Therapeutic Evaluation of *Foeniculum vulgare* Derived Selenium Nanoparticles in Arthritic BALB/c Mice Model

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Nothing is deserving of worship except Almighty "ALLAH" the most merciful, compassionate and gracious. All praises for Him. All respects for HIS HOLY PROPHET HAZRAT MUHAMMAD (Peace BE Upon Him) who taught me the right path and enabled me to recognize the oneness of my creator.

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Dedicated to

My Parents

For their endless love, support and encouragement

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LIST OF ABBREVIATIONS

RA	Rheumatoid Arthritis
ACPA	Anti-Citrullinated protein Antibodies
RF	Rheumatoid Factor
ESR	Erythrocyte Sedimentation Rate
SDI	Socio demographic Index
TNF	Tumor Necrosis Factor
IL	Interleukin
MMPs	Matrix Metalloproteinases
NF-κB	Nuclear Factor Kappa b
COX	Cyclooxygenase
HIF	Hypoxia Inducible Factor
ROS	Reactive Oxygen Specie
RNS	Reactive Nitrogen Specie
SE	Shared Epitope
MHC	Major Histocompatibility Complex
HLA	Human Leukocyte Antigen
ARA	American Rheumatism Association
DMARDs	Disease Modifying Anti-rheumatic Drugs
NSAIDs	Non-steroidal Anti-inflammatory drugs
HPA	Hypothalamic-pituitary-adrenal axis
IFX	Infliximab
HCQ	Hydroxychloroquine
GI	Gastrointestinal
MTX	Methotrexate
SeNP	Selenium Nanoparticle
SPS2	Selenophosphate synthetase 2
TRx	Thioredoxin reductase
GSH-Px	Glutathione peroxidase
UV	Ultra violet
CIA	Collagen induced Arthritis
CFA	Complete Freund's Adjuvant
BSA	Bovine Serum Albumin
ANOVA	Analysis of Variance

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease affecting diarthrodial joints. It is characterized by erosive synovitis, cartilage and bone destruction, systemic complications affecting 1% of the world population. Current treatment modalities for Rheumatoid arthritis, though effective, but their use is limited by multiple side effects. Therefore, researchers are exploring better and safer treatment options. Pro-inflammatory transcription factor, nuclear-factor-kappa B (NF-κB) controls immune responses, proliferation and inflammation in rheumatoid arthritis. Hypoxia is a hallmark of rheumatoid arthritis that occurs due to an imbalance of oxygen supply and demand in the synovial tissue and results in the inflammation, angiogenesis and even cell death. Therefore, NF-κB pathway induced gene Hypoxia-inducible factor 1 alpha (Hif1-*α) can be used as a therapeutic target in rheumatoid arthritis.* This study aimed to investigate the therapeutic efficacy of Biogenic *Foeniculum vulgare* derived selenium nanoparticles to ameliorate oxidative stress and inflammation in rheumatoid arthritis due to their antioxidant potential. The selenium nanoparticles were synthesized using seed extract in which sodium selenite was used as a precursor. The therapeutic potential of selenium nanoparticles to target Hypoxia-inducible factor 1 alpha (Hif1-*α)* was checked by In Silico Molecular Docking analysis in which Selenium was used as a Ligand and HIF1- alpha as a target protein. The Arthritic mice model was successfully constructed using Collagenase type 2 and Freund's adjuvant to check the effectiveness of biogenic selenium nanoparticles. The results of In-silico Molecular Docking showed that there is a binding affinity between Selenium nanoparticles and Hif1-*α*. *These results will be a step toward the further testing and evaluation of biogenic Foeniculm vulgare derived selenium nanoparticles in wet lab to study their antioxidant and anti-arthritic potential in arthritic mice models and cell lines.*

INTRODUCTION

1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder primarily affecting joints. It is characterized by cartilage and bone destruction. The arthritis is originated from a Greek word meaning "joint inflammation" (Kesharwani, Paliwal, Satapathy, & Paul, 2019). RA is characterized by progressive synovitis, autoantibody production and joint destruction, diarthrodial joints being affected first (Alamanos, Voulgari, & Drosos, 2006; Scheel et al., 2006). In RA patients hand and feet joints are affected first, consequently affecting other joints of the body (K. L. Ong, Wu, Cheung, Barter, & Rye, 2013). Several autoantibodies have been associated with RA such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA).

Rheumatoid arthritis significantly decreases patients' functional capacity, increases the morbidity and mortality rates, and results in significant costs for the health and social care systems (Lajas et al., 2003). The prevalence of RA is 1% of the worldwide population and women are more affected (Ming Di, Zhou, Guang Li, & Zhou, 2011). Although the onset is more frequent during the fourth and fifth decades of life, RA can occur at any age. The clinical signs of RA involve puffiness, rigidity and pain in ankle and knee joints leading to deformity and immobility in later stage (Q. Zhang et al., 2018). Patients have a higher risk of other autoimmune and cardiovascular disorders (Gibofsky, 2012). The incidence of secondary complications and cardiovascular diseases increases with age (K. L. Ong et al., 2013). The exact etiology of the disease is unknown, but several genetic and environmental factors are associated with its progression and severity (Barton & Worthington, 2009).

1.2. Prevalence of rheumatoid Arthritis

It is estimated that RA approximately affects 0.24 to 1% of the worldwide population and the incidence is twice in women as compared to men (Safiri et al., 2019). The risk of RA development is 1.7% in men and 3.6% in women (Crowson et al., 2011). According to the Global Burden of Disease study conducted in 2010, the prevalence of RA is higher in northern European countries and the United States ranging from 0.5 to 1% of their population(Aljary, Czuzoj-Shulman, Spence, & Abenhaim, 2020; Myasoedova, Davis, Matteson, & Crowson, 2020). In northern European countries and the United States an annual estimation of RA incidence is 40 per 100,000 persons approximately (Klareskog, Rönnelid, Saevarsdottir, Padyukov, & Alfredsson, 2020). As most of the RA epidemiological studies are conducted in northern European countries and the United States, therefore statistics of prevalence and incidence of RA largely come from these countries.

According to GBD 2017 study, the detailed data of RA prevalence from 1990 to 2017 is given based on global, national and regional prevalence, and incidence in terms of sex, age and Socio demographic Index (SD1). The Age-standardized incidence rate was comparatively higher in females, which increases with age and found to be among 70 to 74 age groups of females and 75 to 79 of males. This study indicated that the number of affected increased with age and was maximum at 60 to 64 age groups of males and females, and the trend declined after this age (Safiri et al., 2019).

1.3. Pathogenesis of Rheumatoid Arthritis

Inflammation, Synovial hyperplasia, autoantibody production and joint destruction are the hallmarks of rheumatoid arthritis (Alamanos et al., 2006). The synovium of RA is a multicellular

tissue which contains different cell types such as T cells, dendritic cells, synovial fibroblast and macrophages. These different cells interact with each other to promote inflammation (Fearon, Hanlon, Wade, & Fletcher, 2019). The activation of T cells, B cells, plasma cells, dendritic cells and macrophages cause sinusitis, but the severity of inflammation and joint degradation depends on the level and number of activated macrophages in the synovium.

It is a multifactorial disease that results in bone and cartilage destruction. The combined infiltration of adaptive and innate immune cells along with a complex cytokine network into the synovial joint leads to synovitis (Bartok & Firestein, 2010). Activated T cells release a complex network of pro inflammatory cytokines such as TNF-α, IL-1 beta, into the synovial membrane shown in Figure 1.1. Macrophages are then activated to secrete various pro-inflammatory cytokines and thus creating an amplified pro-inflammatory loop (Su et al., 2015). Differentiated memory B-cells produce ACPA and RF autoantibodies. Synovial fibroblast-like cells releases metalloproteinases (MMPs) that lead to bone erosion by dissolving cartilage and activating osteoclast at the interface of pannus-cartilage (Sabeh, Fox, & Weiss, 2010; Tolboom et al., 2002).

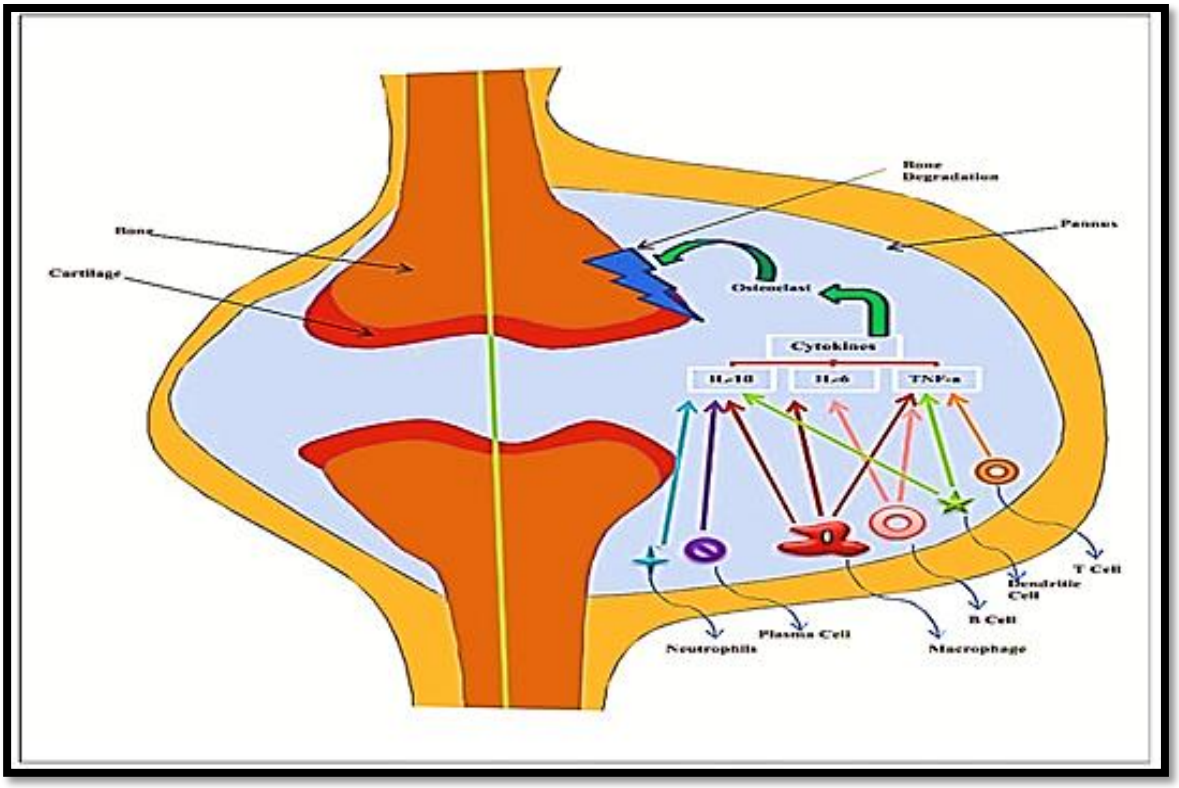

Figure 1.1: Pathophysiology of RA joint

The hyperactivation of pro-inflammatory cytokines such as interleukin (IL) -1β, IL-6, TNF-α, metabolic enzyme COX, and suppression of anti-inflammatory cytokines IL-10 and IL-4 lead to the pathogenesis of the disease. Another pro-inflammatory transcription factor, nuclear-factor-kappa B (NF-κB) controls immune responses to the proliferation and inflammation. The expression of NF-κB is significantly higher in RA patients (Miller et al., 2010). Free radicals and reactive oxygen species(ROS) are also involved in the RA pathogenesis (Afonso, Champy, Mitrovic, Collin, & Lomri, 2007). NF-κB has a vital role in the inflammatory and degradative pathways of RA. It controls the level of transcriptional synthesis of various inflammatory signals including IL-1β, NOS, TNF-α and matrix metalloproteinases. These pro-inflammatory signals

trigger the activation of complex signal transduction mechanisms which lead to the progression of RA, e.g., cytokine synthesis and cartilage destruction.

1.4. Risk factors associated with Rheumatoid arthritis

There are multiple genetic and environmental factors associated with the development and progression of the RA. The disease progression is complex to understand especially of seropositive RA, in which an elevated circulation of autoantibodies is seen prior to the development of the disease. This period is called "preclinical RA", which suggests that there is a subset of environmental and genetic factors acting years before the onset of first RA (fig 1.2). The preclinical stage is usually present in seropositive RA, which can be determined by the presence of RA related autoantibodies including ACPA, RF and other autoantibodies (Deane & El-Gabalawy, 2014). The understanding of the genetic and environmental factors along with oxidative stress associated with the development of RA is essential to find the pharmacological targets.

