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Evaluating the Effects of Nano-Formulated Chrysin on The Inflammatory and Oxidative Markers Levels in Iraq Women with Rheumatoid Arthritis (*in vitro* study)

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala, in Partial Fulfilment of the Requirements for the Degree of Master in [Clinical Chemistry]

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بسم الله الرحمن الرحيم

﴿فَتَعَٰلَى ٱللَّهُ ٱلْمَلِكُ ٱلْحَقُّ ۗ وَلَا تَعْجَلْ بِٱلْقُرْءَانِ مِن قَبْلِ أَن يُقْضَىٰٓ إِلَيْكَ وَحْيُهُۥ ۖ وَقُل رَّبِّ زِدْنِى عِلْمًا﴾

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Supervisor Certification

We certify that this thesis entitled" **Evaluating the Effects of Nano-Formulated Chrysin on The Inflammatory and Oxidative Markers Levels in Iraq Women with Rheumatoid Arthritis (in vitro study)** " was carried under our supervision at the College of Medicine, University of kerbla, as a partial fulfillment for the requirement of the degree of Master of Science in Clinical Biochemistry.

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We the examining committee, certify that we have read this M.Sc. thesis entitled:

Evaluating the Effects of Nano-Formulated Chrysin on The Inflammatory and Oxidative Markers Levels in Iraq Women with Rheumatoid Arthritis (in vitro study)

We have examined the student **" Sara Nadhuim Sahib "** in its content and our opinion; it meets the standard of thesis for the Master's degree in **"Clinical Chemistry"** with an appreciated **"Excellent"**.

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Dedication

To my Creator, the Messenger of Mercy, and my Imams

My father and mother

To my brothers and sisters

To my dear children

To everyone who stood by me on my difficult path

Sarah, 2024

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Summary

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joints, leading to pain, swelling, and progressive joint destruction. Oxidative stress and inflammation play a crucial role in the pathogenesis of RA. Chrysin, a natural flavonoid, has shown promising anti-inflammatory and antioxidant properties in various studies. However, the therapeutic potential of HA-encapsulated nano-formulated chrysin in RA has not been widely explored, especially in Iraqi women. This in vitro study aimed to evaluate the effects of HA-encapsulated nano-formulated chrysin on inflammatory and oxidative markers in Iraqi women with RA.

Aims The research endeavor is focused on formulating and assessing the effect of chrysin nanoparticles on inflammation, oxidative stress, and gene expression levels in patients afflicted with RA.

Methods A case-control study was conducted at Imam Hassan Al-Mujtaba Hospital and Marjan Medical Hospital from mid-October 2023 to March 2024, which included 130 females divided into 70 rheumatoid arthritis cases and 60 apparently healthy controls. The rheumatoid arthritis group was split into 35 non-obese and 35 obese. Age groups matched the study and 5 ml of venous blood was collected from rheumatoid arthritis patients. The study was divided into three parts. The **first part** evaluated the levels of TNF-α, IL-10, NO, SOD, FRAP and lipids in rheumatoid arthritis. The blood lipid profile was examined and ELISA system to detect the level of TNF-α, NO, IL-10, SOD and FRAP. The **second part,** after blood component separation using Ficoll and extraction of peripheral blood cells, ***in vitro*** investigated the structural features of chrysin-loaded and hyaluronic acid-coated nanoparticles as anti-inflammatory and antioxidant agents and demonstrated

their effects on the study markers of peripheral blood cells of rheumatoid arthritis patients. The properties of the nanoparticles were measured using scanning electron microscopy (SEM), Fourier transform infrared (FTIR), various AFM, MTT and DLS techniques. The **third part** was to evaluate the effect of chrysin-loaded and hyaluronic acid-coated nanoparticles on the gene expression of various genes including MPP9 and TIMP1 Statistical analysis was performed using Statistical Package for Social Sciences version 21.

Result The study showed an increase in lipid profile levels, especially in the obese group with rheumatoid arthritis, as well as an increase in TNF-α, IL-10, NO, and MMP9 genes. In addition, patients showed a decrease in HDL-C, antioxidants, and TIMP1 gene levels. The effect of hyaluronic acid-coated chrysin nanoparticles was clear on the study markers as well as gene expression, as chrysin nanoparticles reduced inflammatory and oxidative stress, increased TIMP1 gene expression, and decreased MMP9 gene expression.

Conclusion The study concluded that inflammatory, oxidative, and antioxidant markers are valuable biomarkers for diagnosing RA. Specifically, it highlighted the therapeutic efficacy of hyaluronic acid-coated chrysin nanoparticles in significantly reducing inflammatory and oxidative markers while enhancing antioxidant markers. This underscores the potential of such nano-formulations in managing RA by addressing the underlying oxidative stress and inflammation associated with the disease.

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List of Abbreviations

Abbreviation	Meaning
ACR	American College of Rheumatology
ACPA	Anti-Citrullinated Protein Antibodies
AFM	atomic force microscopy,
Blank Nio NPs	Blank niosomal nanoparticles
BMI	Body Mass Index
B-cell	B lymphocyte
CAT	Catalase
CBC	Complete Blood Count
cDNA	Complementary DNA
CD44	Cluster- determinant 44
CHR	Chrysin
CNS	Central nervous system
CVD	Cardiovascular disease
DLS	Dynamic Light Scattering
DLS	Dynamic light scattering system
DMARDs	Disease-modifying anti-rheumatic drugs
DMSO	Dimethyl sulfoixide
DNA	Deoxyribonucleic Acid
EULAR	European Alliance of Associations for Rheumatology
FLSs	Fibroblast-like synoviocytes
FRAP	Ferric reducing ability of plasma
FT- IR	Fourier-Transform Infrared Spectroscopy

GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GC	Glucocorticoid
GPx	Glutathione peroxidise
GSH	Glutathione
GSTs	Glutathione *S*-transferases
GWAS	Genome-Wide Association Studies
Hb	Haemoglobin
HLA	Human Leukocyte Antigens
HLA-DRB1	Human Leukocyte Antigens, DR beta 1
H Nio-chrysin NPs	Chrysin-loaded hyaluronic acid-coated niosomal NPs
HA	hyaluronic acid
IL-10	Interleukin - 10
ILD	Interstitial lung disease
INF- ɣ	Interferon-gamma
MDA	Malondialdehyde
MENA	Middle East North Africa
MHC	Major histocompatibility complex
MMP9	Matrix metalloproteinase 9
MMPs	Matrix metalloproteinase
mRNAs	Messenger RNA
MIR-22	MicroRNA-22
NADH	Nicotinamide adenine dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate(Reduced)
Nio-chrysin NPs	Chrysin-loaded niosomal NPs
NO	Nitric oxide

NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDI	Poly dispersity index
PLGA-PEG	Poly lactide co glycolide –poly ethylene glycol
PUFA	Poly unsaturated fatty acid
PBMCs	Peripheral Blood Mononuclear Cell
RA	Rheumatoid arthritis
RBC	Red Blood Cell
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
RPM	Rotation per minute
SEM	Scanning electron microscopy
SLNs	Solid lipid nanoparticles
SOD	Superoxide dismutase
Span 60	Sorbitan Monostearate
STAT	Signal transducer and transcription
TIMP1	Tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptors
TNF-α	Tumors necrosis factors- alpha
hTERT	Human telomerase reverse transcriptase
WHO	World Health Organization
LPO	Lipid peroxidatioin
CI	Confidence interval

Chapter One

Introduction and Literature Review

1. Introduction

1.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a heterogeneous disorder caused by an abnormal autoimmune response initiated by the complex interactions of genetic and environmental factors that contribute to RA etiology **(Zamanpoor, 2019)**. The disease affects (0.5-1) % of the population worldwide. The disease mainly involves the joints which may lead to functional disability but can also show extra-articular manifestations in 40% of cases (**Motta *et al*., 2023**). RA affects women who are three times more likely to develop the disease than men (**Deane *et al*., 2023**). Common to many autoimmune disorders, there is a female predominance **(Raine and Giles, 2022**).

Overproduction of pro-inflammatory cytokines like TNF-α and interleukins leads to synovial cell proliferation joint cartilage destruction, and bone erosions due to the proliferation of synovial cells (**Nattagh-Eshtivani *et al*., 2021).**

The inflammatory cascade activates oxidant-generating enzymes (NADPH oxidase, xanthine oxidase, myeloperoxidase), promoting the production of reactive oxygen and nitrogen species. Inflammatory mediators and reactive species directly or indirectly impact the pathophysiology of RA (**Mateen, *et al.* 2019**).

1.1.1. Symptoms of Rheumatoid Arthritis

Joint injuries cause deformities, bone erosion, pain, and discomfort, typically affecting individuals aged 35-60, with symptoms including morning rigidity, fatigue, fever, weight loss, swollen joints, and rheumatoid nodules (**Bullock *et al*., 2019)**.

