* in carrageenan-induced mice. The plant was found to be highly active in reducing inflammation and pain as compared to the standard drug diclofenac

(Muhammad et al., 2019). These findings suggest a robust potential of the plant to be used as a medicinal drug for inflammatory diseases. In-vivo analysis of the extract was performed by constructing collagen-induced (CIA) mice models. The protocol followed in the study induces chronic arthritis in mice that sustains the immunological response and stays erosive for around 60 days in comparison to the other experimental models (Boe et al., 1999a). The acute toxicity test was performed that proved the protective and safe nature of the plant as described earlier. The mice models were subjected to oral treatments of leflunomide (commercial drug) and *Dodonaea viscosa* to find their potential to treat RA. During arthritic induction and treatment, paw sizes were measured to assess the degree of inflammation. An appreciable increase in paw was observed in the arthritic group in comparison to the other groups. However, the treatment and positive control group exhibited reduced paw size than the arthritic group suggesting the plant to be a good therapeutic intervention. Many studies suggested the role of pro-inflammatory cytokines such as TNF, IL-1, and IL-6 in the aggravation of RA (Feldmann et al., 1996). This study also focuses on the qualitative and quantitative interleukin-6 expression analysis in all mice groups. For qualitative analysis, sandwich ELISA was performed to establish a possible correlation between IL-6 & arthritis. The kinetics of IL-6 in the serum were found to be parallel with the severity of symptoms in the arthritic group. However, reduced arthritic symptoms and IL-6 serum levels were observed in the treated groups. The quantitative analysis was also performed through real-time PCR to determine IL-6 mRNA expression. The arthritic group showed several folds higher expression of IL-6 as compared to the normal and treatment groups. The findings suggest IL-6 association in the development of the disease. This phenomenon correlates with another study on knocked-out IL-6 mice where the severity of arthritic symptoms was reduced in the absence of IL6 (Williams et al., 1992; Joosten et al., 1996). Another study corresponds to the results where enhanced IL-6 levels in synovial fluid and sera were observed in human RA patients. Thus, this study supports the notion of using serum IL-6 as a parameter to monitor disease progression (Takahisa Sugita et al., 1993). Based on these findings, we can also speculate that IL-6 induced arthritic development in CIA and that the suppressive effect of *Dodonaea viscosa* may be because of IL-6 signal transduction inhibition at some stage. Further studies could be performed to confirm this hypothesis. Taken together, this study indirectly suggests the use of *Dodonaea visocsa* as an IL-6 blockade to suppress IL-6 expression during the treatment of inflammatory arthritis. (Ohshima et al., 1998b) In addition, during the expression studies, the *Dodonaea viscosa*-treated group showed elevated IL-6 levels in comparison to the leflunomide-treated group. Based on these results, we can speculate that the dose and time (2 weeks) of the extract administration were not sufficient to suppress IL-6 and CIA development. The therapeutic efficacy was evaluated by clinical parameters, spleen indices, liver catalase activity rheumatoid factor, and paw x-rays for radiological evaluation of joints. The radiological examination of the arthritic paw revealed severe inflammation of soft tissues, bone erosion, and joint destruction as compared to the reduced symptoms in treated mice paws. There is evidence in support of the role of IL-6 in osteoclast formation that contributes to osteoclastic bone resorption. It is possible that the increased IL-6 levels might have