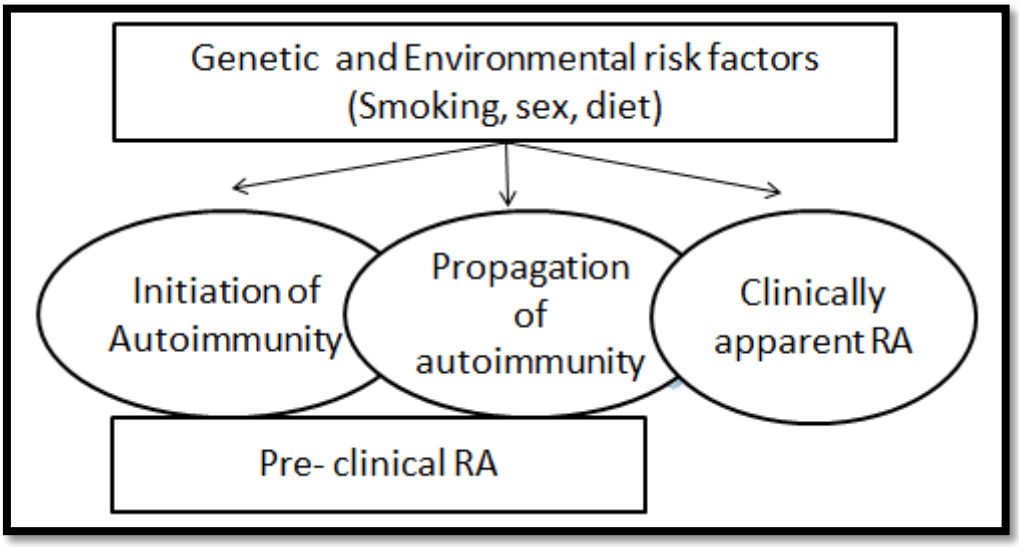

Fig.1.2: A general model of RA development

1.4.1 Genetic risk factors

Genetics have a major impact on the progression and development of RA. These factors suggest increased RA prevalence within families with approximately 40 to 50 percent of seropositive RA (Frisell, Saevarsdottir, & Askling, 2016). In addition to families, increased RA prevalence is seen in different ethnic groups for example, North Americans with 5 to 7 % of prevalence(Ferucci, Templin, & Lanier, 2005). The strongest genetic risk factor is a set of alleles in major histocompatibility complex (MHC) which encodes amino acid to predict the structural similarity of human leukocyte antigen binding groove termed as "shared epitope" (SE) (Gregersen, Silver, & Winchester, 1987; Raychaudhuri et al., 2012). SE alleles contribute to approximately 40% risk of RA development(Frisell et al., 2013). There are several alleles containing SE in the HLA DRB1 region, among them HLADRB1*0404 is a high risk allele (Du Montcel et al., 2005). There are two major genes associated with RA development; the HLA-DRB1 gene and protein tyrosine phosphatase 22(PTPN22). There are multiple variants of the HLA-DRB1 which are linked with the increased risk of RA development. Another genetic association lies in the gene protein tyrosine phosphatase non-receptor 22 (*PTPN22*) (Begovich et al., 2004). The risk is increased when other environmental factors interact with the already present gene in the individual.

1.4.2 Environmental factors

Other than genetics, various environmental factors such as air pollution, smoking, dietary intake, exposure to infections and lifestyle are associated with higher risk of RA development (Källberg et al., 2007). However, their effects are limited to ACPA positive RA (Liao, Alfredsson, & Karlson, 2009). Other risk factors associated with RA are coffee intake, alcohol intake and low socioeconomic status (Mao, Zhang, & Yin, 2018).

1.4.3 Smoking

Multiple studies have confirmed that smoking is a major environmental risk factor associated with ACPA-positive rheumatoid arthritis and it has very low effects on autoantibody negative RA (Klareskog et al., 2006; Padyukov, Silva, Stolt, Alfredsson, & Klareskog, 2004; Pedersen et al., 2007). Several studies have shown that smoking contributes 20 to 30% of the environmental risk of RA as the odd ratio of association was found greater than 2 between RA and smoking. There are several features that show strong association of smoking with RA development, primarily of ACPA positive RA. However, its site of action in the RA development is yet to be determined (Klareskog, Gregersen, & Huizinga, 2010). An important association between smoking and HLA-DR alleles was found in ACPA positive RA patients in one North American (Lee et al., 2007) and 3 European studies (Klareskog et al., 2006).

1.4.4. Dietary factors

Various dietary factors such as medications and supplements are also associated with RA. The higher intake of red meats, iron, protein, sugar and lower vitamin D intake is linked to high risk of RA development (Benito-Garcia, Feskanich, Hu, Mandl, & Karlson, 2007; Merlino et al., 2004; Pattison et al., 2004). According to a large scale study in the United States, it is shown that a healthy diet has a lower risk of seropositive RA (Hu et al., 2017). Furthermore, various dietary studies have shown that a higher intake of omega-3-fatty acids and fish have a decreased risk for RA (Athena Linos et al., 1999; A Linos et al., 1991). It was also reported that a higher intake of omega-3-fatty acids diet is associated with a lower risk of ACPA positivity and RF (Gan et al., 2017; Gan et al., 2016).

1.4.5 Sex linked RA

Gender is an important risk factor associated with RA, as females have two to three-fold higher risk (Alamanos & Drosos, 2005; Kvien, Uhlig, ØDEGÅRD, & Heiberg, 2006; Lawrence et al., 1998). Other genetic factors such as sex hormones and other environmental factors also differ in two genders. The female sex hormones also play an important role in the expression and development of RA (Alpízar-Rodríguez, Pluchino, Canny, Gabay, & Finckh, 2017; Cutolo et al., 2004). The estrogen concentration is higher in both sexes of RA which is attributed to hydroxylated forms, usually 4-hydroxyestradiol and 16a-hydroxyestrone (Cutolo et al., 2004). In another study, the level of urinary 2-hydroxylated estrogens was found to be ten times lower in patients of RA than healthy controls. Another study has shown that androgen receptor exon 1 has fewer number of tandem CAG repeats are present in patients with early onset of RA as compared to late-onset RA male patients (Yong, Lim, Qi, Ong, & Mifsud, 2000).

1.4.6 Air pollution

A suspension of different gases such as sulfur dioxide (SO2), carbon monoxide, ozone and nitrates contribute to the risk of RA development (Essouma & Noubiap, 2015). The major sources of these pollutants include industry, forest fires, stationary fuel burners, solid fuel combustion and heavy traffic. Reactive oxygen species released from these pollutants when entering into the respiratory tract, activate NF-ƙB which is a pro-inflammatory cytokine in RA development (Murphy & Hutchinson, 2015). These cytokines stimulate other monocytes, auto-antigens, T lymphocytes which cause the destruction of synovial joints leading to RA development.

1.4.7 Oxidative stress

Reactive oxygen species (ROS) are of great importance in the pathology of RA (Smith, Smith, & Seidner, 2011). ROS are free radicals such as hydroxyl radicals, peroxide, reactive nitrogen

species (RNS) and superoxide. ROS can be produced because of inflammation and results in the neutrophils degranulation, destruction of bone and cartilage (Stamp et al., 2012). ROS are highly reactive in nature and have the ability to destroy proteins, DNA and lipids in joint tissues. To control their production, various antioxidant defense systems are present in human body. The imbalance of antioxidant and oxidants production results in oxidative stress which damages the biological membranes (Öztürk, Çimen, Çimen, Kacmaz, & Durak, 1999). Due to highly reactive chemical nature of RNS and ROS, they cannot be measured directly in vivo, therefore their effects on proteins, nucleic acids and lipids can be measured (Vasanthi, Nalini, & Rajasekhar, 2009). Oxidative stress is found to be involved in the joint destruction and inflammation in RA patients and arthritic animal models (Leonavičienė, Bradūnaitė, Vaitkienė, Vasiliauskas, & Keturkienė, 2008). The oxidative status is altered in serum and synovial fluid of RA patients (Bracht et al., 2016).

1.4.8 Reactive Oxygen Species and hypoxia

Hypoxia is an important feature of RA synovial membrane. It was first identified in 1970 in the synovial tissue of RA patients in which oxygen tension was lower as compared to health controls (Falchuk, Goetzl, & Kulka, 1970; Lund-Olesen, 1970). Hypoxia is a hallmark of rheumatoid arthritis which occurs due to imbalance of oxygen supply and demand in the synovial tissue and results in the inflammation, angiogenesis and even cell death. The increased neovascularization of immature blood vessels in synovium is incapable of supplying enough oxygen to meet the desired demand of cells (Distler et al., 2004).

In order to explain the hypoxia in RA synovium, two theories are proposed. The first one suggests that a high metabolic energy demand creates a gap in the nearby blood vessels and proliferating cells which results in decreased supply of oxygen than required level (Larsen,

Akhavani, Raatz, & Paleolog, 2007). The second theory explains that hyperplasia, joint movement and synovial fluid effusion generates intra-articular pressure in the joint capsule, which collapse the capillaries network and hence results in the insufficient blood supply in synovial tissue (Jawed, Gaffney, & Blake, 1997).

To cope up with the hypoxic condition, cell has various mechanisms; one of them is the activation of hypoxia inducible factor (HIF)(Semenza & Wang, 1992). HIF is a transcription factor and key regulator of this process which regulates different genes related to cell migration, angiogenesis, energy metabolism, apoptosis etc. (Semenza, 2009). HIF was detected in human (Hep3B) hepatoma cell line nuclear extract, which is composed of two subunits; HIF 1 alpha and HIF1 beta. HIF-1β is constitutively expressed while HIF-1α is regulated by oxygen levels (G. L. Wang & Semenza, 1995). Under hypoxic condition cells activate pro-inflammatory transcription factor NF-kB signaling pathway in addition to the HIF-1α. NF-kB is involved in the regulation of IL-8, IL-6, TNF-α, matrix metalloproteinase, vascular endothelial growth factor (VEGF) and different chemokines which control inflammation, bone destruction and angiogenesis (Drexler & Foxwell, 2010) shown in fig 1.3. Therefore, NF-kB works in a synergistic and additive manner with HIF and promotes the inflammatory mechanism in RA (Oliver, Taylor, & Cummins, 2009;

Quiñonez-Flores, González-Chávez, & Pacheco-Tena, 2016).

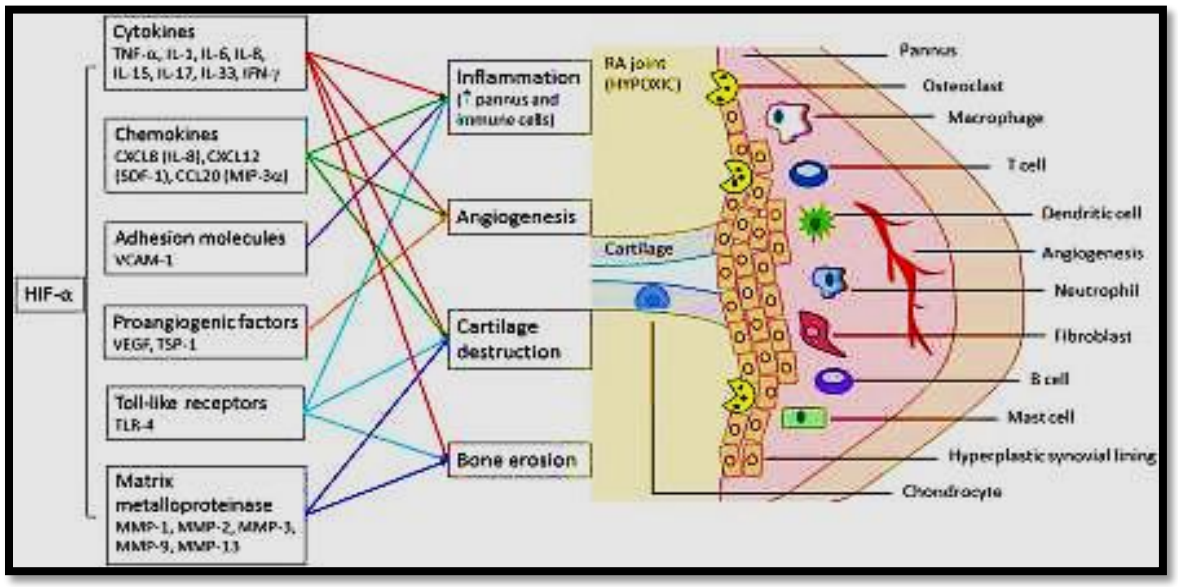

Fig. 1.3: Role of oxidative stress induced hypoxia in Pathogenesis of RA

1.5 Clinical Diagnosis of Rheumatoid arthritis

There is not a single test or criteria to diagnose RA. Patient's history and clinical findings are important and challenging because the disease symptoms are quite similar to some other inflammatory diseases (Weinblatt & Kuritzky, 2007). However, there are specific tests that are used for enhanced diagnostic and disease progression level. The American college of Rheumatology along with American Rheumatism Association(ARA) presented 7 different diagnostic criteria for clinical diagnosis of RA in 1987 (Costenbader & Kountz, 2007) which were updated in 2010 classification criteria of European League Against Rheumatism (EULAR)/ ACR. According to EULAR/ ACR, any person having at least 4 of the specified symptoms for six weeks or even longer is said to have RA (Arnett et al., 1988).

1.5.1 Laboratory tests

For correct diagnosis of RA different parameters are tested in laboratory including complete blood cell count having differential rheumatoid factor, C-reactive protein more than 0.7 pg/mL, erythrocyte sedimentation rate (ESR) of more than 30 mm/h. Other hepatic and renal functions are also monitored throughout the treatment (Edward & Emery, 2005). Rheumatoid factor is an immune complex of IgG and autoantibody and positive RF does not always indicate RA. As 20% of the RA patients may be negative for RF, on the other hand about 5 to 10% healthy individuals may be RF positive and also positive in some other diseases (Birch & Bhattacharya, 2010). Therefore, some other diagnostic tests are performed which are given below:

1.5.2 Detection of Anti-Citrullinated peptide (Anti-CCP)

The diagnostic test is based on the detection of Anti-Citrullinated Peptide IgG, the antibody which is produced in the early stage of RA development. The B cells present in synovium produce anti-CCP which is more than 98% specific and its sensitivity is increased when used with RF in a combination. During the early stage of disease it might be negative as one study found its association with advanced stage RA with longer duration (Heidari, Abedi, Firouzjahi, & Heidari, 2010; Samanci et al., 2005).

1.5.3 Plain Film Radiography

Plain film radiography is a method of choice to evaluate and examine soft tissue and bone damage at the initial stage. The changes in the oft tissue might be a source of differential diagnosis in RA. According to the severity of the disease, radiography is able to expose joint space narrowing and soft tissue swelling as a result of cartilage destruction and joint space widening which indicates joint effusion (Firestein, Panayi, & Wollheim, 2000).