Rheumatoid arthritis begins with minor joints and progresses to larger joints causing function loss in various joints (most frequently the hands, wrists, and knees) (**Prasad *et al*., 2023)**. The lining of the affected joint becomes inflamed, resulting in tissue

injury, and chronic pain (**Iqbal and Rattu, 2019**). The disease, symmetrical in clinical manifestations, affects various joints, causing redness, swelling, pain, and joint dysfunction in early stages and rigidity and deformity in later stages (**Fresneda Alarcon *et al*., 2021**). The extra-articular involves the eyes, nerves, epidermis, kidney, lungs, liver, and heart (**Ding *et al*., 2023**). Early diagnosis can prevent joint damage, with significant differences in permanent damage occurring within the first two years of disease onset (**Cheng *et al.,* 2021**). Reliable biomarkers are essential for early diagnosis, accurate prognosis, and improved disease management of RA, especially during the first three to six months (**Mueller *et al*., 2021**).

RA, despite its pathological hallmark of synovitis, is characterized by complex, chronic, inflammatory, and autoimmune features, leading to increased morbidity and premature mortality **(Figus *et al*., 2021).**

1.1.2. Risk factors of Rheumatoid arthritis

Genetic and environmental factors, some of the most important causes of RA, contribute significantly to its development **(Dedmon, 2020).** Environmental factors like smoking, and eating habits which directly or indirectly alter genes through epigenetic processes, are crucial in the development of RA (**Nemtsova *et al*., 2019)**. Autoimmune tolerance disorders may be exacerbated by changes in the relative amounts and expression of encoded proteins caused by the interplay of environmental variables, susceptibility genes, and epigenetics **(Ding *et al*., 2023).**

1.1.2.1 Genetic Factors

Studies have shown that around one-half to one-third of RA cases are attributed to genetic factors (**Carnero-Montoro *et al*., 2019)**. Genetic variants can also influence treatment response and the development of side effects and comorbidities in RA patients **(Nemtsova *et al*., 2019**). Overall, genetic factors contribute to the complex etiology of RA, interacting with environmental and

individual factors to influence disease susceptibility and progression **(Wysocki *et al*., 2020).**

1.1.2.2. Environmental Factors

Several environmental variables are risk factors for the development of RA, but the most powerful and reliable one is smoking cigarettes (**Cush, 2022**).

1.1.2.2.1. Smoking

Cigarette smoking is a significant external factor linked to RA triggers (**Croia *et al*., 2019)**. Increasing the likelihood of seropositive RA by up to 35% dowing to autoimmunity within the pulmonary mucosa and the secretion of inflammatory cytokines (**Ren *et al*., 2023**). Passive smoking during infancy also increases the risk of developing RA by 34%(**Zhang *et al*., 2023**).

1.1.2.2.2. Traffic Pollution

Research suggests air pollution may initiate autoimmune conditions like RA, as particles can transform into antigens within airway tissues, creating a protective barrier and stimulating autoimmune responses (**Sigaux *et al*., 2019**).

The World Health Organization (WHO) highlights air pollution as a major health concern, with exposure to smoking and silica causing inflammatory stress and RA risk (**Zhang *et al*., 2023**) . The most harmful gases include sulfur dioxide, nitrogen dioxide, carbon monoxide, and ozone (**Alsaber *et al*., 2020**). air pollution triggers pro-inflammatory immune responses and disrupts oxidation-reduction balance in the respiratory mucosa (**Prisco *et al*., 2020**).

1.1.2.3. Sex and Hormones

Sex is a significant epidemiological factor linked to the beginning of RA, as women have higher innate and adaptive immunological responses **(Intriago *et al*., 2019)**. Hormonal and sex-related variables are disease risk factors **(Chancay *et al*., 2019).** Hormone imbalance in RA patients is often caused by pro-inflammatory estrogens

(Romão and Fonseca, 2021), while progesterone and androgens have decreased anti-inflammatory effects (**Benagiano *et al.*, 2019**). Factors like menarche, pregnancy, postpartum period, menopause, and hormone replacement therapies affect this transition (**Dupuis *et al*., 2021**)**.**

1.1.2.4. Vitamin D Deficiency

Vitamin D is a steroid hormone and one of the most important immune endocrine mediators **(Saponaro *et al*., 2020).** In humans, 7-dehydrocholesterol undergoes a series of modifications mediated by ultraviolet light to generate vitamin D **(Harrison *et al*., 2020)**. Vitamin D is known to have anti-inflammatory effects on multiple immune cells **(Ao *et al*., 2021)**. Vitamin D inhibits T cell growth and synthesis of cytokines such as TNF-α, IL-2, and interferon-gamma (INF- ɣ) which are important pathogens in autoimmune disorders **(Aslam *et al*., 2019).**

Research indicates vitamin D deficiency is common in RA patients, prompting further investigation into its potential protective role in this condition (**Romão and Fonseca, 2021)**. The active form of vitamin D is [1,25$(OH)_2$D], which affects both innate and adaptive immune system components (**Bikle and Chrysinistakos, 202**0). It suppresses inflammation by blocking the development of Toll-like receptors (TLR) 2 and 4 (**Dupuy *et al*., 2021**)**.**

1.1.2.5. Obesity

Obesity increases the risk of autoimmune disorders and RA in women due to pro-inflammatory adipocytokines **(De Figueiredo, *et al*., 2023**)

High levels of adipokines, such as leptin, resistin, and visfatin, and low levels of anti-inflammatory adipokines, may increase inflammation and disease activity in obese patients with RA (**Guimarães, *et al*., 2019**).

1.1.2.6. Age

The impact of age on rheumatoid arthritis (RA) holds notable importance. A study showed that age was associated with the non-achievement of clinical and functional remission criteria in RA patients **(Aoki *et al.*, 2020)**. Individuals diagnosed with RA before age 50 had a significantly higher risk of fractures compared to matched controls, emphasizing the importance of bone health management in younger RA patients **(Erwin *et al*., 2021)**

1.1.2.7 Family History

The study indicates that individuals with affected first-degree relatives are at a higher risk of developing seropositive RA **(Sitorukmi *et al*.,2020)**, influenced by family history, social support, and lifestyle choices (**Kim *et al.*, 2023)**.

1.1.3.Pathophysiology

Rheumatoid arthritis pathogenesis is the activation of the innate immune response that includes the activation of dendritic cells by exogenous material and autologous antigens **(Pabón-Porras *et al.*, 2019).** Antigen-presenting cells, including dendritic cells, macrophages, and activated B cells, present arthritis-associated antigens to T cells. T-cell activation and B-cell activation result in increased production of cytokines and chemokines (**Thorarinsdottir, 2019**). Macrophages contribute to osteoclastogenesis, producing cytokines like TNF-α, IL-1, and IL-6, while synovial membrane-activated fibroblast-like synoviocytes produce inflammatory cytokines and prostaglandins and matrix metalloproteinases (MMPs) **(Jalalvand *et al*., 2024)**. Pro-inflammatory cytokines are involved in the pathogenesis of RA (**Ponist *et al*., 2019**).

Autoimmunity and immune complexes result from a state of cellular activity in the joints and other organs that precede RA **(Weyand and Goronzy, 2021)**. The main clinical indicators of the illness are inflammation of the synovium in joints that

degenerate and the fibroblast cells comparable to synoviocytes, play a major part in the damage **(Nygaard and Firestein, 2020 ; ALivernini *et al* ., 2022).**

1. The first phase, is brought on by non-specific inflammation.
2. Second phase (resulting from T-cell proliferation and activation)
3. Third phase (resulting from cytokine IL1, TNFalpha, and IL6)-induced tissue damage.