triggered IL-6-induced osteoclasts differentiation resulting in joint destruction in arthritic mice (Yokota et al., 2021). Moreover, the spleen indices were compared between different groups. The results showed a significant difference indicating spleen enlargement in the arthritic group. The data suggests that CIA induction and increased IL-6 levels might disturb systemic immunological responses in arthritic mice. Thus, the restored IL-6 levels in treatment groups also restored the spleen size. Further studies are required to understand the underlying mechanism (Boe et al., 1999b). Similarly, RF values were obtained in the normal range for the treated groups as compared to the arthritic group. It has been hypothesized that IL-6 triggers B-cell differentiation. It is reasonable that elevated IL-6 levels induce B cell hyperactivation in RA that might result in the over-production of autoantibodies such as rheumatoid factor (Boe et al., 1999b). It can also be speculated that the extract might be capable of influencing lymphocyte differentiation during cell-mediated responses in the treatment group. Recently, Tong et al. demonstrated the antioxidant effects of *Dodonaea viscosa* against carbon tetrachloride oxidative damage in the liver, spleen, and kidney (Z. W. Tong et al., 2021). In the present study, the liver catalase test showed excessive ROS production in the arthritic group as compared to the treated groups. No observable morphological changes were seen in either treated groups supporting the previous study. Thus, the results are relevance signifying the protective use of *Dodonaea viscosa* against liver toxicity (Z. W. Tong et al., 2021).

CONCLUSION AND FUTURE PERSPECTS

In conclusion, the ethanolic extract of *Dodonaea viscosa* showed presence of multiple phytochemical compounds. The extract possesses antioxidant and anti-inflammatory potential. Moreover, *Dodonaea viscosa* extract was safe up to concentrations of about 5000mg/kg suggested through LD_{50} analysis. The study suggests the use of *D.viscosa* to suppress IL-6 expression during RA treatment.

However, further studies are required to understand the value of the plant as a potential for RA treatment. Further in-vivo studies are required on larger sample size to validate these results. In-silico analysis must be performed to pinpoint the bioactive compounds involved in RA treatment.

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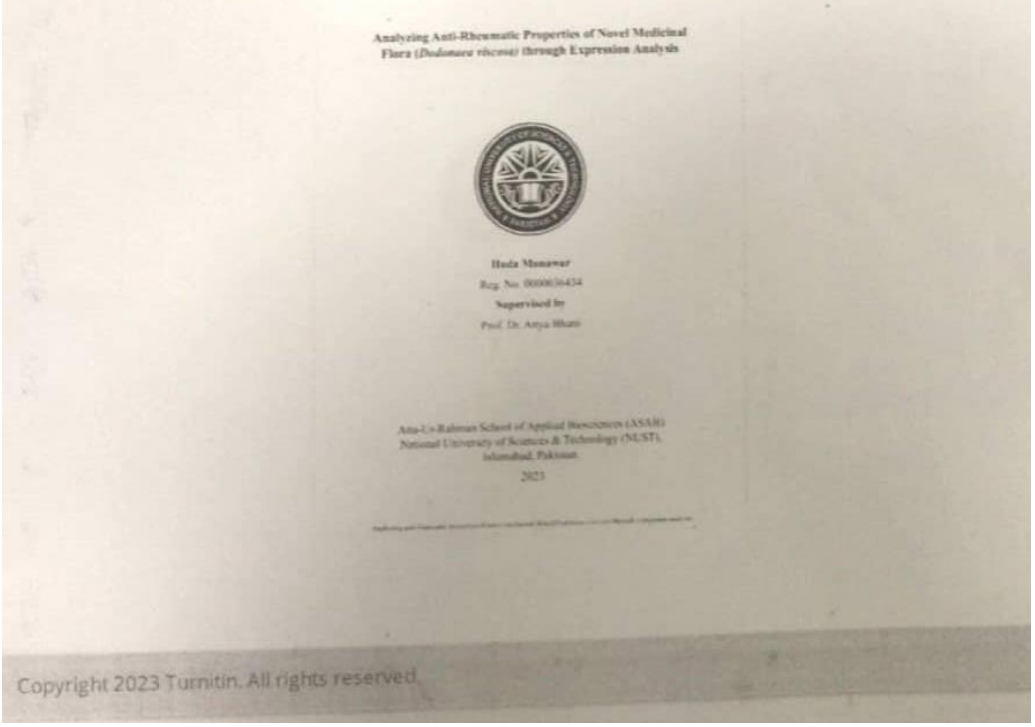