1.6. Current Therapies for Rheumatoid arthritis

There are three general classes of drugs for the treatment of rheumatoid arthritis which are given below:

- Non- steroidal anti-inflammatory drugs (NSAIDS)
- Glucocorticoids
- Disease modifying anti-rheumatic drugs (DMARDS)

1.6.1 First line treatment of Rheumatoid Arthritis

The first line treatment aims to decrease inflammation and relieve pain; it includes medications which are fast-acting and include Non-steroidal anti-inflammatory drugs(NSAIDS) and corticosteroids.

1.6.1.1 Non-steroidal anti-inflammatory drugs

NSAIDS involve ibuprofen, etodolac, naproxen and acetylsalicylate (Aspirin). Aspirin is the oldest NSAID which is used for joints pain. It is an affective anti-inflammatory drug when used at higher doses against RA because it inhibits prostaglandins. However, its high doses have some side effects such as gastric intolerance, hearing loss and tinnitus. Other than Aspirin newer NSAIDS are available in the market and as effective as aspirin. These drugs are required at a lower dose per day. They prevent prostaglandins synthesis by inhibiting cyclo-oxygenase. Potential side effects are abdominal pain, gastrointestinal (GI) bleeding, ulcers and nausea. However, these side effects can be lowered if these drugs are taken with food, misoprostol (Cytotec), proton pump inhibitors and antacids. Celecoxib (Celebrex) is a newer NSAID which is a selective inhibitor of Cox-2 and it has lower risk of gastrointestinal side effects (C. Ong, Lirk, Tan, & Seymour, 2007).

1.6.1.2 Corticosteroids

Corticosteroids are stronger anti-inflammatory drugs other than NSAIDs, but they have their potential side effects. That is why they can be used for a short time period and at a low dose. Corticosteroids can be given by intra articular injections for the treatment of local inflammation (Combe et al., 2017). Their mode of action includes limiting the phospholipids release and decreasing eosinophils action, thus lowering the inflammation. The potential side effects are immunosuppression, diabetes, weight gain and bone-thinning. So, it is important to take vitamin D and calcium supplementation to prevent bone thinning. The dose can be reduced gradually as the patient's health improves, but the abrupt discontinuation of the dose can cause suppression of hypothalamic-pituitary-adrenal axis (HPA) (Liu et al., 2013).

1.6.2 Second-Line treatment:

The second line treatment management includes disease modifying ant-rheumatic drugs (DMARDS), which promote healing by stopping or slowing the process of joint destruction. Medications are slow acting which take weeks to months for their effects. DMARDS help in reducing lymphoma which is associated with RA (Smolen et al., 2010). DMARDS are further categorized into biological and non-biological agents

1.6.2.1 Non-biologic DMARDs

Non-biologic DMARDs include Azathioprine (AZA), MTX, SSZ, Minocycline, Gold salts, Cyclosporine, D-penicillamine, Hydroxychloroquine (HCQ) and Leflunomide. Methotrexate is the first choice within this group as it can be used in monotherapy as well as in combination with other DMARDS (Fiehn et al., 2018). Patients with positive RF and high disease activity have a risk of methotrexate failure because of its inefficacy (Verschueren et al., 2017). In such cases when MTX is failed, sulfasalazine or leflunomide can be used. Both of these showed same efficacy in a placebo-controlled randomized trial (RCT) (Smolen et al., 1999). The mechanism of

action of MTX involves depletion of purine and thymidine residues and cell cycle arrest during S1 phase(Brown, Pratt, & Isaacs, 2016). It is folic acid analog which inhibits dihydrofolic acid (FH2) binding to the enzyme which converts FH2 to folinic acid (FH4). In the absence of FH4, the purine and pyrimidine metabolism is impaired, thus amino acids and polyamine synthesis is stopped. MTX is an immunosuppressant and blood tests are required due to its side effects such as cirrhosis, bone marrow deterioration and other liver problems (Tian & Cronstein, 2007).

Hydroxychloroquine is an antimalarial drug which is also used for RA treatment. This drug lowers the secretion of pro-inflammatory cytokines. Its side effects involve skin, central nervous system and GI tract problems. High dose particularly affects eyes and patients require regular checkup with an ophthalmologist (Silva et al., 2013).

Sulfasalazine is an effective drug for the treatment of RA which is a combination of anti-inflammatory and antibacterial agent. Its mechanism of action is not completely understood, however its pharmacological effects are on the inflammatory cells functions, immunological processes and gut microbiota (Smedegård & Björk, 1995).

Leflunomide works by reversibly inhibiting dihydroorotate dehydrogenase (DHODH), which is mitochondrial enzyme and rate limiting step in the pyrimidine synthesis. The DHODH inhibition prevents the activated lymphocytes entry from G1 to the S phase, thus trigger apoptosis(Breedveld & Dayer, 2000). Side effects include nausea, diarrhea and elevation of liver enzymes. Some people develop hypertension, therefore it is recommended to monitor their blood pressure during treatment (Krüger & Bolten, 2005).

1.6.2.2Biological DMARDS or biologics

Biological DMARDS or biologics are effective in reducing the joint damage and deformity in RA. They are thought to be more “defined, targeted and direct” approach for treatment of RA

(Bullock et al., 2018). Despite the fact that biologics have some side effects for example, high risk of infections and other neurological diseases like lymphoma and multiple sclerosis (den Broeder, van Herwaarden, & van den Bemt, 2018; Rein & Mueller, 2017; Tovey & Lallemand, 2011).

Biologic DMARDs are TNF inhibitors which bind TNF and help in restricting interaction to its receptors. TNF alpha inhibitor includes Infliximab, Golimumab, Adalimumab, Certolizumab and Etanercept. Infliximab (IFX) is the first TNF-α-inhibitor approved in 2000, which binds to the membrane bound and soluble TNF-α receptors thus blocking its cellular response (Emery, 2001). Etanercept, adalimumab and certolizumab are approved as a monotherapy and methotrexate combination (R. Fleischmann et al., 2017; Gaubitz et al., 2017; Keystone et al., 2018) . As we know that TNF- α plays a major role in immune system during certain infections, therefore this treatment is associated with higher risk of infections such as bacterial infections, opportunistic infections, tuberculosis, herpes zoster and pneumonia (Singh et al., 2011).

Interleukin 1 Inhibitor includes Anakinra approved in 2001 in US but it showed moderate effectiveness against RA (Konttinen et al., 2006; Singh, Noorbaloochi, & Singh, 2010). Side effects include pain, redness and itching at the site of injection. One to ten percent patients showed decreased white blood cells or platelets and severe infections (R. M. Fleischmann et al., 2006).

While Interleukin-6 (IL-6) inhibitors are Tocilizumab (TCZ) and Sarilumab, which bind to the membrane-bound and soluble IL-6 receptor thus blocking its action which results in decreased inflammatory response in RA (X. Zhang et al., 2013). Their side effects are hyperlipidemia, liver enzyme elevations and severe infections (Genovese et al., 2013).

1.7. Need for an alternative therapy

RA has been under investigation for its successful treatment for more than two centuries and despite decades of scientific research it is still a challenge for existing medicines. Currently available drugs only improve the quality of life of patients to some extent but do not reverse the damage. Therefore, we need to discover some alternative therapeutic options for RA treatment. Nano-therapy has gained attention due to its enhanced therapeutic efficacy; therefore, the present study was conducted to assess the potential of essential micronutrients such as Se at Nano-scale due to its antioxidant and anti-inflammatory properties that could ameliorate symptoms of rheumatoid arthritis.

Hypothesis

The proposed study is focusing on the antioxidant and anti-rheumatic potential of selenium nanoparticles synthesized from local plant *Foeniculum vulgare.* Oxidative stress activates inflammatory cytokines and various transcription factors such as hypoxia-inducible factor-1α to help in the disease progression. An understanding of the complex interactions involved in these pathways might allow the development of novel therapeutic strategies for rheumatoid arthritis. Therefore, we decided to validate the potential of *Foeniculum vulgare* synthesized selenium nanoparticles to target the genes involved in the inflammatory and oxidative pathways of RA by In Silico docking analysis.

Objective of the study

The project was aimed to analyze the therapeutic potential of "Foeniculum vulgare" synthesized selenium nanoparticles in arthritic mice model by measuring the level of oxidative stress markers such as TNF alpha, HIF-1 alpha, antioxidant enzymes and other clinical characteristics of the disease.

Impact of study

Since rheumatoid arthritis is much prevalent within Pakistan, and standard drugs are not very effective. This study provides a product from local plant for its anti-rheumatic activity. As selenium nanoparticles have good antioxidant activity and less side effects, so it can be considered as a future Nano medicine after further testing to treat Rheumatoid arthritis. As these nanoparticles can directly be isolated from a local plant thus it will be a very cost-effective drug and help to provoke the pharmaceutical industry

REVIEW OF LITERATURE

2.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder primarily affecting joints. It is characterized by cartilage and bone destruction. Inflammation, Synovial hyperplasia, autoantibody production and joint destruction are the hallmarks of rheumatoid arthritis (Alamanos et al., 2006). The prevalence of RA is 1% of the worldwide population and women are more affected (Ming Di et al., 2011). Although the onset is more frequent during the fourth and fifth decades of life, RA can occur at any age. The clinical signs of RA involve puffness, rigidity and pain in ankle and knee joints leading to deformity and immobility in later stage(Q. Zhang et al., 2018). Patients have a higher risk of other autoimmune and cardiovascular disorders (Gibofsky, 2012). The incidence of secondary complications and cardiovascular diseases increase with age (K. L. Ong et al., 2013). The exact etiology of the disease is unknown, but several genetic and environmental factors are associated with its progression and severity (Barton & Worthington, 2009). The current available therapies for RA have several side effects and secondary complications associated with their use. Therefore, an alternative therapeutic option for RA treatment need to be discovered.

2.2 Biological roles of selenium

Selenium is an essential microelement which possesses wide range of biological functions in humans and animals. Selenium was discovered by J.J. Berzelius in 1818 who evaluated the influence of Se on living organisms(Berzelius, 1826). Selenium is an essential trace element in the body due to its antioxidant potential. Selenium is originated from a Greek word “Selene”,

meaning moon goddesses. It is found in various oxidation states such as selenate and selenite, which has +6 and+4 oxidation states respectively. Selenium is a potent antioxidant micronutrient for mammalian cells playing crucial roles by incorporating selenoproteins. Selenoproteins are enzymatic structures in which selenium is present as a cofactor. For nutritional supplementation, highly bioavailable forms of selenium are Selenocysteine and selenomethionine figure 2.1. In humans, 30 selenoproteins are identified which play essential role in immunomodulation and redox balance. Glutathione peroxidase (GSHPx) was the first selenoprotein to be identified, which reduces hydroperoxide to alcohols (Back, 2013).

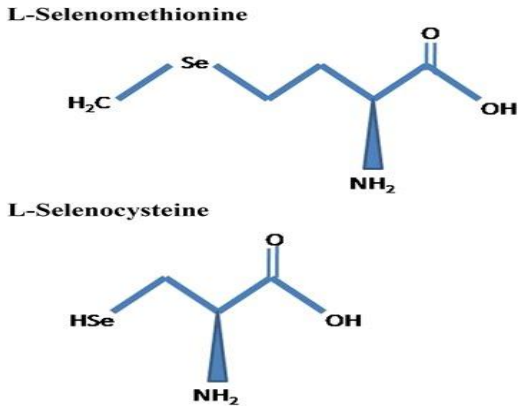

Figure 2.1: Schematic diagram of bioavailable Selenium

The biological roles of Selenium are mainly due to its antioxidant potential which protects against free radicals generated during oxidative stress. It regulates homeostatic mechanism and cell proliferation in body. Selenium is an integral part of enzymes and biomembranes that protect the cell damage and overoxidation during oxidative stress. Antioxidant defense mechanism that inhibits cell damage by free radicals during inflammation, stressors and infections are categorized into nonenzymatic and enzymatic components. Enzymatic components are usually immune system related selenoproteins such as thioredoxin reductases (TXNRDs), selenophosphate synthetase 2 (SPS2) and glutathione peroxidases (GPXs), while the

nonenzymatic are melatonin, vitamin E, carotenoids, glutathione and thioredoxin. Selenium being part of antioxidant enzymes such as thioredoxin reductase (TRx), deiodinases and glutathione peroxidase helps in scavenging reactive oxygen species which protects DNA and cells from oxidative damage it is a part of glutathioneperoxidase, iodothyroindineiodinase, glutathionereductase as selenomethionine and selenocysteine (Maysenok, Pityuk, & Maysenok, 2002; Tutelyan et al., 2002). The major role of selenium in metabolism is associated with its involvement in glutathione peroxidase (GPx) for the reduction of various peroxides produced during biochemical reactions, also the prevention of cellular biomembranes from oxidative damage (Oberlis, Kharland, & Skal'nyy, 2008).

Selenium has medicinal applications for its effectiveness in disease management. Various organic and inorganic forms of selenium for example, selenomethionine, selenium chloride, selenocycteine and sodium selenite are used as nutritional supplements for adults in minor doses less than 200 g/day along with other medicines (Rayman, 2000).

Selenium act as potent anti-carcinogenic agent, antioxidant, anti-aging and also has a protective role against muscular dystrophy in humans. In a study by it is reported that HIV-1 virus encodes selenoproteins homologous to human GPx, which deprives the host cell of selenium because of these viral proteins synthesis. Therefore, selenium is thought to be a drug supplement in AIDS (Moghadaszadeh & Beggs, 2006). Selenium has medicinal applications for its effectiveness in disease management. Various organic and inorganic forms of selenium for example, selenomethionine, selenium chloride, selenocycteine and sodium selenite are used as nutritional supplements for adults in minor doses less than 200 g/day along with other medicines (Rayman, 2000).