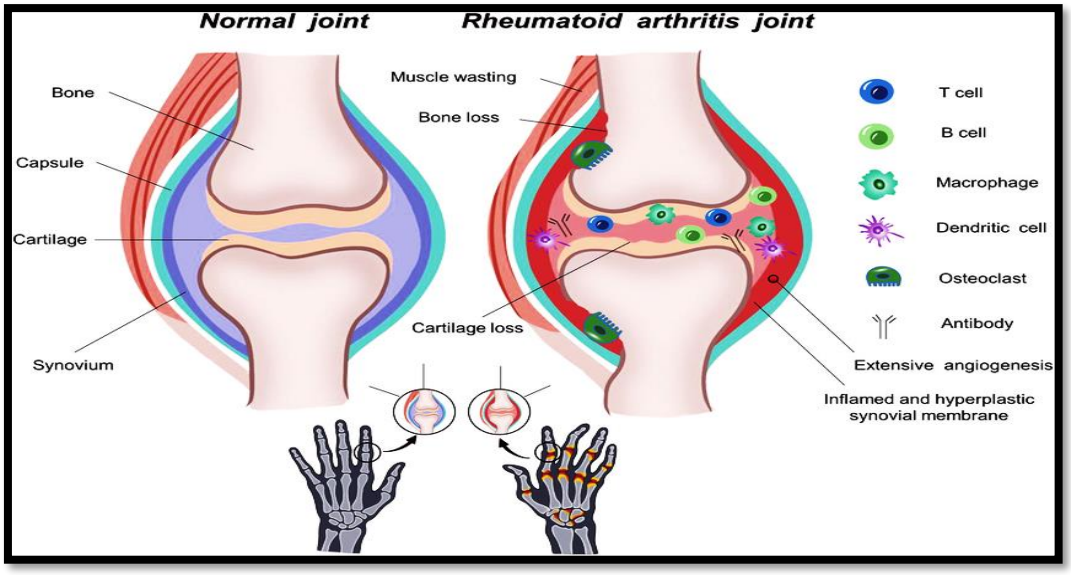

Fig. (1-1): **Normal and Rheumatoid Arthritis Joints (Ding *et al*., 2023)**

1.1.4. Epidemiology

Epidemiologic variations in RA incidence and prevalence are influenced by ethnicity and geography (**Nair *et al*., 2019).** Global prevalence estimates range from 0.24% to 1% **(Almoallim and Cheikh, 2021).** The epidemiology in the Middle East and North Africa remains inadequately characterized (**Bedaiwi *et al*., 2019**), with 0.16% of the Middle East and North Africa area(MENA) population impacted **(Yip and Navarro-Millán, 2021**). In Iraq, RA prevalence rose from 1.6% in 2001 to 2.2% in 2019 (**Al-Badran et al., 2022).**

1.1.5.Diagnoses of Rheumatoid Arthritis

Rheumatoid arthritis diagnosis follows the 2010 ACR-EULAR criteria (*American College of Rheumatology-European League Against Rheumatism*) (**Aletaha D1 *et al*., 2010**). The 2010 RA Classification Criteria establishes a scoring system incorporating history, physical examination, and biomarkers for RA identification in clinical trials. These criteria exhibit a sensitivity of 84% and a specificity of 60% for RA classification. Rheumatologists commonly utilize these criteria to substantiate RA diagnoses in clinical settings (**Shapiro, 2021).**

The diagnosis of RA relies on clinical symptoms, laboratory tests, and imaging results, including RF, CRP, and ESR. These markers are non-specific and indicate only transient inflammatory conditions. It is essential to investigate novel markers for enhanced clinical diagnosis of RA(**He *et al*., 2020**).

Rheumatoid arthritis patients often experience tender joints, stiffness, and abnormal laboratory tests. Early diagnosis is crucial for treatment, preventing disease progression and disability **(Lin *et al*., 2020)**. Diagnostic methods include symptoms, doctor's examination, risk factors, family history, joint assessment, and ultrasound sonography **(Earwood *et al*., 2021)**.

Both ultrasound (US) and Magnetic Resonance Imaging (MRI)are recommended for disease activity monitoring, with ultrasound being cost-effective and MRI limited by cost and capacity (**Bullock *et al*., 2019)**. The new criteria for defining "definite RA" include the presence of synovitis in at least one joint, the absence of alternative diagnoses, and a total score of 6 or greater in four domains from individual scores as indicated in Table (1-1) **(Aletaha *et al*., 2010).**

Table (1-1): **ACR/EULAR 2010 classification criteria for RA.**

Criteria		
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**
Acutephase reactants	**Normal CRP/ESR** **abnormal CRP/ESR**	**0** **1**
Symptom duration	**< 6 week** **> 6 week**	**0** **1** **Total= overall score**

RA is diagnosed when a patient's joint score is ≥ 6, indicating clinical synovitis, with symptoms categorized based on regional norms and serology results (Dekkers,2019)

1.1.6. The Role of Cytokines in Rheumatoid Arthritis

Cytokines, soluble signaling proteins, are essential for balancing innate and adaptive immunity, secreted by most cells, especially leukocytes, in response to inflammatory stimuli **(Mantovani *et al.*, 2019)**. Cytokines are regulators of host responses to infection, immune responses, inflammation, and trauma (**Kany *et al.*, 2019)**. Cytokines are Synthesized by B-cells, T-cells, macrophages, dendritic cells, and natural killer cells. The cytokine family includes Interleukins(ILs), chemokines, TGFs, IFNs, TNFs, and adipocytokines (**Shekhar *et al.*, 2022**). ILs are a class of cytokines that were initially thought to be exclusive to leukocytes but have since

been found in various body cells (**Carbone and Failla, 2021**). ILs, divided into pro- and anti-inflammatory cytokines, are essential for immune cell proliferation, maturation, migration, adhesion, activation, and differentiation, controlling growth and differentiation during inflammatory and immune responses (**Vaillant and Qurie, 2022)** . The figure below shows the classification of interleukins**.**

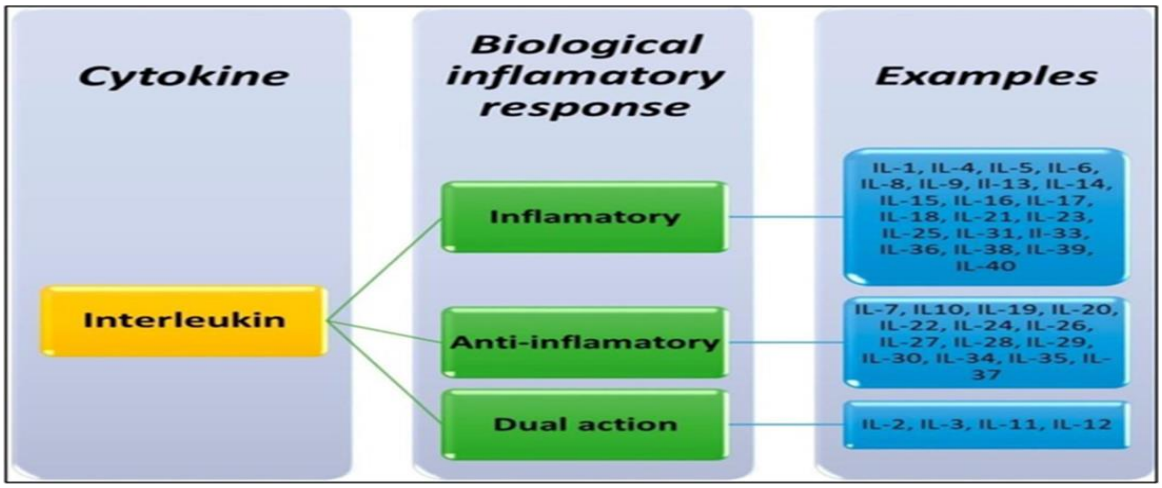

Fig. (1-2): Classification of ILs (Shekhar, Yadav *et al*. 2022)

1.1.6.1. Pro-Inflammatory Cytokines in RA

Some cytokines act to make the disease worse (proinflammatory) such as tumor necrosis factor (**Kany *et al.*, 2019)**. Pro-inflammatory cytokines play a crucial role in the pathogenesis of RA **(Mohammadian Haftcheshmeh *et al.*, 2021)**. Pro-inflammatory cytokines trigger signaling pathways that promote immune-mediated inflammation, leading to joint destruction **(Vasilev, *et al*. 2021)**. In RA patients, elevated levels of cytokines such as IL-6, IL-17A, IL-23, IL-18, TNF-α, and IL-12 have been observed **(Lozada-Mellado *et al.*, 2022)**.

1.1.6.1.1. Tumor Necrosis Factor -Alpha

Tumor Necrosis Factor -Alpha (TNF-α) is a homotrimer protein consisting of 157 amino acids, It is functionally known to trigger a series of various inflammatory molecules, including other cytokines and chemokines (**Jang *et al*., 2021)**. TNF-α is a potent pro-inflammatory cytokine that plays a key role in cell metabolism, including glucose and lipid metabolism **(Coras *et al*., 2020).** TNFα is mainly produced in activated macrophages and natural killer cells, whereas lower expression is found in a variety of other cells, including fibroblasts, smooth muscle cells, and tumor cells **(Wang and He, 2020).** Human TNFα is translated as a 26-kDa membrane-associated form and is then cleaved in the extracellular domain through the action of matrix metalloproteases to release a mature soluble 17-kDa protein **(Liang *et al* ., 2013).** TNF-α serves as a pivotal regulator in the pathogenesis of RA. The TNF–α signaling pathway triggers the activation of endothelial cells and the recruitment of proinflammatory cells such as synovial fibroblasts and macrophages **(Kondo *et al*., 2021).** TNF-α is a pro-inflammatory cytokine that plays a crucial role in various inflammatory disorders, including autoimmune diseases (**Subedi *et al* ., 2020**). It functions through two main receptors, TNFR1 and TNFR2, with different roles in inflammation and cell survival. While TNFR1 activation is pro-inflammatory and apoptotic, TNFR2 activation has a dual role, The correct functioning of TNF requires effective communication with TNF receptors (TNFRs). **(Rolski *et al* ., 2020).** TNF-α activities increase synovial inflammation, angiogenesis, cartilage, and bone resorption, inhibit regulatory T cells, and enhance pain, also amplifying osteoclast activation (**Sikorska *et al*., 2019)**.