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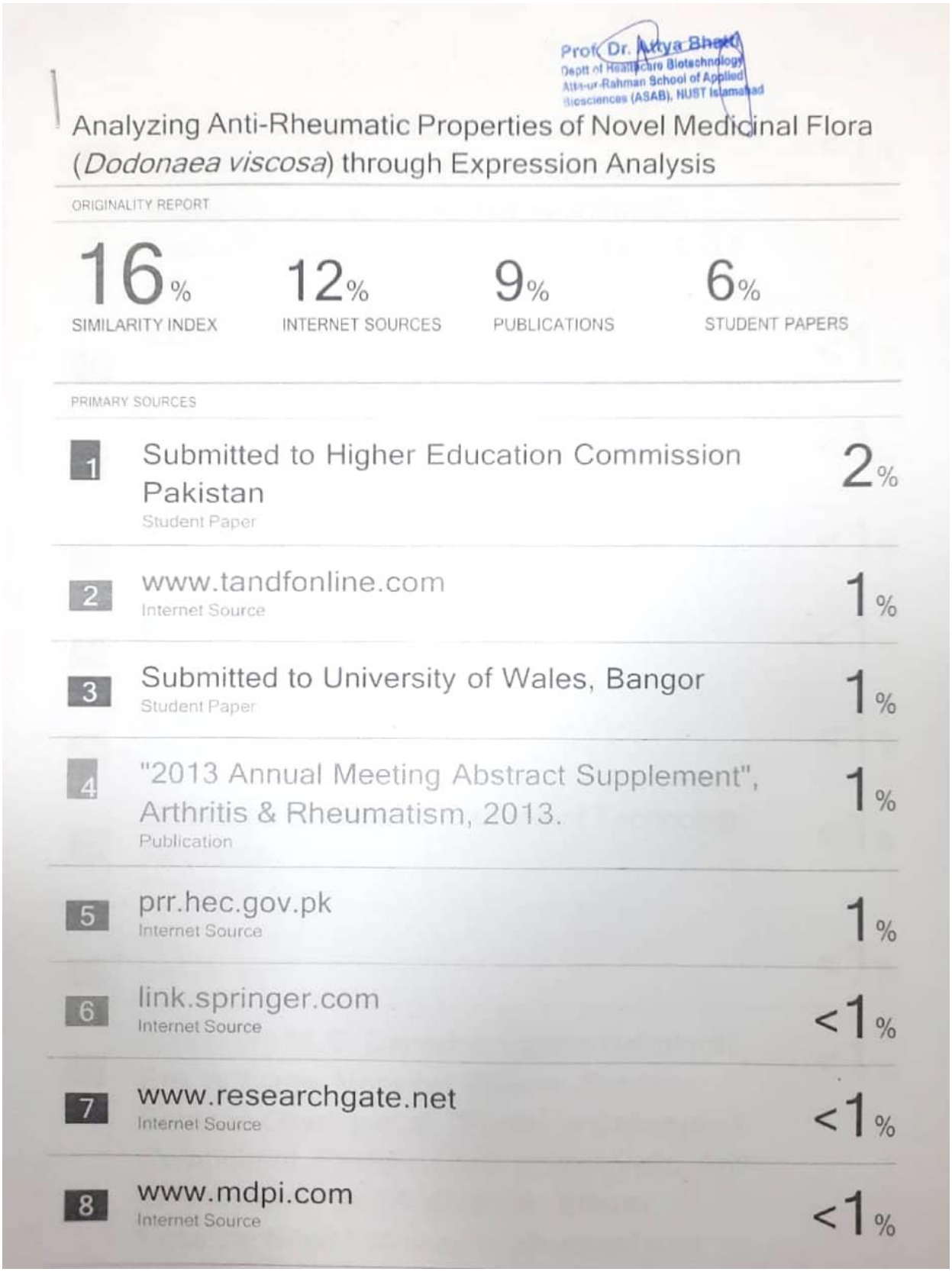

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