Selenium acts a carrier of many redox enzymes, which manifest the use of selenium for treating various autoimmune disorders. Selenium helps in activation of oncogenes and inhibit the transformation of normal cells into malignant upon a specific dosage. Selenium consumption affects the expression and development of nonspecific immune responses. Selenium supplements of low doses results in restoration and augmentation of immune functions. Various inorganic and organic forms of Se are used for oral supplementation which has multiple health benefits (Zeng & Combs Jr, 2008).

2.2.1 Selenium deficiency in Rheumatoid arthritis and other diseases

Studies reported that being a trace element, selenium deficiency causes various health problems. Selenium deficiency in diet has adverse effects on human health. Dietary selenium deficiency is affecting 0.5–1 billion people of the world (Berzelius, 1826). World Health Organization (WHO) recommends that daily selenium intake must not exceed than 70 μg per day (Kieliszek & Błażejak, 2013). The recommended dietary selenium content is far from average household consumption in European countries ranging between 30 and 50 μg per day (Rayman, 2000; Tinggi, 2003).

Selenium deficiency is associated with decrease expression and synthesis of selenoproteins, which affects their targeted biological processes leading to degeneration of tissues and organs (Pedrero & Madrid, 2009). Moderate deficiencies have impact on human health by causing disorders related to joints and heart muscles, prostate cancer and neurological disorders. It is also associated with a kashin-Beck disease or endemic osteoarthropathy which expresses itself by rheumatoid arthritis. Oxidative damage leads to cartilage and bone destruction known as hyaline cartilage degeneration (necrosis) (Yao, Pei, & Kang, 2011). Other disorders associated with Se

deficiency are cardiomyopathy, asthama, AIDS, stroke, cardiac arrhythmia (Patelski & Dziekonska, 2012).

Various organic hydroperoxides and hyperoxides are reduced by glutathione peroxidase in the presence of glutathione (GSH) which is a reducing agent. The end products of this reaction are alcohols,oxidized glutathione(GSSG) and water. Phospholipid hydroperoxide (PH-GSH-Px) exhibits has an additional role in the synthesis of prostaglandins (Yaroshenko, Dvorska, Surai, & Sparks, 2003). Selenium has an important role in cell signaling systems and activation of transcription factors(Hendrickx, Decock, Mulholland, Fairweather-Tait, & Bao, 2013). Glutathione peroxidase(GSH-Px) prevents oxidation of lipid membranes. It helps in reducing organic peroxides (ROOH) and hydrogen peroxide (H2O2) by reduced glutathione (GSH) (Jovanović et al., 2013). GSH-Px was the first characterized selenoprotein. It has tetrameric structure with a Se atom in each subunit (Ruseva, Himcheva, & Nankova, 2013; Zarczynska, Sobiech, Radwinska, & Rekawek, 2013). Selenium supplementation needs special care to avoid its negative effects as selenium is toxic element even in its small quantities, but also an essential micronutrient, therefore the range between effective and toxic dose of Se is very narrow (Kieliszek & Błażejak, 2013; Navarro-Alarcon & Cabrera-Vique, 2008).

2.3. Nano selenium

Despite the fact that selenium has got so many medical applications, it is found to be essential element but toxic at high dose(Reid et al., 2004). Therefore, a specific concentration of selenium is required for human health. The toxicity associated with high dose of selenium puzzles scientists and they are carrying out research on its mode of action in order to enhance the efficacy and decrease its toxicity (Papp, Lu, Holmgren, & Khanna, 2007). Researchers are

aiming to achieve this strategy of reducing toxicity and enhancing efficacy of selenium by Nanotechnology.

Nanotechnology deals with manufacturing and exploiting systems, devices and materials at nanometer scale roughly 1-100nm in size. At Nano scale level, systems, devices, and materials exhibit unique electrical, magnetic, biological, chemical, mechanical, photoelectrical and optical properties which are far different and better than their bulk form properties. Nanotechnology has a potential to benefit the society by manipulating and designing different devices and materials. It opens the doors to advancement in the field of manufacturing, energy and medical treatments. These Nano materials serve as building blocks of larger complex structures with unique properties (Walsh, Balbus, Denison, & Florini, 2008).

The Nano materials have various applications in sensors, solar cells and nanowire electronics. Nano selenium as a supplement has gained attention for the treatment of different disease of enhanced efficacy and properties of nanomaterial. Nano selenium has shown decreased toxicity, better efficacy and biocompatibility as compared to other inorganic and organic forms of selenium. Therefore, Nano selenium can be viewed as a better option over the bulk form due to its unique properties such as better reactivity, low dosage and low toxicity (Chhabria & Desai, 2016).

Owing to the unique features of selenium nanoparticles, there is an emerging trend in their synthesis for different nanotechnology applications. Research is being done on the potential applications of selenium nanoparticles in different fields such as use of Se nanoparticles for capturing mercury and precipitating on the surface of nanoparticle as HgSe from gaseous phase (Fellowes et al., 2011; Johnson et al., 2008). Selenium nanoparticles are being exploited for

medical applications such as antioxidant, antifungal, anticancer and antimicrobial. Therefore, selenium nanoparticles are considered better and safe candidates for medical applications.

2.4. Immunomodulatory Role of Nano-selenium

Oxygen is essential for aerobic living system but its higher concentration leads to generation of reactive oxygen species which causes oxidative stress (Bhattacharjee, 2005). Oxidative stress is either the result of decreased antioxidant defense system or overproduction of ROS. Oxidative stress is a common cell response which needs to be maintained by antioxidant defense systems in the body. Antioxidant defense systems are categorized as enzymatic and non-enzymatic defense mechanisms. Enzymatic defense systems for ROS detoxification include enzymatic cascades which directly react with ROS for detoxification or act as redox regulators. For example, Catalase is not only involved in the hydrogen peroxide detoxification but also in adaptation to lipid peroxidation and endogenous oxidative stress. On the other hand, non-enzymatic systems are not much specific like enzymatic ones, but they act as a first line of antioxidant defense mechanism like vitamin C that quenches free radicals and generate a less oxidative ascorbyl radical, vitamin E is involved in modulating different signal transduction pathways and transcription factors such as NF κB (Zingg, 2007).

By considering the role of antioxidant defense system in oxidative stress, a feedback mechanism can be examined within the cell to maintain the oxidative homeostasis. For this purpose, a multifunctional antioxidant system can be synthesized for maintaining redox balance in the body for monitoring both health and disease condition. Nano selenium has a potential to perform these versatile roles in multifunctional immunomodulatory phenomenon. The potential of selenium nanoparticles was checked in tissues of albino rats in which oxidative stress was induced by

acetaminophen (Mohammed & Safwat, 2013). Considering the results of different studies, the role of SeNPs in immunomodulation is presented in a schematic model in figure 2.2.

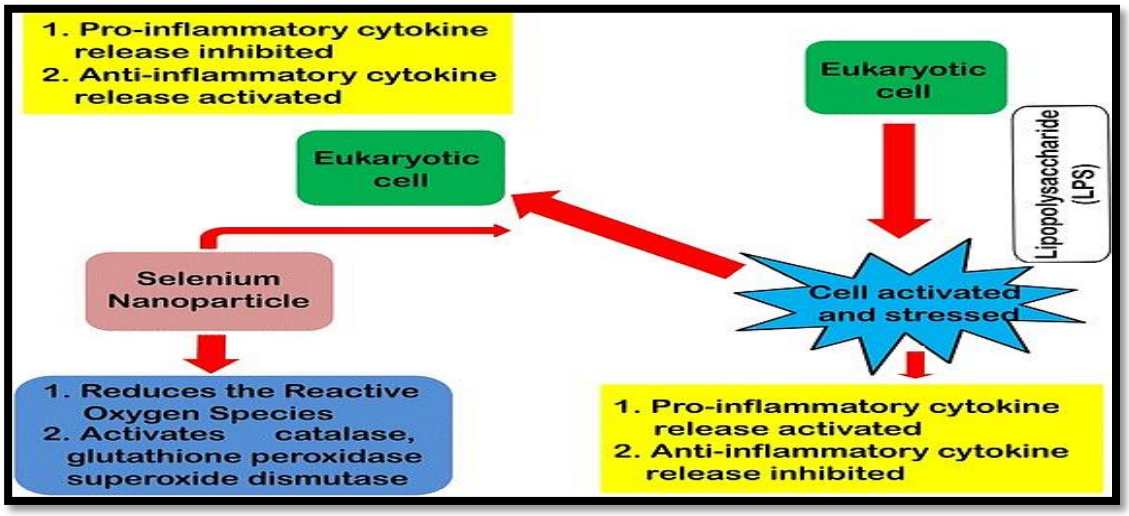

Figure 2.2: Schematic model depicting the immunomodulatory role of selenium nanoparticles

2.5 Biogenic Synthesis of Selenium Nanoparticles

There are different physical and chemical methods for the synthesis of selenium nanoparticles. The physical methods include laser ablation, hydrothermal techniques and UV radiations (Iranifam et al., 2013; Quintana, Haro-Poniatowski, Morales, & Batina, 2002), while chemical methods involve acid decomposition, precipitation method, reduction by using sodium dodecyl sulfate, ascorbic acid and sulfur dioxide etc (Dwivedi, Shah, Singh, Kumar, & Bajaj, 2011; Lin & Wang, 2005; Y. Zhang, Wang, & Zhang, 2010). All these methods need high temperature, acidic pH and harsh chemicals which make them unsafe for medical applications (Wadhwani, Shedbalkar, Singh, & Chopade, 2016). Therefore, an alternative method is biosynthesis of nanoparticles from natural organic sources such as plants (Menon, KS, Agarwal, & Shanmugam, 2019), fungi (Henglin Zhang et al., 2019) and bacteria (Wadhwani et al., 2017). Different

biomolecules from these organic sources such as enzymes, sugars, phenols, flavonoids and proteins help in reducing ionic forms of selenium into selenium nanoparticles.

2.5.1 Significance of Biogenic Selenium nanoparticles

Biosynthesized nanoparticles have gained much value due to their biomedical applications such as anticancer(Ali, El-Sonbaty, & Salem, 2013), antibacterial(Hariharan, Al-Harbi, Karuppiah, & Rajaram, 2012), anti-inflammatory(Khurana, Tekula, Saifi, Venkatesh, & Godugu, 2019), antifungal (Kazempour, Yazdi, Rafii, & Shahverdi, 2013) and antioxidant (Forootanfar et al., 2014) properties. Biogenic selenium nanoparticles are preferable because they are ecofriendly, economical and do not produce any toxic byproduct (Wadhwani et al., 2016). Biomolecules from organic sources act as reducing agents and stabilizers in the synthesis of nanoparticles to prevent them from aggregation over time. For example, proteins are involved in the capping of selenium nanoparticles in some organisms such as bacteria- Bacillus sp, Zooglea ramera(Srivastava & Mukhopadhyay, 2013), plants-Diopyros Montana (Kokila, Elavarasan, & Sujatha, 2017) and fungus-Mariannaea sp.HJ (Henglin Zhang et al., 2019) likewise; polysacharrides are involved in stabilization of selenium nanoparticles in different organisms such as mushroom-Pleurotus tuber-regium (H. Wu et al., 2012) and cyanobacteria- Spirulina platensis (Yang et al., 2012). Some secondary metabolites such as alcohols, flavonoids, tarpenes, lignin and phenols are involved in selenium nanoparticles synthesis from different plants like vitis vinifera(Sharma et al., 2014), Psidium guajava (Alam, Khatoon, Raza, Ghosh, & Sardar, 2019) and ginger (Menon et al., 2019) etc.

Biologically synthesized selenium nanoparticles have gained more stability because of the natural coating of organic material on their surface that prevents nanoparticles from being aggregated over the time(Park, Hong, Weyers, Kim, & Linhardt, 2011). The production of

nanoparticles from plant extracts is found to be more beneficial as compared to microbial synthesis as it eliminates long procedures for maintaining microbial cultures. Although various plants are reported, but for the phytogenic synthesis of selenium nanoparticles a few reports are available. The studies reported synthesis of SeNPs using seed extract of fenugreek(Ramamurthy et al., 2013), leaf extract of Terminalia arjuna (Prasad & Selvaraj, 2014), lemon, Capsicum annuum(Li et al., 2007), Leucas lavandulifolia (Kirupagaran, Saritha, & Bhuvaneswari, 2016), Clausena dentate, Diospyros Montana(Kokila et al., 2017), Psidium guajava, green tea extract(W. Zhang et al., 2018), aqueous extract from Allium sativum(Anu, Singaravelu, Murugan, & Benelli, 2017), dried fruit extract from Vitis vinifera and flower extracts from Bougainvillea spectabilis (Deepa & Ganesan, 2015). Biogenic synthesis of SeNPs from reported plants is given in the Table 2.1 (Wadhwani et al., 2016).

Table 2.1: Reported biogenic selenium nanoparticles

Plant	Size (nm)	Shape	Location
Vitis vinifera(Sharma et al., 2014)	3–18	Spherical	Fruit extract
Terminalia arjuna(Prasad & Selvaraj, 2014)	10-80	Polydispersed	Leaf extract
Lemon(Prasad, Patel, Patel, Patel, & Selvaraj, 2013)	60–80	Spherical	Leaf extract
Trigonella foenum-graecum (fenugreek)(Ramamurthy et al., 2013)	50–150*	Spherical	Seed extract
Capsicum annuum(Li et al., 2007)	200–500*	Polygonal	Leaves extract

*Size ≥100 nm, but reported as nanoparticles

2.6. Therapeutic potential of selenium nanoparticles

As compared to Selenium, SeNPs are found to be effective against various diseases due to their low toxicity and higher bioavailability. Due to their antioxidant and pro-oxidant nature, they are involved in multiple pathological conditions. The therapeutic potential of SeNPs is attributed to their anti-diabetic, anticancer, anti-inflammatory and antibacterial activity.