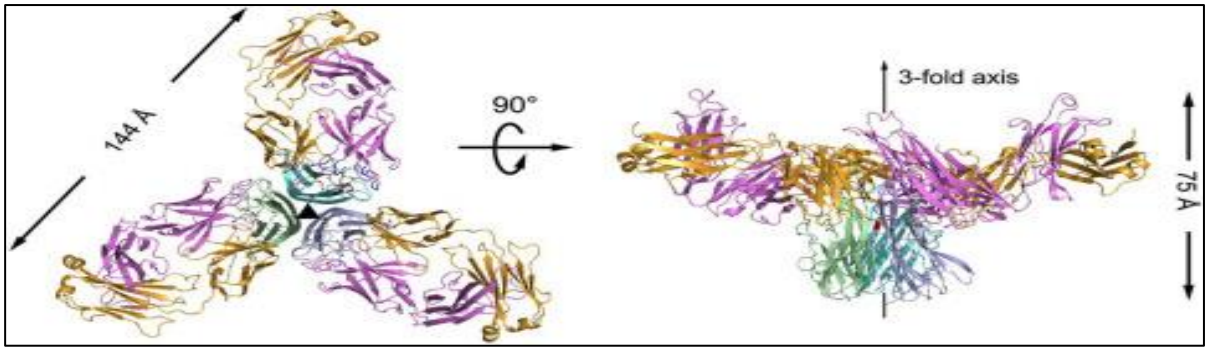

Figure (1-3) Structure of human TNF-α (Liang *et al* ., 2013)

1.1.6.2. Anti-Inflammatory Cytokines in RA

Anti-inflammatory cytokines might effectively inhibit arthritis, either by affecting innate immune cells (for example, by skewing macrophage polarization) or by interfering with the activation of B cells or T cells (**Chen *et al.,* 2019)**. IL-4 and IL-10 act antagonistically to reduce the inflammatory response by preventing the production of IL-1 and TNF-α pro-inflammatory cytokines (**Gandhi *et al*., 2021)**.

1.1.6.2.1. Interleukin-10

IL-10 is a cytokine with anti-inflammatory and immunostimulatory effects, downregulating genes, promoting cytokine production, inhibiting chemokines. Its anti-inflammatory effects limit inflammatory responses, making it beneficial for inflammatory diseases like RA (**Trivella *et al* ., 2010**).IL-10 is a homodimer with a helical structure that functions through the IL-10R1 and IL-10R2 receptor complexes (**Wei *et al* ., 2019**). dimeric IL-10 molecules bind to the high-affinity IL-10R1 receptor and this complex recruits the low-affinity receptor IL-10R2 (**Josephson *et al* ., 2001**). Interleukin-10 (IL-10) is a crucial cytokine produced by immune cells like macrophages and T cells, known for its anti-inflammatory properties, It regulates the immune system by inhibiting excessive reactions and preventing prolonged inflammation and tissue damage (**Novianti and Nur'aeny, 2024)**. plays a protective role in the pathogenesis of RA, inhibits the activity of

natural killer cells, and promotes the differentiation of B cells, thereby producing antibodies (**Qu *et al.*, 2019)**. Strong anti-inflammatory cytokine interleukin-10 (IL-10) effectively inhibits phagocyte activation, which makes IL-10 a promising therapeutic candidate **(Ben-Khemis *et al.*, 2023).** IL-10 is important in regulating the differentiation and proliferation of various immune cells, such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes (**Zheng *et al.*, 2020**). It is interesting to note that various innate and adaptive immune system cells, such as neutrophils, B cells, T cells, mast cells, eosinophils, dendritic cells, monocytes, and natural killer cells, can produce IL-10 (**Neumann *et al.*, 2019)**. IL-10 has both pro- and anti-inflammatory effects, influencing cytokine secretion and immune cell functions(dual role) (**Wang *et al* ., 2022**).

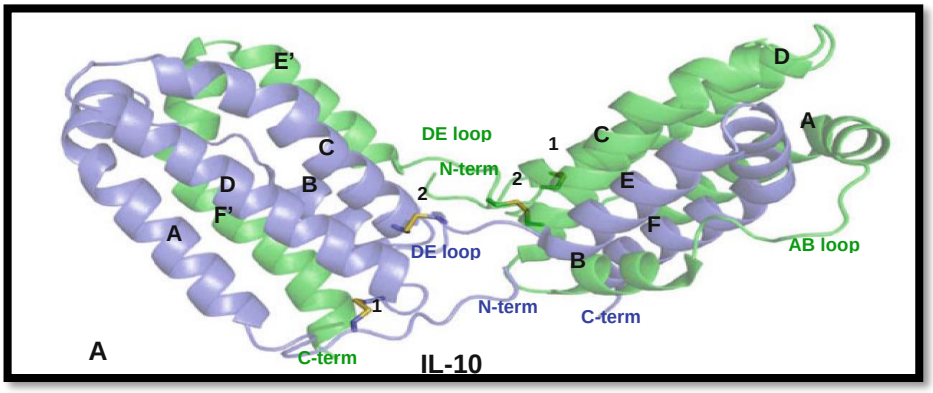

Figure (1-4) Structure of humanIL-10 (Trivella *et al* ., 2010)

1.1.7. Rheumatoid Arthritis and Inflammatory Anti- Inflammatory Balance.

RA is affected by the balance of pro- and anti-inflammatory cytokines, which can cause tissue damage or chronic inflammation, associated with the pathophysiology of arthritis (**Bedković *et al.*, 2023**). During cartilage invasion and bone erosion, there will be an imbalance between pro-inflammatory factors and anti-

inflammatory factors (**Haridas *et al*., 2019**). Imbalance also promotes the onset of autoimmunity and chronic inflammation in RA patients **(Uttra *et al*., 2019**).

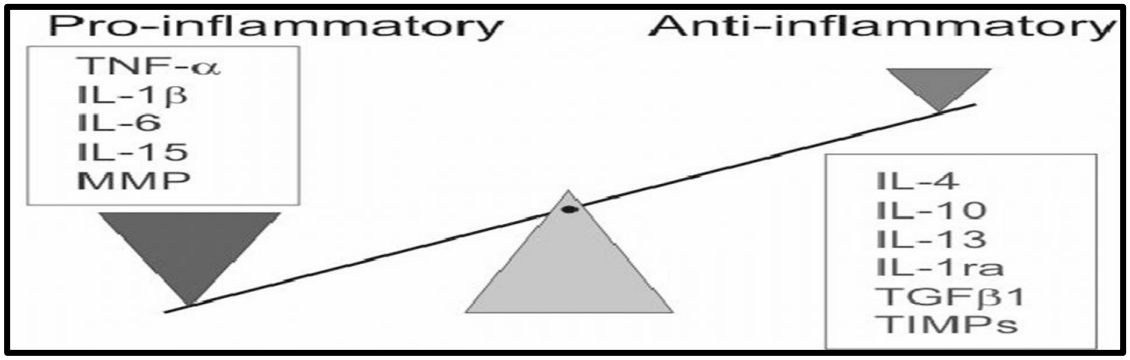

Fig. (1-5): An imbalance between pro- and anti-inflammatory mediators (Kramer *et al*., 2003)

1.1.8. Role of Oxidant and Anti-oxidant in Rheumatoid Arthritis

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS) are free radicals that can oxidize substrates**(Ali *et al*., 2020)**. Oxidative stress, a harmful state where the balance of oxidative molecules is imbalanced, can lead to a pro-inflammatory environment in RA patients (**Zamudio-Cuevas *et al*., 2022**). Biomarkers of oxidative stress such as nitric oxide have helped to explore the relationship between oxidative damage to macromolecules (proteins, lipids, and DNA) and disease progression (**Caliri *et al*., 2021**). Increased production of superoxide and hydrogen peroxide initiates the pro-inflammatory events in RA. Although these reactive species directly contribute towards the destructive, proliferative synovitis evident in RA **(Datta *et al*., 2014)**. The role of oxidative stress is strongly mooted owing to the identification of an enhanced presence of pro-oxidants, pro-inflammatory cytokines (TNF-a, IL-6, IL-17, IL-23, IFN-c, IL-1b), and pro-inflammatory enzymes (NADPH oxidase, myeloperoxidase, and xanthine oxidase) **(Ashour and Al-Mashhadani, 2024**).

1.1.8.1. Nitric Oxide (•NO**)**

Nitric oxide an unstable, highly lipophilic, free radical molecule with an unpaired electron, exhibits poor solubility in water but higher solubility in lipid membranes and nonpolar solvents(**Goshi *et al.*, 2019)**. Reactive nitrogen species are chemically reactive species that can damage cells through nitrosative stress (**Hameister *et al.*, 2020**). Nitric oxide targets are currently under active investigation to determine whether •NO itself is sufficiently cytotoxic or if its derivatives are more potent. •NO in target cells is known to generate active intermediates such as nitrosonium (NO+), nitroxyl (NO−), and peroxynitrite (ONOO−). Some researchers posit that most of the cytotoxic effects attributed to •NO stem from ONOO−, which forms through a reaction with superoxide (O_2−). Indeed, peroxynitrite is significantly more reactive; it extensively nitrosylates proteins and can serve as a source of highly toxic hydroxyl radicals (-OH) through reactions (**Kwon *et al.*, 2021; Sharma *et al* ., 2019)**

•NO · + O_2− a ONOO− + H+ a ONOOH a ONO + · OH

OH causes lipid peroxidation and other phenomena associated with oxidative stress (**Engwa *et al*., 2022)**. it was discovered that a family of enzymes called nitric oxide synthases (NOS) is responsible for •NO production. These enzymes catalyze the conversion of L-arginine into •NO and L-citrulline, simultaneously utilizing NADPH and reducing oxygen to water (**Yeo *et al*., 2019)**. Nitric oxide, plays a vital role in the human body particularly in conditions of excessive production caused by physiological disorders and severe inflammatory diseases such as RA (**Yeo *et al*., 2019)**. •NO has an average lifetime of 5–30 s, during which it rapidly interacts with its targets, primarily thiols and transition metals, or undergoes oxidation to form inactive nitrate and nitrite, for instance, via cytochrome C oxidase **(Ismail *et al*., 2016)** •NO has been reported to inhibit osteoclastic bone resorption, yet potent

stimulators of bone resorption, such as IL-1 and TNF-α are known to stimulate •NO production **(Ralston *et al.*, 2020).** Overproduction of •NO can trigger the release of inflammatory cytokines, accumulate free radicals, and trigger oxidative stress, potentially contributing to the pathogenesis of RA (**Huang *et al*., 2023)**.

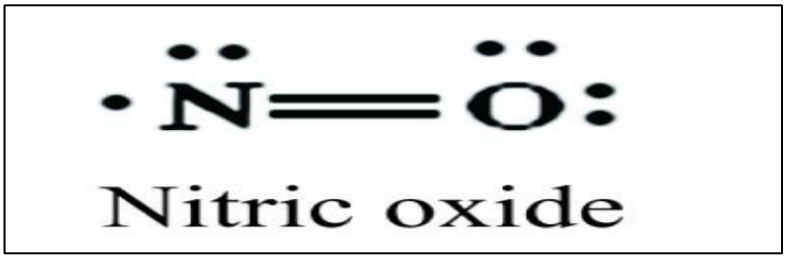

Figure (1-6) structure of nitric oxide (Belenichev *et al* 2024)

1.1.9. Antioxidants

Antioxidants stabilize free radicals by converting them into stable species, quench singlet oxygen, decompose peroxides, chelate metal ions, and inhibit oxidative enzymes (**Deshmukh and Gaikwad, 2024**). The increase in ROS and RNS production or the decrease in antioxidant mechanisms generates a condition called oxidative stress (**Taysi *et al.*, 2019)**. Antioxidants are divided into two main important types: enzymatic and non-enzymatic antioxidants

1.1.9.1. Non-Enzymatic Antioxidants

Non-enzymatic antioxidants, such as vitamins E, A, C, flavonoids, and polyamines, protect against oxidative damage from free radicals and non-radical oxidants **(Moussa *et al*., 2019).** On the other hand, the continuous production of free radicals in inflamed joints gives rise to the failure of the antioxidant system and further tissue damage (**Chaudhary *et al*., 2023**).

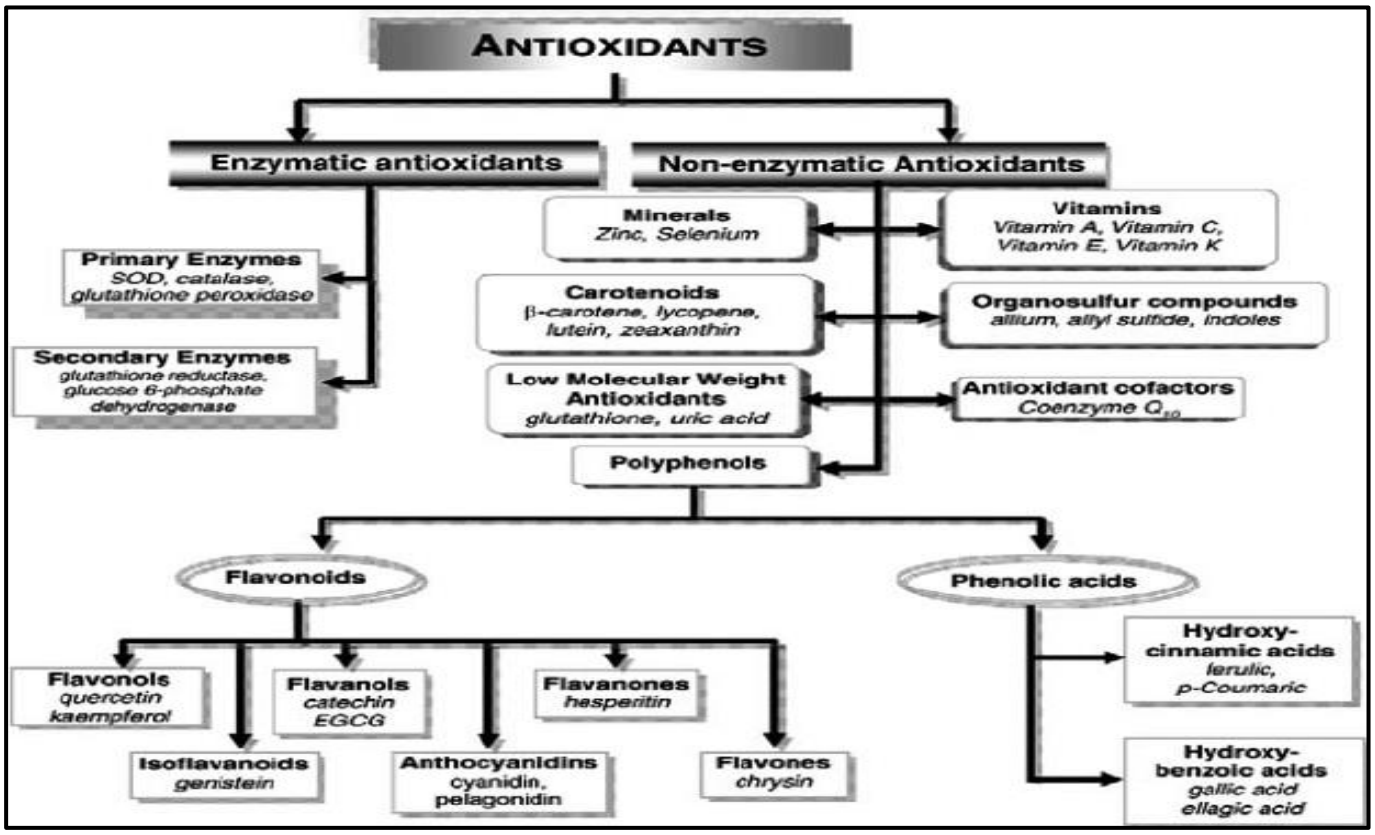

Fig. (1-7): **Different classes of antioxidants (Bunaciu *et al*., 2012)**

1.1.9.1.1. Chrysin

Chrysin (CHR), a natural flavonoid present in various sources, exhibits multiple pharmacological effects such as anti-inflammatory, antibacterial, antioxidant, and antitumor properties. It mitigates the synthesis of inflammatory mediators, and its antioxidant and disease-preventing capabilities are attributed to its structural variation in ring A and lack of oxygenation in rings B and C (**Naz *et al.,* 2019**).

Chrysin, a flavonoid with potent antioxidant properties due to its -OH group at carbon 5, is a hepatoprotective agent against d-galactosamine-induced hepatotoxicity, influenced by the presence and quantity of -OH groups **(Deldar *et al* ., 2018**) .

Chrysin's medical application is limited due to its hydrophobic nature, poor bioavailability, permeability, and water solubility. Its metabolism occurs quickly, preventing it from entering blood circulation, and prompting ongoing research on vesiculation strategies (**Ferrado *et al.*, 2019).** Chrysin demonstrated a potent antioxidant effect by decreasing lipid peroxidation, increasing antioxidant defense mechanisms by increasing superoxide dismutase activity, and reducing glutathione content (**Kseibati *et al*., 2020**).