Selenium nanoparticles possess several therapeutic applications including anti- inflammatory, anti-diabetic, anticancer and antioxidant potential. The anticancer activity is due to prooxidant potential of selenium nanoparticles which triggers the production of reactive oxygen species (ROS) which results in damaging endoplasmic reticulum, mitochondria and ultimately DNA

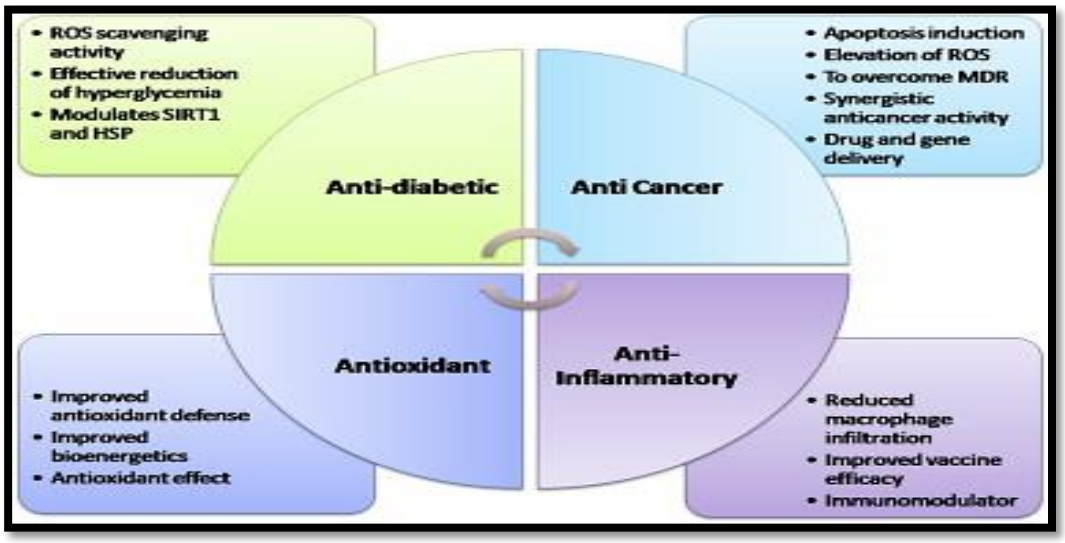

Figure 2.3: Therapeutic applications of selenium nanoparticles

Selenium has a protective role which depends upon its chemical composition and concentration such as selenium-methylselenocysteine , the organic forms of selenium, is effective against cancer with least toxicity, on the other hand anti-cancerous mineral selenite has high toxicity (Björnstedt & Fernandes, 2010; Wadhwani et al., 2017). In the past few years, study and

preparation of selenium nanoparticles has gained much interest due to their lower toxicity, higher bioavailability, and exciting in vitro and in vivo biological properties as compared to Selenium Se (VI) and Se (IV). The SeNPs play important role in antioxidant defense system which is essential for reducing oxidative stress (Iranifam et al., 2013; H. Wang, Zhang, & Yu, 2007). Selenium nanoparticles have antibacterial activity against S. aureus, which causes hospital acquired infections. Selenium nanoparticles are less toxic as compared to silver nanoparticles (Tran & Webster, 2011, 2013).

2.7. Nano-Selenium as an antioxidant

Lack of selenium is linked with many pathological conditions such as increased risk of cancer, rheumatoid arthritis etc. The restoration of selenium with the help of different supplements of selenium is failed because of their low efficacy and therapeutic index. So, it is better to have least toxic supplementation of selenium in Nano form with high potential for therapeutic applications.

P Sonkusre reported the potential of biogenic SeNPs derived from *Bacillus licheniformis* against prostate cancer cell death. SeNPs were used at a minimum concentration of 2 µg Se/ml which results in down-regulation of oncogenes in C3H/HeJ mice and induced lower toxicity as compared to the L-selenomethionine. activityThe results suggest that biogenic selenium nanoparticles are safest supplementation with high anticancer activity (Sonkusre, 2020).

Nano selenium has a better antioxidant activity with decrease toxicity as compared to other chemical forms of selenium. Wang et al., reportd the antioxidant potential of SeNPs which showed less toxicity as compared to Selenomethionine(SeMet) (H. Wang et al., 2007). Zhang et al., reported the effect of Nano selenium on glutathione peroxidase activity in the weanling pigs liver and compared with inorganic selenium. The results showed that animals had higher GPx

activity in the selenium treated group at a concentration of 0.5 and 1.0mg/kg selenium in diet as compared to sodium selenite (Na2 SeO3)(Hongmei Zhang, Xia, & Hu, 2007).

In another study Wu et al., reported that Nano selenium maternal administration has a potential to increase hair follicle and growth of fetus. It is attributed to the antioxidant status of the fetal skin. An increase in antioxidants results in decreasing reactive oxygen species (ROS) production, which results in IGF-1 and IGF-1R upregulation which play a key role in growth and development of fetus (X. Wu et al., 2011)

Cremonini et al., reported the antimicrobial activity of biogenic selenium nanoparticles synthesized from S. maltophilia and B. mycoides which showed strong antimicrobial activity as compared to synthetic SeNPs against P. aeruginosa isolates (Cremonini et al., 2016).

Biogenic selenium nanoparticles derived from *Bacillus licheniformis* has shown effectiveness against prostate cancer cell death at a concentration of 2 μg Se/ml. Histopathological analysis of selenium administration in mice showed lower toxicity as compared to L-selenomethionine. The results suggest that biogenic selenium nanoparticles are safest supplementation with high anticancer activity (Sonkusre, 2020).

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2.7.1 Antioxidant Mechanism of action of Selenium Nanoparticles

Nano-selenium has a potential to restore the levels of superoxide dismutase, catalase, melanodialdehyde and reduced glutathione. It is also involved in increasing the glutathione-S-transferase activity in mice as compared to selenomethionine at supra-nutritional level (J. Zhang, Wang, & Xu, 2008). From these studies and results it can be stated that nano- selenium leads to the formation of selenomethionine and selenomethionine forms selenocysteine using precursor via Se-cystathionine. Seleocysteine serve as an essential component for Se-glutathione formation. Se-glutathione along with glutathione peroxidase plays an important role in ROS and H_2O_2 neutralization. In a study it is reported that nano-selenium stimulate the expression of glutathione peroxidase- a selenium dependent enzyme, through the selenophosphate formation, that is an essential part of tRNA selenocysteiyl (Mizutani, Goto, & Totsuka, 2000). Glutathione peroxidase has a vital role in reducing reactive oxygen species level in the cell. A schematic model is designed in figure 2.4 by considering the above facts, to showcase the SeNps induced amelioration of reactive oxygen species in organisms.

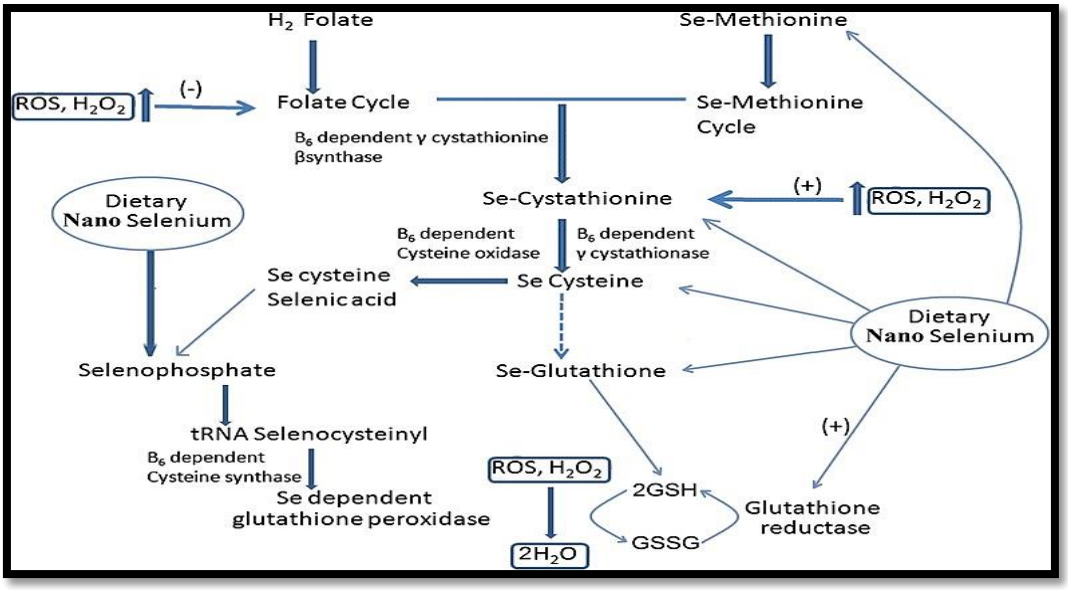

Figure 2.4: Schematic diagram representing the probable antioxidant mechanism of selenium nanoparticles

2.7.2 Effect of Selenium Nanoparticles on oxidative stress parameters

In another study effect of Nano selenium and organically bound selenium was compared on oxidative stress parameters. Nano selenium showed comparable potential with Selenium methionine in enhancing plasma GPx level in mice, but it showed lower toxicity. The study suggested that nano selenium can be administered with reduced risk of selenium toxicity(H. Wang et al., 2007). The effect of nano selenium in the upregulation of selenoenzyme is comparable to the Se-MetSeCys and selenite with reduced toxicity (Bao-hua et al., 2003; J. Zhang, Wang, Yan, & Zhang, 2005; J. Zhang et al., 2008).

Hegedüs et al., reported the potential of nano selenium against FLD in male Wistar rats, which showed low level of inflammation and free radical production in experimental sick group as compared to control group. These results were confirmed by histological analysis of samples (Hegedüs et al., 2012).

The results of various studies showed that elemental Nano selenium has less toxicity and higher efficacy in upregulation of selenoenzymes in the body as compared to other organic and inorganic selenium compounds such as SeMetSeCys and SeMet (J. Zhang et al., 2008).

2.7.3 Anti-inflammatory potential of Selenium Nanoparticles

Inflammation is one of the earliest signs of disease pathology and researchers are investigating the anti-inflammtory potential of SeNPs as a novel therapeutic approach to combat the inflammatory diseases. Selenium nanoparticles are effective in reverting back the inflammatory response caused by Lipopolysaccharide or hydrogen peroxide (H_2O_2). Attributing to their antioxidant potential, SeNP scavenge RNS and ROS. It performs its anti-inflammatory activity by modulating the NFκB and p38 MAPK (Mitogen activated protein kinase) pathways to reduce the inflammatory cytokines expression such as IL-2, IL-1, IL-6 and TNF-α. The Anti-inflammatory mechanism of action of SeNPs is shown in the figure 2.5.

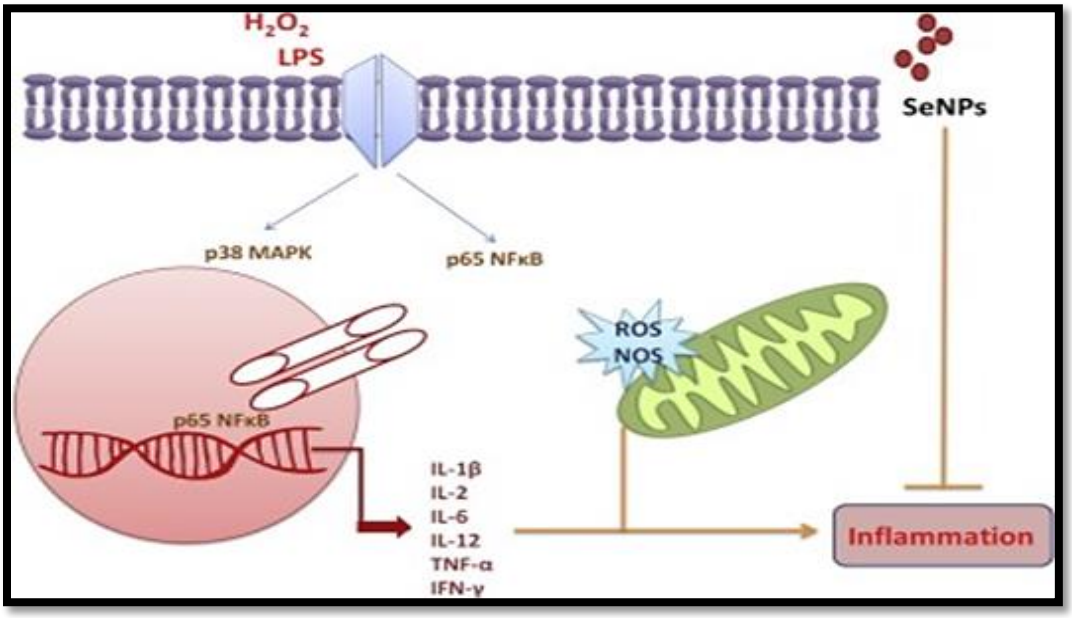

Figure 2.5: Anti-inflammatory mechanism of action of SeNP

Selenium nanoparticles coated with polysaccharide of Ulva lactuca showed effectiveness in reducing colitis by inhibiting the expression of proinflammatory cytokines TNF-α, IL-6 and NFκB signaling pathway (Zhu et al., 2017).