Flavonoids like chrysin can control synovial inflammation, and immune cell function, decrease synoviocyte proliferation, balance apoptosis, reduce angiogenesis, and prevent bone and cartilage degradation, all common features of RA **(Rufino *et al*., 2024)**. Antioxidant capacity refers to compounds that protect biological systems from reactive species' harmful effects. Plant extracts like chrysin are a significant source of chemical compounds with high antioxidant activity **(Rojas and Buitrago, 2019).**

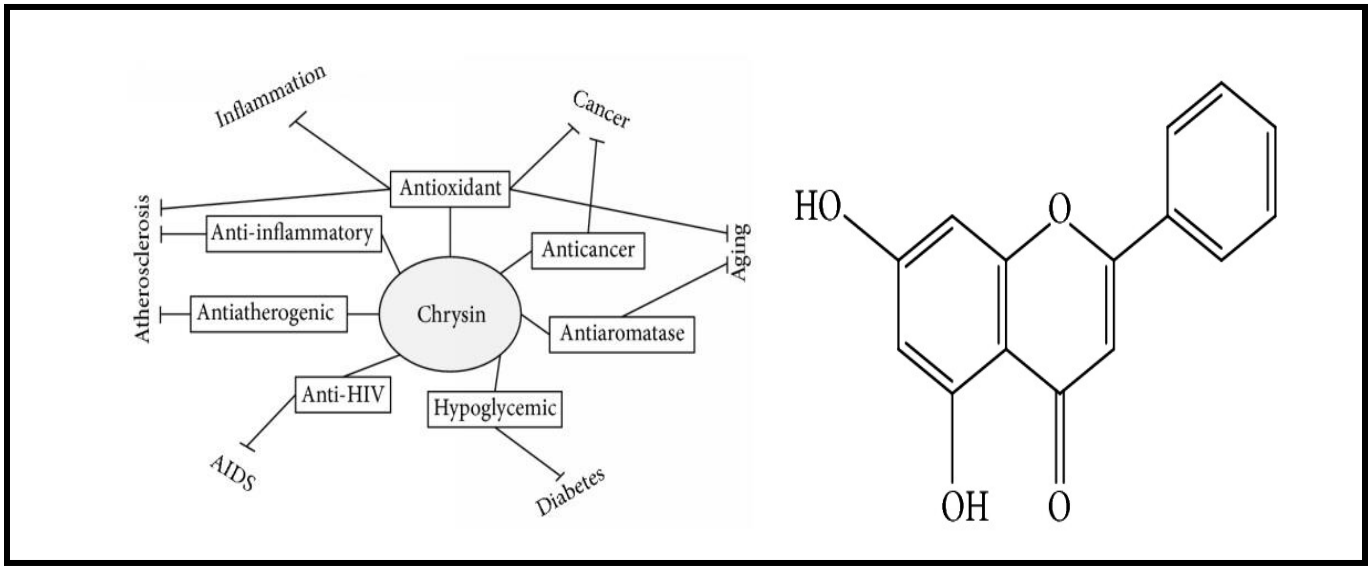

***Fig. (1-8)*: A schematic diagram of the chemical structure of chrysin and its pharmaceutical applications (Naz *et al.*, 2019).**

1.1.9.1.2. Ferric-Reducing Ability of Plasma

Antioxidant activity against ROS is achieved through hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms. HAT stabilizes free radicals, inhibiting oxidation, while SET converts radicals into cations (**Garcia *et al*., 2021**). Antioxidant activity methods primarily use HAT and SET-based assays, involving competition for peroxyl radicals and assessing antioxidant capacity for one-electron redox reactions, including FRAP TAC reactions (**Munteanu *et al* ., 2021**).

The ferric-reducing ability of plasma FRAP measures antioxidants' ability to reduce ferric ions, indicating antioxidant potential. Research shows an inverse association between FRAP total antioxidant capacity and RA risk (**Moradi *et al*., 2022**). Total antioxidant capacity (TAC) is a key indicator of the body's ability to counteract oxidative stress, with higher levels associated with a lower risk of RA (**Mititelu *et al*., 2020**). It's influenced by external factors like diet and behavior. **(Pellegrini *et al*., 2020)**. The FRAP assay assesses oxidative stress in health conditions like cardiovascular diseases and diabetes, with higher values indicating greater antioxidant capacity and improved health outcomes **(Mic *et al*., 2021).**

Strengths and Limitations for FRAP Assay

Strengths:

1. Simple and inexpensive instrumentation (**Berker *et al*., 2007**).

2. Highly reproducible and sensitive.

3. Can screen a wide spectrum of biological samples including plasma, blood, serum, saliva, tears, urine, cerebrospinal fluid, exudates, transudates, and aqueous and organic extracts of drugs, foods, and plants.

4. Good correlation is usually observed with H_2O_2 scavenging assay **(Choirunnisa *et al*., 2016).**

Limitation:

1. Non-specific this assay is non-specific. This is because if any species present in the reaction mixture possesses a redox potential lower than that of Fe (III) (<0.70 V), that species will be responsible for the reduction in [FeIII(TPTZ)2]3 **(Bibi sadeer *et al.*, 2020)**

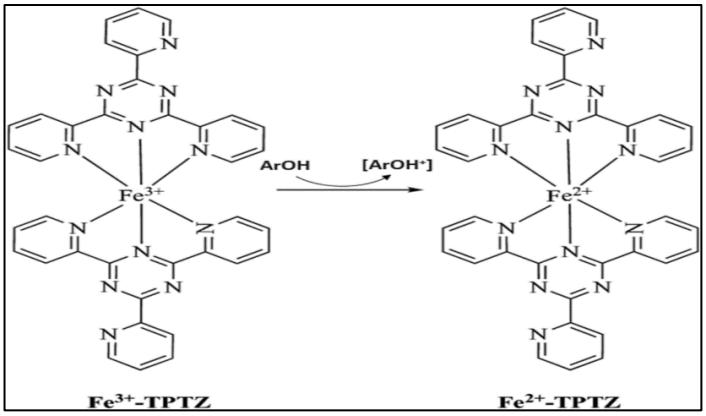

Figure (1-9) FRAP reaction mechanism, ArOH(phenolic anti-oxidant)(Bibi sadeer *et al* ., 2020)

1.1.9.2. Enzymatic Antioxidants

Enzymatic antioxidants play a crucial role in protecting cells from oxidative damage. Enzymatic antioxidants include superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase **(Maciejczyk *et al.*, 2019**).

The activity of Superoxide dismutase (SOD), Catalase (CAT), and Glutathione peroxidase (G-Px) constitute the first line of antioxidant defense which plays a key role in the total defense mechanisms of the host biological system **(Ighodaro and Akinloye 2018)**.

1.1.9.2.1. Superoxide Dismutase

Superoxide dismutase (SOD) is an essential antioxidant enzyme in animals and plants, protecting against free radicals and reactive oxygen species, and playing a crucial role in inflammatory diseases (**Nguyen *et al.*, 2020**). Mammals have three types of superoxide dismutase: Cu/ZnSOD, Mn-SOD, and EC-SOD, each with

different binding metal cofactors and cellular localization **(Thao *et al.*, 2023)**. Superoxide dismutase (SOD) is a metalloenzyme that converts superoxide anion-free radical O2•− into H2O2, liberating molecular oxygen and found in various biological kingdoms (**Zelko *et al.*, 2002**). SOD requires cofactors like iron, manganese, copper, and zinc for free radical detoxification, with a metal coordination site between domains and amino acid side chains (**Zheng *et al.*, 2023**). These cofactors exhibit a propensity to donate electrons to O_2•−, thereby undergoing regeneration throughout the catalytic process. (**Rosa *et al.*, 2021**). SOD, a component of the endogenous antioxidant defense system, undergoes CAT, GPx, and Fenton reactions to convert harmful substances into harmless ones**(Islam et al., 2022).** Overall, SOD is crucial in combating oxidative stress and inflammation in RA, making it a potential target for therapeutic interventions **(Ponist *et al* ., 2019).**