Literature review showed low selenium concentration in rheumatoid arthritis patients, which can be compensated by administration of biogenic SeNPs. Biochemical properties of phytochemicals serve as a medium for NP dispersion. In a study by Shi-Xiang Ren et al., the anti-inflammatory potential of SeNPs dispersed in 1% *P*-Coumaric acid (CA) was studied in complete Freund's adjuvant induced rats. The administration of SeNP at a dose of 500 μg/Kg which significantly restored COX-2 activity, antioxidant enzyme activity, inflammatory cytokines IL-6, IL-1β, MCP-1, TNF-α and thiobarbituric acid. The results also showed that mRNA levels of antioxidant enzymes GPx1, CAT, MnSOD was down regulated and COX-2 was up regulated in RA group, while SeNPs treated group reverted back to the normal expression. So it clearly indicates that biogenic SeNPs have effectiveness against inflammatory disorders like rheumatoid arthritis (Ren, Zhang, Lin, Ma, & Yan, 2019).

M.A. El-Ghazaly et al., reported the analgesic and anti-inflammatory effect of selenium nanoparticles in irradiated rats. They measured nociceptive threshold and paw volume in hyperalgesia and carrageenan-induced paw edema model. Nano selenium was administered in2.55 mg/kg dose in gamma irradiated and non-irradiated rats which results in reducing the paw volume in both groups and nano selenium decreased also reverted back the elevated levels of TNF-α, prostaglandin E2, total nitrate/nitrite (NOx), leukocytic count and thiobarbituric acid reactive substances in irradiated and non-irradiated rats. Thus Nano selenium showed significant anti-inflammatory potential in irradiated rats (El-Ghazaly, Fadel, Rashed, El-Batal, & Kenawy, 2017).

Na Yu et al., reported the relation between serum selenium (Se) levels and rheumatoid arthritis (RA) through a meta-analysis approach. In this study they reported the meta-analysis from 14 case control studies which include 716 participants which showed significant association between RA and low serum selenium concentration (Yu et al., 2016).

Glenn A. Jacobson et al., reported Plasma glutathione peroxidase level in rheumatoid arthritis in a case control study. They measured the concentration of GSH-Px using ELISA and found that it was increased in RA patients as compared to healthy controls. The elevated GSH-Px concentration suggests the protection against oxidative stress (Jacobson, Ives, Narkowicz, & Jones, 2012).

Lokanadhan Gunti et al., describes the synthesis of biogenic phytofabricated-SeNPs from E. officinalis aqueous fruit extract. These PF-SeNPs showed potent antioxidant activity by radical scavenging in ABTS and DPPH assays. The concentration of PF-SeNPs used was directly proportional to their antioxidant activity. Also the antioxidant potential of these phytofabricated SeNP was higher than standard ascorbic acid antioxidant (Gunti, Dass, & Kalagatur, 2019).

Jianguo Wang et al., reported that selenium nanoparticles coated with sulfated polysaccharides from *Ganoderma lucidum* has shown the inhibition of LPS-stimulated production of nitric oxide by macrophages and decreasing the mRNA expression of pro-inflammatory cytokines TNF-α and interleukin-1(IL-1). On the other hand increased the expression of anti-inflammatory cytokine interleukin-10(IL-10) (Ding, Zhou, Zhang, & Xu, 2010; J. Wang, Zhang, Yuan, & Yue, 2014).

2.8. Foeniculum vulgare

Foeniculum vulgare is an aromatic, flowering plant that belongs to Apiaceae family. It is commonly called fennel but also known by several other regional names. Although it is originated in Mediterranean regions but due to naturalization it is now present in almost every country of the world (Muckensturm, Foechterlen, Reduron, Danton, & Hildenbrand, 1997). Castroviejo et al., discriminate two subspecies of fennel on the basis of seed taste, *piperitum* (bitter seeds) and *vulgare* (sweet seeds), however there is no clear morphological difference between the subspecies (Castroviejo et al., 2005). Funnel seeds finds its use in culinary due to its characteristics anise order and nutritional value (Díaz-Maroto, Díaz-Maroto Hidalgo, Sánchez-Palomo, & Pérez-Coello, 2005). Fennel is also a medicinal plant and its therapeutic potential is widely studied.

Figure 2.6: Foeniculum vulgare (a) plant (b) flowers (c) seeds

Phytochemical including phenols, fatty acids, hydrocarbons, volatile compounds and several other secondary metabolites have been isolated from different parts of plant. Essential oil is the major repertoire of these phytochemicals. Fennel seed essential oils contain several compounds and their composition varies with the developmental stage of plant. Chemical composition also varies with species origin and adopted extraction method. Tran-aenothole, fenchone and

estragole are the major essential oil components of Feoniculum vulgare (Díaz-Maroto, Pérez-Coello, Esteban, & Sanz, 2006). More than 87 volatile compounds have been identified in fennel essential oil (Badgujar, Patel, & Bandivdekar, 2014).

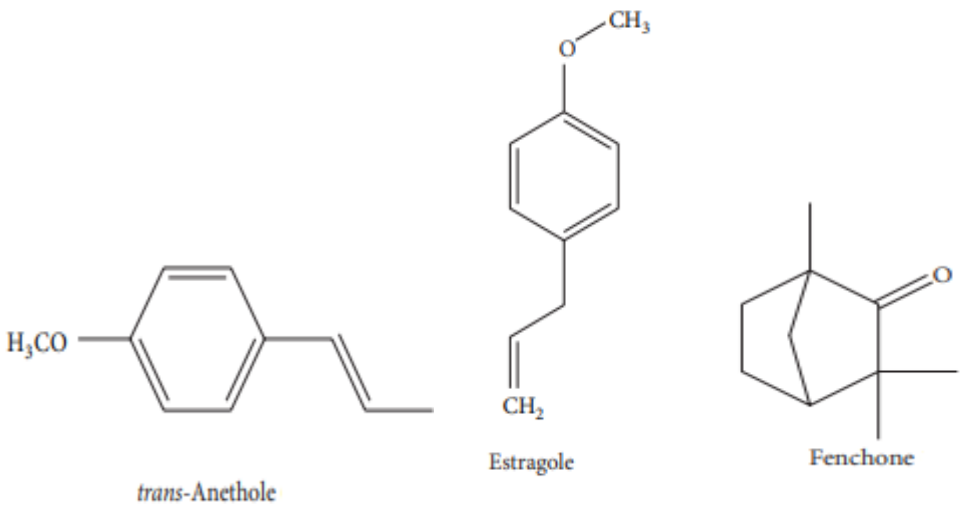

Figure 2.7: Chemical structure of some isolated phytochemicals of fennel

2.8.1 Therapeutic potential of fennel

The medical significance of fennel was known from centuries and it has been used in traditional medicine. Foeniculum vulgare has been used for more than 40 different diseases in different parts of the world (Badgujar et al., 2014). Romans and Greeks used fennel for stomach problems and vision enhancement. In Italian regions it is recommended for mouth ulcer, milk enhancement during breastfeeding, diabetes, cough and kidney stones. It is also used in fever, respiratory and urinary tract infections (Akbar, 2018). In Chinese traditional medicine, it is used for abdominal discomfort, diarrhea and vomiting (Hsü, 1980). Sugar coated seeds of fennel are used as mouth freshener in India and Pakistan.

Various studies have been performed on fennel extracts to evaluate and identify the drug like properties namely, antibacterial, antioxidant, anti-inflammatory, antiviral, antifungal, antidiabetic, antistress, cardiovascular, antitumor activities. Some of the studies are briefly described here.

Fennel essential oil, aqueous and organic extract showed antibacterial activity against human pathogenic bacteria, foodborne pathogens, Campylobacter jejuni, Halicobacter pylori and various other bacteria (Kaur & Arora, 2008; Mahady et al., 2005; Mohsenzadeh, 2007). Another study evaluates the aqueous extracts of Foeniculum vulgare and eleven other plants of Apiaceae family. Their results showed the antibacterial activity of fennel against Agrobacterium tumefaciaciens, Pseudomonas, Erwinia carotovora, Staphylococcus aureus and Salmonella typhi (Duško, Comiæ, & Sukdolak, 2006). Antiviral effects of fennel fruit essential oils was evaluated by Orhan et al., and results showed the effective antiviral response against Herpes simplex type-1 virus (Orhan, ÖZÇELİK, Kartal, & Kan, 2012). Several studies also demonstrate the antifungal effect of fennel essential oil, aqueous and alcoholic seed extracts against various fungal species (Abed, 2007; Anwar, Ali, Hussain, & Shahid, 2009; Martins, Tinoco, Almeida, & Cruz-Morais, 2012; Rahimi & Ardekani, 2013).

In-vivo studies also demonstrate the effectiveness of fennel in lowering blood pressure (Bardai, Lyoussi, Wibo, & Morel, 2001). Hypoglycemic or antidiabetic activity of fennel is also investigated by researchers and several in-vivo studies showed positive results (Dongare, Arvindekar, & Magadum, 2010; El-Soud et al., 2011). Kopula el al., reported the memory enhancing effect of fennel extracts (Koppula & Kumar, 2013).

2.8.2 Antioxidant Activity

Antioxidant activity of fennel plant makes it a good medicinal plant. Several studies have been performed to evaluate the antioxidant potential of *Foeniculum vulgare.* Faudale at el., reported the higher antioxidant acitivity of wild fennel plant than edible plant (Faudale, Viladomat, Bastida, Poli, & Codina, 2008). Damage caused by oxidative stress can be protected by using natural antioxidant (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005).

Ruberto at el., evaluated the antioxidant potential of fennel essential oil by two lipid model system and their study showed a strong antioxidant activity of oils as compared to reference alpha tocopherol and BHT (Ruberto, Baratta, Deans, & Dorman, 2000). Sahat at el., studied the antioxidant activity of ethanol and water extracts of fennel plants. Both ethanol and water extracts showed the efficient reducing power and scavenging of free radical, hydrogen peroxide and superoxide anions. Inhibition of peroxidation by 100ug of water and phenol extract was 99.1% and 77.5% respectively. However when the same dose was used for alpha tocopherol inhibition was 36.1% (Shahat et al., 2011).

Another study reported the antiradical scavenging activitiy of phenolic compounds isolated form Foeniculum vulgare. The evaluated compounds were 3-caffeoylquinic acid, kaempferol-3-O-rutinoside, quercetin-3-O-galactoside, kaempferol-3-O-glucoside (Parejo et al., 2004). Reported antioxidant activity of essential oils is more than phenol and water extracts (Díaz-Maroto et al., 2005).

2.8.3 Anti-inflammatory Activity

Inflammation is a part of host immune response and vital for defense against injurious agents however uncontrolled inflammation can damage the health of individual (Medzhitov, 2008). Many natural products with anti-inflammatory activity are used to relief the symptoms of

inflammation. Methanol extracts of Foeniculum vulgare fruit were used to evaluate the anti-inflammatory effect. 200mg/kg orally administrated methanolic extract to rat and mice showed inhibitory effect against acute and subacute inflammatory disease. Induced arthritis, paw edema and ear edema was screened to evaluate the anti-inflammatory effect (Choi & Hwang, 2004). Ammara Arif et al., reported the therapeutic potential of selenium nanoparticles against rheumatoid arthritis in Balb/c mice model. SeNp were synthesized from *foeniculum vulgar* Mill and they showed significant results by reducing paw volume at a dose of 10mg. Their antioxidant potential was confirmed by DPHH assay. So, it is clear that selenium nanoparticles derived from *Foeniculum vulgare* Mill have significant antioxidant and anti-arthritic potential and provide an alternative therapeutic approach for rheumatoid arthritis (Arif & Attya Bhatti, 2019). Therefore, we decided to further check the therapeutic efficacy of foeniculum vulgare derived selenium nanoparticles to target the potential NF-KB pathway involved genes involved in rheumatoid arthritis.

METHODOLOGY

3.1 Molecular Docking

In Silico molecular docking was performed to check the interactions between selenium and target protein HIF1- alpha. The 3D structure of HIF1- was retrieved from Protein Data Bank having PDB ID: 4ZPR and prepared for docking analysis in Pymol software to remove attached DNA and then final result is shown in figure 4.1 (a) The selenium as a ligand was taken from Chemspider Database with ID 6326970 in .sdf format and converted into pdb format using Discovery Studio to visualize 3D structure. Form the results of PatchDock software; the one with best docking score showing greatest binding affinity was selected and 3D visualization was done using Pymol software. The 2D visualization of blind docking to see the interacting residues and distance was done using LigPlot software.

3.2 Primer Designing:

The primers for HIF1-α and TNF- α were designed through Primer3 Plus. The sequence homology of all primers was confirmed using Primer BLAST and in silico software UCSC genome browser In Silico PCR.

3.3 Synthesis of Biogenic Selenium Nanoparticles

3.3.1 Collection and identification of *Foeniculum vulgare* Seeds

Foeniculum vulgare seeds were purchased from the local market. The seeds verification and identification was done by analyzing their morphology comparing to the available literature.

Figure 3.1 *Foeniculum vulgare* seeds

3.3.2 Preparation of seed extract

The plant extract was prepared by taking 7 grams of grinded seed powder in 100 ml of deionized water. The solution was then kept for incubation for 24 hours in a shaking incubator at a speed of 2000 rpm and 40 in dark. After incubation, the plant extract was centrifuged in a pre-cooled centrifuge machine at 4 and 6000 rpm. Then centrifuged extract was filtered by using Whattman filter paper.

3.3.3 Synthesis of selenium nanoparticles

For the synthesis of selenium nanoparticles, a 10mM 100ml solution was prepared by adding 173mg sodium selenite (Na_2SeO_3) salt to the 90ml deionized water. Then kept the reaction mixture on hot plate to dissolve the salt completely by shaking the flask constantly. After dissolving the salt in water, 10ml of plant extract was added to the mixture. After that reaction mixture was placed in a shaking incubator for 72 hours at 2000 rpm and 4c in a dark

environment. During incubation period, the reaction mixture kept on changing its color from yellow on first day to orange red after 24 hours, which turned red after 48 hours and dark brick red after the completion of incubation at 72 hours.