SOD

$2O_2^{\bullet-} + 2H^+$ ----------► $H_2O_2 + O_2$

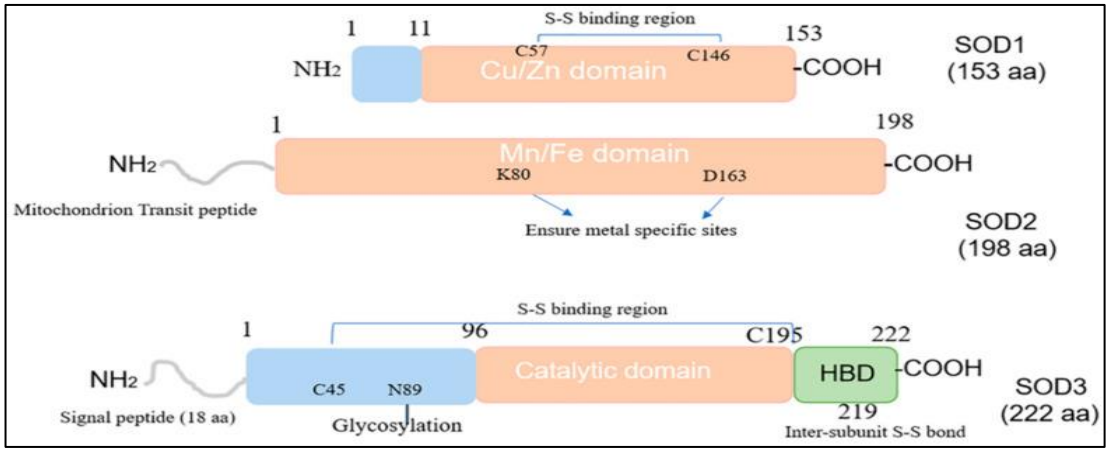

Figure (1-10) Composition of SOD1, SOD2, and SOD3. HBD: heparin-binding domain (Zheng *et al* ., 2023).

1.1.10. Rheumatoid Arthritis and Oxidants Anti-Oxidants Balance

Oxidant and antioxidant balance play a crucial role in RA (**Agina *et al*., 2021)**. Studies have shown increased ROS production and oxidative damage in RA patients, leading to an imbalance in oxidant-antioxidant levels **(Chaudhary *et al*., 2023).** This imbalance contributes to the pathophysiology of RA, with oxidative stress being a significant factor in the condition **(Singh *et al*., 2023).**

1.1.11. Lipid peroxidation in rheumatoid arthritis

Lipid peroxidation (LPO) occurs when reactive oxygen species attack phospholipids or PUFA, generating aldehydes, ketones, alkanes, carboxylic acids, and polymerization by-products. These compounds are reactivity indicators of LPO and are produced within cellular membranes (**Gianazza *et al*., 2021).** Oxidative stress and LPO are involved in rheumatoid arthritis (RA) development and pathogenesis, leading to the formation of electrophilic reactive lipid species and changes in the common lipoprotein profile (**Ferreira *et al*., 2021**). Changes in the lipoprotein profile in RA patients also contribute to cardiovascular diseases and a chronic inflammatory environment (**Greanious *et al*., 2021**). Lipid oxidation leads to the formation of unstable lipid hydroperoxides, which can break down to produce bioactive aldehydes such as 4-hydroxynonenal and malondialdehyde (MDA). These aldehydes can interact with proteins, causing alterations in their functionality **(Gęgotek and Skrzydlewska, 2019).**

1.2. Obesity and Rheumatoid Arthritis

Obesity represents a notable risk factor for RA due to its propensity to elevate the likelihood of enduring chronic systemic inflammation (**Johri *et al*., 2023**). Adipose tissue secretes adipokines, potentially influencing immune responses and inflammatory processes, increasing susceptibility to RA in individuals with an elevated body mass index **(Kadhim *et al*., 2023).** Increased BMI was associated

with an increased risk of RA, Understanding the association between BMI and RA might benefit the prevention or control of RA **(Feng *et al.,* 2019**). Obesity is an inflammatory disease, and studies have shown that women with high BMI have higher levels of the pro-inflammatory cytokines tumor necrosis factor and interleukin 6 **(Al-Yasiry *et al*., 2022**). Excessive production of various factors, such as TNFα, IL6, or IL1, which can enhance inflammatory responses commonly observed in various rheumatic diseases, has been associated with an accumulation of adipose tissue (**Alonso-Pérez *et al*., 2022)**.

1.2.1. Effect of RA Chemotherapeutic Agents on Lipid Profile

RA medications fall into two main categories with different purposes. Non-steroidal anti-inflammatory drugs (NSAIDs) aim to alleviate symptoms without preventing joint damage. Examples include aspirin and ibuprofen, while glucocorticoids like prednisolone are potent anti-inflammatory agents. Disease-modifying anti-rheumatic drugs (DMARDs), on the other hand, are meant to hinder joint deterioration and are further divided into non-biological (e.g., methotrexate), biological (e.g., TNF-α inhibitor, B-cell depleting), and targeted synthetic DMARDs (**Lin *et al.*, 2020**).

In patients with RA experiencing active disease, there is a tendency for reductions in Total Cholesterol (TC), low-density lipoprotein (LDL-C), and high-density lipoprotein levels (**Venetsanopoulou *et al*., 2020)**. This phenomenon, known as the [**lipid paradox**], contradicts the traditional belief that an atherogenic lipid profile comprises elevated TC, LDL-C, TG, and reduced HDL-C (**Myasoedova,2017**).

The development of lipid metabolomics has revealed changes in lipid small molecules and potential metabolic pathways, making lipid metabolism in RA patients and systemic changes after treatment more comprehensive **(Lei *et al*., 2023).**

alteration in the level of lipids is more closely related to CRP changes than those of DAS28 for RA, comprising clinical and laboratory data for evaluation of disease activity (**Behl *et al*., 2020**). The treatment of RA patients reduces the inflammatory process but increases TC, LDL-C, and decrease HDL-C, which is not associated with a rise in CV events (**Venetsanopoulou *et al.*, 2020)**.

1.3. Nanotechnology and Nanomedicine

Nanotechnology, an interdisciplinary field, focuses on the synthesis, management, and application of materials smaller than 100 nm **(Zahra *et al.*, 2020)**. Nano biotechnology linked to data science and cognitive abilities will create new opportunities in industries such as agriculture, environment, medicine, and economics **(Odda *et al*., 2022**). Nanotechnology is a broad field with applications in various scientific disciplines, including nanomedicine, focusing on disease diagnosis and treatment (**Domingues *et al*., 2022**). Nanotoxicology emphasizes studying the negative effects of nanomedicine, such as nanoparticles delivering chemotherapy drugs to cancer cells (**Ho *et al*., 2020.**Nanomedicine recognizes the site of human disease and delivers medications, diagnostics, and therapeutics to the target biological cell. Initially, the diagnosis was founded on the idea of cell theory, but research has progressed to the atomic and molecular levels **(Jackson *et al*. 2021**). The main objective of nanomaterials in delivery applications is to carry the desired molecules to their target sites while minimizing the side effects and maximizing their therapeutic effects. (**Sharma *et al*., 2019**).

1.3.1. Nanoparticle Structure

Nanoparticles (NPs), controlled or manipulated particles with higher surface area, exhibit unique chemical and physical properties, including optical, magnetic, electrical, thermal, and mechanical behaviour (**Talib *et al*., 2022**). NPs have a high surface area volume ratio, resulting in unique properties (**Khan *et al*., 2019)**. The

surface of an NP is crucial to the material, and even the simplest NP has a different surface chemistry from the core material (**Xie *et al*., 2019)**. Organic nanoparticles, like carbon NPs, are the first group, followed by inorganic ones like noble metal nanoparticles **(Li and Wang, 2020**).

Nanoparticles are produced through two main techniques: top to bottom (grinding, scraping, or laser) and bottom-to-top (sol-gel or aerosol) **(Srivastava *et al*., 2022*)***. Top to bottom involves reducing large materials to tiny parts, while bottom to top collects separated atoms and molecules. Chemical methods convert materials into nanoscale sizes but are characterized by high cost, toxicity, purification issues, and time consumption (**Abdul-Karim and Hussein,2022**).

1.3.2. Application of Nanoparticles in Medical Fields

1. *Drug Delivery*: Nanoparticles, such as polymeric nanoparticles and liposomes, are used for targeted drug delivery in cancer treatment, enhancing the release and bioavailability of chemotherapeutics **(Malik *et al*.,2023; Dobson *et al* ., 2024**).

2. *Imaging and Diagnostics*: Nanoparticles aid in detecting tumors and abnormalities in MRI and fluorescence imaging, while quantum dots provide detailed insights into biological processes in specific cell and tissue imaging **(Murthy and Shashik,2007; Dobson *et al*., 2024**).

3.*Gene Delivery*: Nanoparticles facilitate the delivery of genetic material into cells, which is crucial for gene therapy applications. They can protect nucleic acids from degradation and enhance cellular uptake, thereby improving the efficacy of gene-based treatments (**Harisinghani *et al*., 2003; Murthy and Shashik,2007**).

4. *Thermal Therapy*: Certain nanoparticles can be used for hyperthermia treatment, where they are targeted to tumor sites and heated using an external magnetic field. This localized heating can destroy cancer cells without harming surrounding healthy tissue (**Dobson et *al*., 2024**).