3.3.4 Purification of Selenium nanoparticles

Freshly prepared nanoparticles were in the solution form that were purified by centrifugation. The solution was centrifuged for 20 minutes at 6000 rpm in 15ml falcon tubes. After centrifugation the supernatant was discarded and nanoparticles in the pellet were settled down. Then 12ml of deionized water was added in the falcon tubes and centrifuged for 20 minutes at 6000 rpm. The supernatant was discarded and again 12 ml deionized water was added to the falcon tubes. It was centrifuged at 6000 rpm for 20 minutes. After that supernatant was removed and nanoparticles in the form of pellet were settled down. The hard nanoparticles pellet was isolated in a china dish ad allowed for drying at 150 on hot plate. The dried nanoparticles were collected in an eppendrof and stored in dark condition to avoid degradation.

For characterization of synthesized selenium nanoparticles different concentrations were prepared by adding deionized water and sonicated the samples for UV visible spectroscopy analysis.

3.4 Acquisition and Acclimatization of Animal

Female Balb/c mice were procured from Laboratory Animal House (LAH) of ASAB, NUST. All these 50 mice kept at Laboratory Animal House were provided with a pathogen free and temperature regulated environment. All the procedures and protocols used in this study were approved by Institutional Review Board (IRB) of Atta-ur-Rehman School of Applied Biosciences (ASAB), NUST. Prior to experimentation, mice were observed for physiological and pathological abnormalities and grouped into specific cages for one week in order to acclimatize

them to the environment. They were given standard laboratory feed during this period. All the testing and experimentation was done according to the recommendations and guidelines of National Institute of Health for handling of animals.

3.4.1Animal model specifications:

The criteria of including animals in the present study is given in table 3.1

Table 3.1: Animal inclusion criteria

MODEL	Balb/c Mice
GENDER	Female
WEIGHT	30-35 Grams
AGE	8-12 weeks
SAMPLE SIZE	Total 50 Mice (10 mice/group)

3.4.2 Experimental groups:

Mice were divided into 2 groups each containing 10 mice for analysis and induction of rheumatoid arthritis. All the groups were placed in a separate cage.

Table 3.2: Experimental grouping of mice

Group No.	Group Type	No. of Mice
1	Healthy mice (Positive control)	10
2	Arthritic mice (Negative control)	10

3.5 Induction of Collagen induced arthritic in mice

For the induction of rheumatoid arthritis in mice, Bovine type 2 Collagen and complete Freund's adjuvant was used. 0.1M acetic acid was prepared and then mixed with Hartman solution in 2:1. After that, to make 1mL of collagenase type 2 solutions, 2mg of collagenase type 2 was added to

the above prepared solution of acetic acid. This freshly prepared mixture was kept in a shaking incubator overnight at 4°C. Bovine serum albumin (BSA) was then dissolved in 1:1 in Hartman solution. Then collagenase type 2 was added in 1:1 in the complete Freund's adjuvant and the mixture was vortexed for 2 to 3 minutes. Bovine serum albumin was mixed in 2:1 in the previous solution. At the end, the solution was mixed thoroughly with the help of pipette in an Eppendrof tube. When the immunization mixture is prepared, 0.2mL or 2 µl of this solution was filled into insulin syringes. First three injections containing this mixture were administered in the mice tail subcutaneously on day 0, 7 and 14. Then fourth and fifth injections were administered on day 21 and 28 in the hind paws of mice, containing 150 µL complete Freund's adjuvant. The protocol was completed in 28 days. During the induction period, mice paw volume was measured using vernier caliper at each injection day respectively (0, 7, 14, 21, 28). At the end of protocol induction, difficulty in motility and swelling in paw were observed in mice. On the basis of swelling, the severity of disease was graded and mice scoring grade 3 and 4 were used for further experimentation.

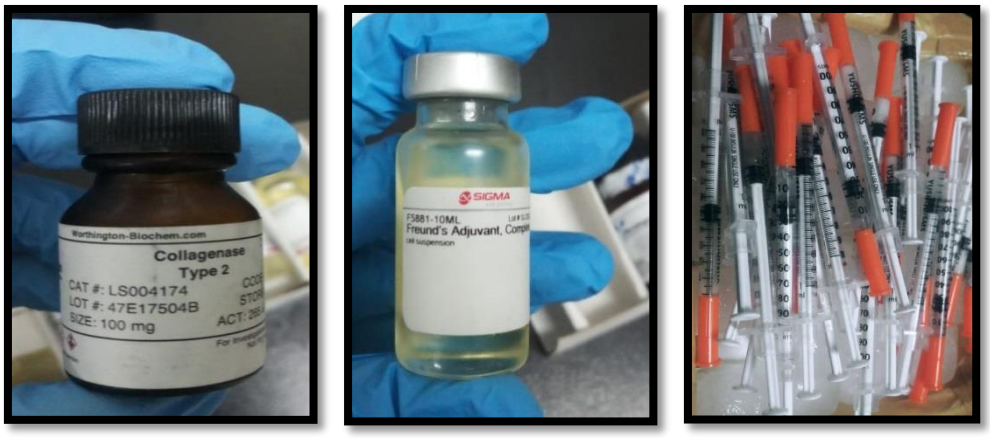

Figure:3.2: (A) Collagenase type 2 (B) Freund's adjuvant (C) Insulin syringes filled with immunization mixture

Table 3.3: Grading criteria for animal selection (Han et al., 2017)

Grade score	Condition
0	No welling or redness of joints
1	Slight swelling of finger joints
2	Moderate redness and swelling in wrist and ankle joints
3	Redness and severe swelling of whole paw
4	joint deformity, severe abscession and Ankylosis

3.6 Dissection of mice and sample collection

By the end of treatment, mice were given 24 hours fasting overnight and then dissection was carried out. After dissection, mice specimen liver, hind paws and blood was collected for further testing and analysis. Liver and hind paws were preserved in 10% formalin for performing histological analysis and assays to check antioxidant activity of selenium nanoparticles.

3.7 Statistical Analysis:

For data compilation, graph generation and statistical analysis Microsoft Office excel and different softwares were used including SPSS version 21, one way ANOVA and GraphPad Prism 5.

RESULTS

4.1 Molecular Docking of protein and Ligand

The 3D structure of HIF1-α was retrieved from Protein Data Bank having PDB ID: 4ZPR and prepared for docking analysis in Pymol software to remove attached DNA and then final result is shown in figure 4.1 (a) The selenium as a ligand was taken from Chemspider Database ID 6326970 in .sdf format and converted into pdb format using Discovery Studio to visualize 3D structure as shown in figure 4.1 (b)

(a) (b)

Figure 4.1 3D structure of HIF1- and selenium

4.1.1 Docking results

Form the results of PatchDock software, the one with best docking score showing greatest binding affinity was selected and 3D visualization was done using Pymol software as shown in figure 4.2. The 3D visualization of docking to see the interacting residues and distance was done using PDBsum software. The results showed that Se has two interactions with isoleucine residues of protein with a distance of 3.36 and 3.86 angstrom given in the figure 4.3

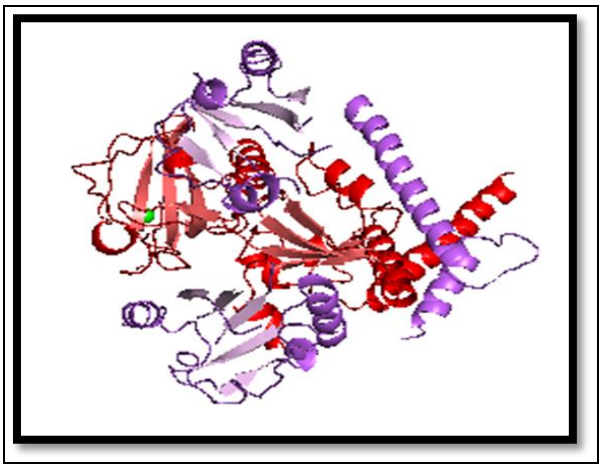

Figure 4.2 3D interactions between protein and ligand

bonded contacts

	<----- ATOM 1 ----->					<----- ATOM 2 ----->				
	Atom no.	Atom name	Res name	Res no.	Chain		Atom no.	Atom name	Res name	Res no.	Chain	Distance
1.	6603	CG2	ILE	233	B	:-:	8378	SE	LIG	1	Z	3.36
2.	6604	CD1	ILE	233	B	:-:	8378	SE	LIG	1	Z	3.86

Number of bonded contacts : 2

Figure 4.3: PDBsum result showing bonded interactions between protein residues and selenium

4.2 Primer Designing

The primer for HIF1- α and TNF- α was designed through Primer3 Plus. The sequence homology of all primers was confirmed using primer BLAST and in silico software, genome browser InSilico PCR. Primer properties and sequence is given below in table 4.4

Table 4.4: primer sequence and properties

Gene	Primer Sequence	Product size	Tm (°C)	GC content (%)	Self-complementarity
HIF1-α	(F) 5' GGTTCCAGCAGACCCAGTTA 3' (R) 5' GCCTTAGCAGTGGTCGTTTC 3'	169 bp	60.1 59.9	55.0 55.0	2.0 0.0
TNF-α	(F) 5' TTGCTCTGTGAAGGGAATGG 3' (R) 5' TTGGACCCTGAGCCATAATC 3'	147 bp	61.2 59.9	50.0 50.0	0.0 1.0

4.2.1 Primer3Plus result

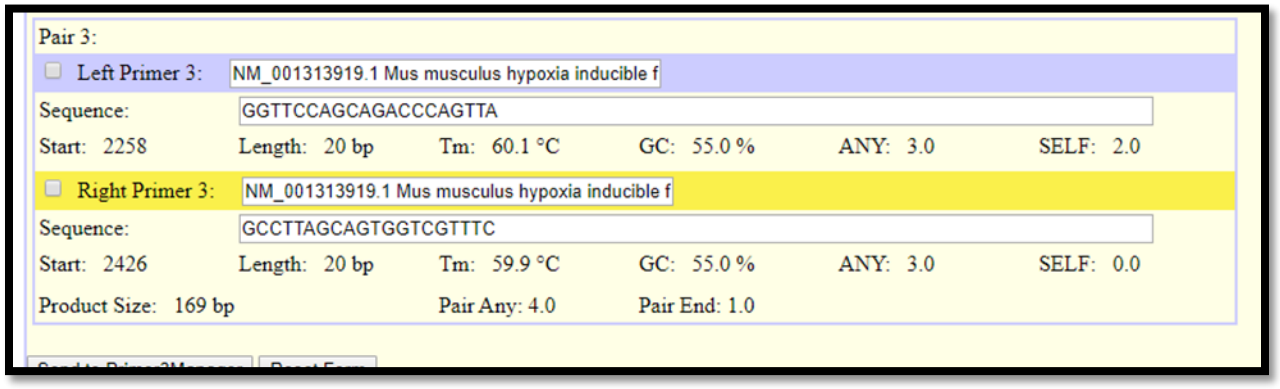

Figure 4.4: Prime3Plus results for HIF1- α

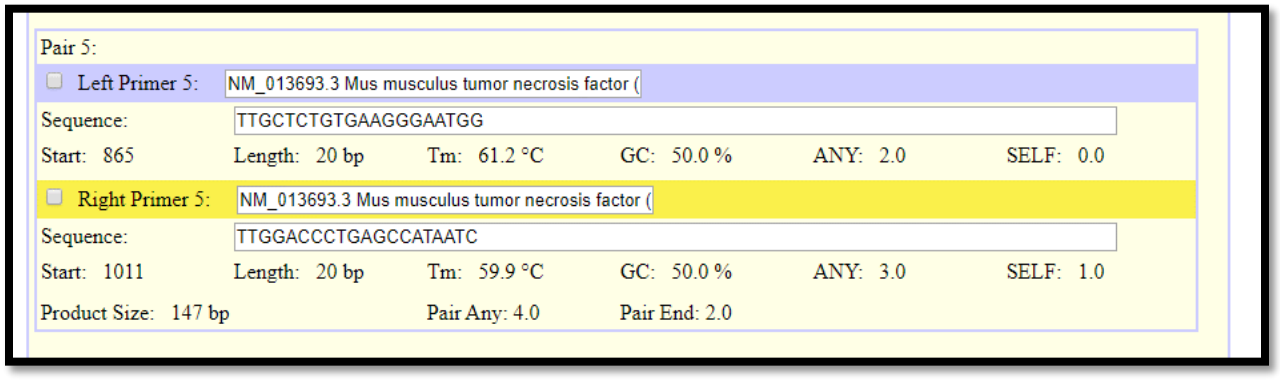

Figure 4.5: Prime3Plus results for TNF- α

4.2.2 UCSC In-Silico PCR results

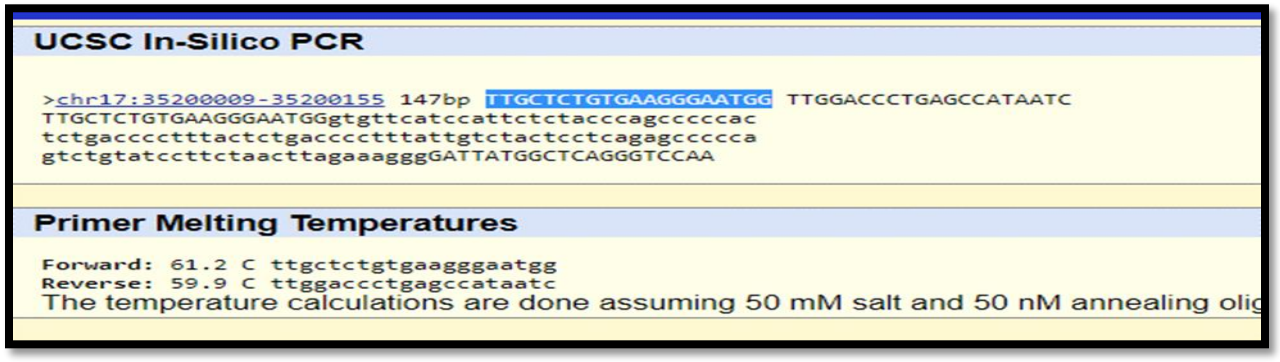

Figure 4.6: UCSC In-Silico PCR result of TNF- α

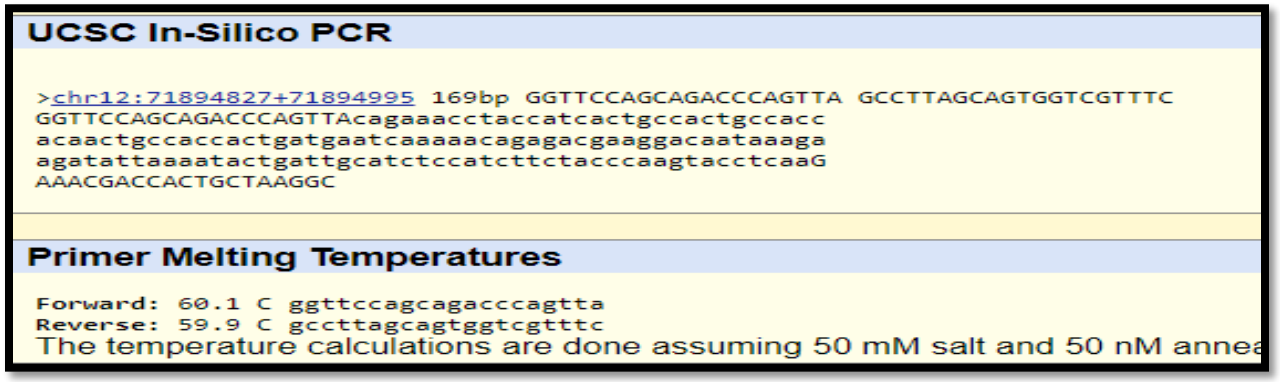

Figure 4.7: UCSC In-Silico PCR result of HIF1- α

4.3 UV visible spectroscopy of selenium Nanoparticles

Nanoparticles dilutions were prepared and their absorbance was checked by UV visible spectroscopy and the results are given in figure 4.8

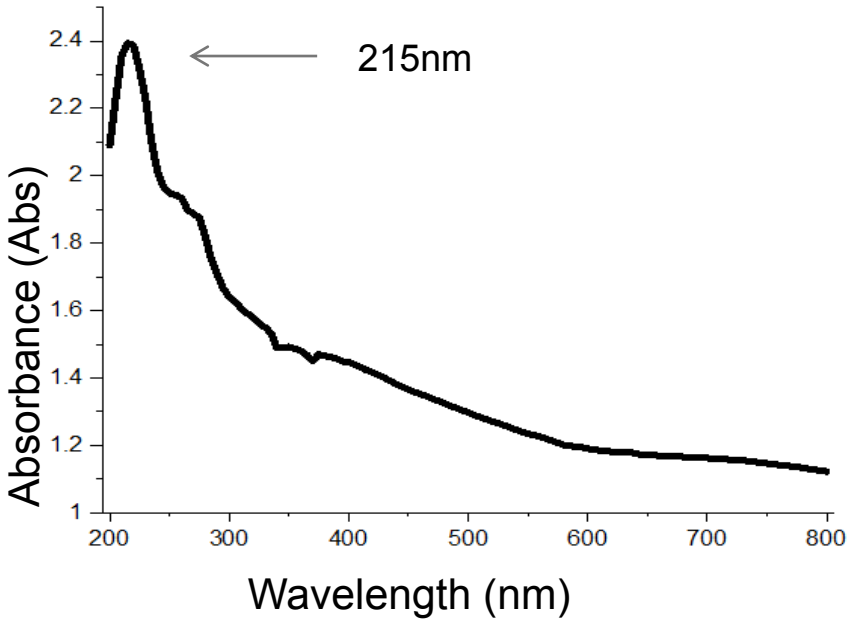

Figure 4.8: UV-visible analysis of SeNP reaction mixture indicting specific SeNP absorbance peak at 215nm

4.4 Induction of Collagen Type-II Arthritis

Mice paw volumes were measured during the induction of arthritis on day 0, 7,14,21,28 using Vernier caliper. An increase in paw volume was observed at day 14 after the administration of first Freund's adjuvant injection in the mice tail. After the administration of second and third injection of Freund's adjuvant a significant increase can be shown in the Figure 4.9, which confirms that arthritis is successfully induced in mice.

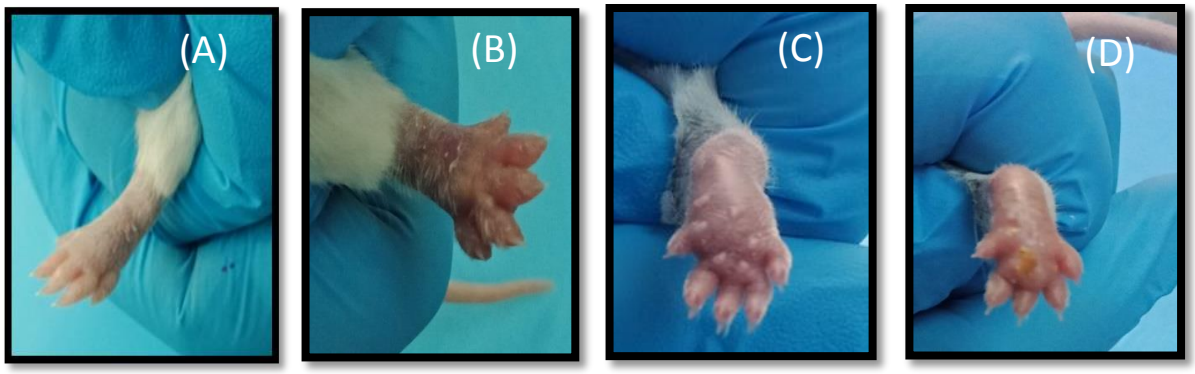

Figure 4.9: Pictorial representation of arthritis induction; **(A)** Ventral view of healthy mice paw, **(B)** Ventral view of Grade-4 arthritic mice paw, **(C)** Dorsal view of Grade-4 arthritic paw showing swelling and ankylosis, **(D)** Ventral view of arthritic mice paw showing abscess formation

At Day-0 and Day-7 there was no significant increase in paw volumes, whereas from Day-14 to 28 after the administration of Freund's adjuvant, the paw volumes were significantly increased as compared to the control mice shown in figure 4.10

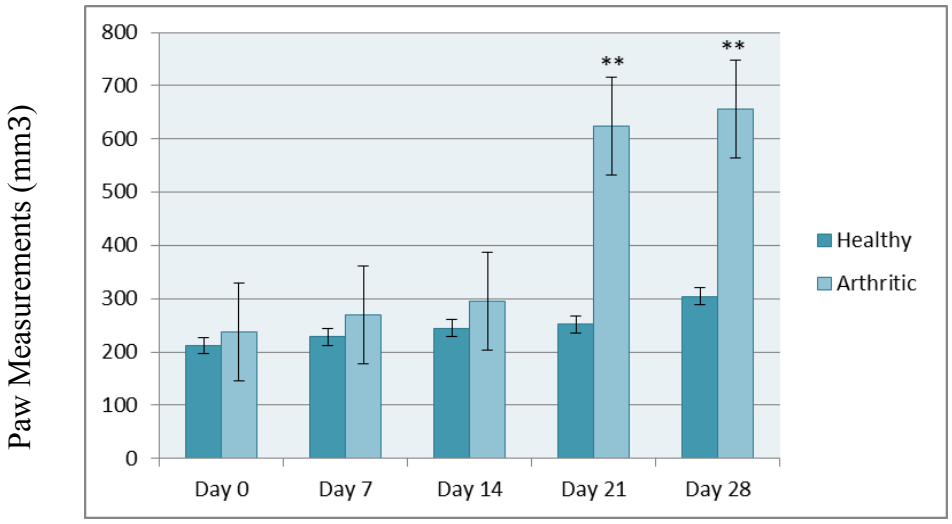

Figure 4.10: Paw volumes during arthritis induction

DISCUSSION

Rheumatoid arthritis has been under investigation for its successful treatment for more than two centuries and despite decades of scientific research it is still a challenge for existing medicines. Currently available drugs only improve the quality of life of patients to some extent but do not reverse the damage. Therefore, we need to discover some alternative therapeutic options for RA treatment. Nano-therapy has gained attention due to its enhanced therapeutic efficacy; therefore, the present study was conducted to assess the potential of essential micronutrients such as Se at Nano-scale due to its antioxidant and anti-inflammatory properties that could ameliorate symptoms of rheumatoid arthritis. Selenium nanoparticles are being exploited for medical applications such as antioxidant, antifungal, anticancer and antimicrobial. Therefore, selenium nanoparticles are considered better and safe candidates for medical applications. Researchers are aiming to achieve this strategy of reducing toxicity and enhancing efficacy of selenium by Nanotechnology. Our first objective was the biogenic synthesis of selenium nanoparticles and checking their therapeutic efficacy on rheumatoid arthritis.

Biosynthesis of nanoparticles involve natural organic sources such as plants(Menon et al., 2019), fungi (Henglin Zhang et al., 2019) and bacteria (Wadhwani et al., 2017). Different biomolecules from these organic sources such as enzymes, sugars, phenols, flavonoids and proteins help in reducing ionic forms of selenium into selenium nanoparticles. Biogenic selenium nanoparticles are preferable because they are ecofriendly, economical and do not produce any toxic byproduct (Wadhwani et al., 2016). Although various plants are reported, but for the phytogenic synthesis of selenium nanoparticles a few reports are available. The studies reported synthesis of SeNPs using seed extract of fenugreek (Ramamurthy et al., 2013), leaf extract of

Terminalia arjuna (Prasad & Selvaraj, 2014), lemon, Capsicum annuum (Li et al., 2007), Leucas lavandulifolia (Kirupagaran et al., 2016), Clausena dentate, Diospyros Montana (Kokila et al., 2017), Psidium guajava, green tea extract (W. Zhang et al., 2018), aqueous extract from Allium sativum(Anu et al., 2017), dried fruit extract from Vitis vinifera and flower extracts from Bougainvillea spectabilis (Deepa & Ganesan, 2015).

Foeniculum vulgare is an aromatic, flowering plant that belongs to Apiaceae family. Funnel seeds finds its use in culinary due to its characteristics anise order and nutritional value (Díaz-Maroto et al., 2005). Fennel is also a medicinal plant and its therapeutic potential is widely studied. Several studies have been performed to evaluate the antioxidant potential of *Foeniculum vulgare.* Faudale at el reported the higher antioxidant acitivity of wild fennel plant than edible plant (Faudale et al., 2008). Damage caused by oxidative stress can be protected by using natural antioxidant (Scalbert et al., 2005). Ruberto at el evaluated the antioxidant potential of fennel essential oil by two lipid model system and their study showed a strong antioxidant activity of oils as compared to reference alpha tocopherol and BHT (Ruberto et al., 2000). Owing to these features of *Foeniculum vulgare* we synthesized the selenium nanoparticles from it to validate their antioxidant and anti-inflammatory potential against rheumatoid arthritis.

Our second objective was the construction of collagen induced arthritic mice model. Different rodent immune mediated arthritis (RMIA) models serve as a standard for understanding of hypothetical immune mediated disease mechanisms and testing the efficacy of novel drug candidates during preclinical trials (Bevaart, Vervoordeldonk, & Tak, 2010; Hegen, Keith, Collins, & Nickerson-Nutter, 2008). For investigation of disease mechanism of arthritis most commonly used RMIA are mice and rats. Rodents as a research subject for rheumatoid arthritis offer many advantages. First of all their small size, receptiveness to group housing and

inexpensiveness reduce the research costs. Second different strains of rodents can be used to evaluate the effect of biological heterogeneity on disease progression. Different strains can modulate severity and extent of immune mediated disease depending on their diverse immunological capabilities (Fox et al., 2007). Modern techniques allow genome alteration of rodents to study the mechanisms regulating joint disease(Bendele et al., 1999; Horai et al., 2000). Fourth, protocols for induction and evaluation of RMIA are well characterized and require inexpensive laboratory equipment. Fifth, mice are the species for immunological research, different cytokines and antibodies are available for research. Finally, animal models of RA are well proven for predicting the efficacy of novel anti-arthritic drugs for humans are performed in rodents. Among RMIA, the closest resemblance to human RA is the joint lesions formed in collagen induced arthritis (Terato et al., 1982; Trentham, Townes, & Kang, 1977).

These experimental models provide better understanding of pathophysiological mechanisms of autoimmune diseases and testing novel therapeutic agents. An ideal RA model must reproduce the complex symptoms and pathogenesis of the disease, inflammatory infiltration, degradation of cartilage, bone erosion, and faster disease progression(Asquith, Miller, McInnes, & Liew, 2009). The use of animal models significantly provides the understanding of inflammatory mediators, cartilage damage and bone erosion, which can be used to access the potential therapeutic drugs to treat these disorders (Lubberts & van den Berg, 2013). Bradford D. Fischer et al. reported that animal models provide insights into the pathophysiology of arthritis and biological therapeutic evaluation. The successful preclinical approach can reproduce its results to treat rheumatoid arthritis (Fischer, Adeyemo, O'Leary, & Bottaro, 2017).

This study provides an insight to the potential role of biogenic selenium nanoparticles to ameliorate oxidative stress and inflammation in rheumatoid arthritis which is confirmed by In

Silico docking analysis which is further needed to be tested in wet lab. In future, these selenium nanoparticles will provide an alternative treatment option for rheumatoid arthritis after further testing on animal models to target specific disease regulating pathways.

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