5. *Regenerative Medicine*: Nanoparticles are being explored for their potential in tissue engineering and regenerative medicine, as they can be incorporated into scaffolds for cell growth and tissue repair (**Dobson et *al.*, 2024**.

6. *Antimicrobial Applications*: Nanoparticles, such as silver nanoparticles, exhibit potent antimicrobial properties and are being integrated into coatings for medical devices to prevent infections (**Malik *et al*.,2023).**

1.3.3. Drug Delivery by Nanotechnology

The disadvantages of conventional medicines were less absorbed, distributed randomly, unaffected areas were damaged, taken out early, and took a long time to treat the disease **(Afzal *et al.*, 2022)**. They were less effective due to many hurdles like their enzymatic degradation or disparity in pH, many mucosal barriers, and off-the-mark effects, and their immediate release enhanced toxicity in the blood (**Alshammari *et al.*, 2023)**. This strategy has exhibited significant promise in effectively managing diseases while minimizing the associated toxic effects (**Hooda *et al.*, 2023).** Understanding nanoparticle interactions, administration methods, bioavailability, and distribution is crucial for effective drug delivery. Nanoparticles improve stability and solubility, making them efficient drug transporters **(Pandey *et al.*, 2020)**. They enhance medication safety and effectiveness, facilitating precise genetic material delivery to cells for protein synthesi (**Hamimed *et al* ., 2022**) .

1.3.4. Chrysin-Loaded Hyaluronic Acid-Coated Niosomal NP

Noisomes, composed of nonionic surfactants and cholesterol, enhance the stability and bioavailability of both hydrophilic and lipophilic pharmaceuticals. Their ability to be tailored for distinct drug release profiles renders them adaptable and biocompatible (**Muzzalupo and Mazzotta,2019; Obeid *et al.*, 2021).** The figure below shows the structure of the niosome molecule

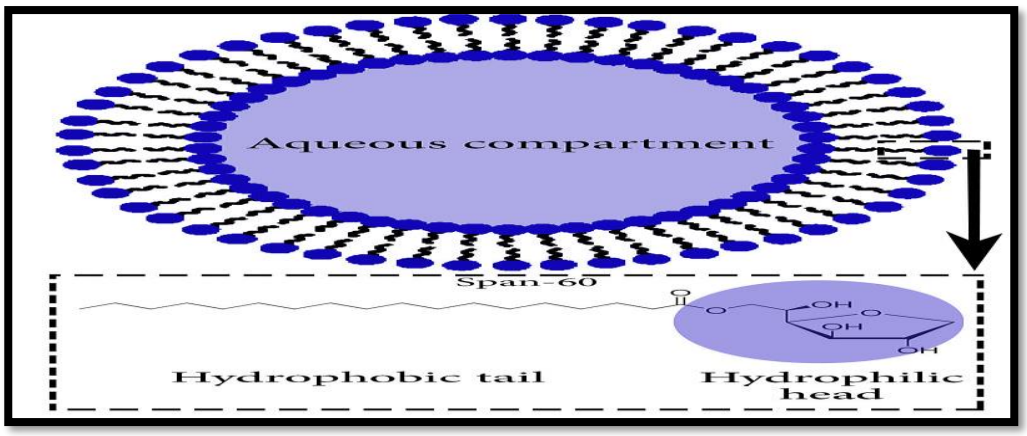

***Fig.* (1-11) Structure of niosomes (Moghassemi *et al* ., 2014)**

Niosomes offer stability, low toxicity, and compatibility with biological systems, making them ideal for drug delivery, entrapping and shielding drugs from the environment **(Shah *et al* ., 2021).** They enhance therapeutic performance and allow sustained drug delivery. Nio synthesis aims for controlled drug release with predictable particle sizes using various techniques **(Obeid *et al*., 2022).**

Hyaluronic acid possesses solubility, biocompatibility, and biodegradability, making it suitable for drug delivery systems (**Harrer *et al*., 2021**). It is a polysaccharide present in the body, contributing to the extracellular matrix in tissues. Its functions involve regulating growth factor interactions, maintaining tissue volume, and offering lubrication (**Elhassan *et al*., 2022**). Research indicates its potential in treating inflammatory conditions, providing pain relief, and demonstrating favorable biocompatibility and biodegradability (**Marinho *et al*., 2021**). The figure below shows the structure of the Hyaluronic acid

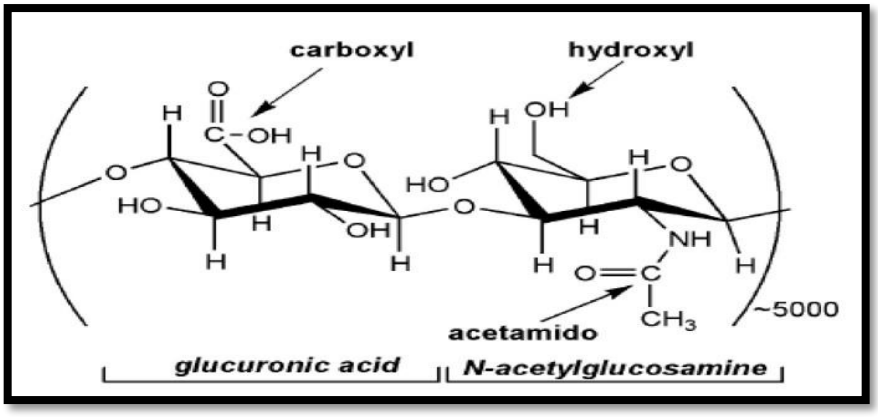

Fig. **(1-12) Structure of the Hyaluronic acid (Machado *et al* ., 2022)**

1.3.5. Peripheral Blood Mononuclear Cell (PBMCs)

PBMCs are composed of lymphocytes, and monocytes, and have a single nucleus. Lymphocytes are the most abundant, followed by monocytes and a small number of dendritic cells (**Zole *et al*., 2019**). They are less dense compared to red blood cells and granulocytes in peripheral circulation (**Karagiannis and Tanya,2021**). The cells can be isolated from whole blood using the ficoll method and gradient centrifugation, which separates blood into plasma, PBMCs, and other cells **(Gill and Prabjot,2019**).

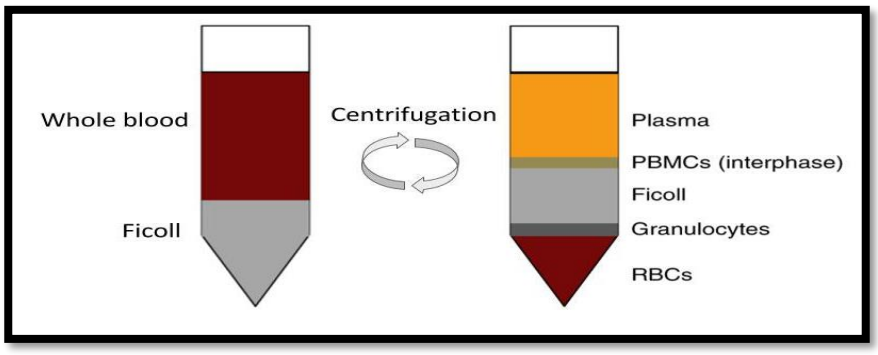

Fig. **(1-13) Schematic illustration of how to prepare the density gradient for isolation of PBMCs from blood (Jaatinen *et al* ., 2007)**

Peripheral Blood Mononuclear Cells are crucial in the immune system for fighting infections and pathogens, and are widely used in research and clinical

applications **(Sen *et al*., 2018).** PBMCs are crucial for drug toxicity analysis, assessing immune system toxicity, and determining dosage limits, making them essential for predictive studies **(Holley *et al*., 2021**). Scientists study normal and diseased PBMCs to understand disease pathways and molecules **(Gill and Prabjot,2019)**.

1.2. Genes

A gene is the basic physical and functional heredity unit passed from parents to offspring. Genes comprise DNA and contain the information needed to specify traits and produce proteins (**Cary *et al* ., 2020**).The exact definition of a gene has evolved: Initially, the term "gene" was coined to denote an abstract "unit of inheritance" without specific material attributes (**Gerstein *et al* ., 2007)** .

In the 1960s, a gene was defined as a continuous segment of DNA sequence specifying a polypeptide chain. However, the discovery of complex patterns of dispersed regulation, pervasive transcription, and non-coding RNAs challenged this notion (**Gerstein *et al* ., 2007** ; **Portin *et al*., 2017**) . A more modern definition is:"A gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products" This definition categorizes genes by their functional products (proteins or RNA) rather than specific DNA loci, with regulatory elements classified as gene -associated regions **(Kuhn and Harold, 2024).** Humans have 19,900 protein-coding genes, with less than 1% having alleles contributing to unique traits. Genes are DNA sequences for proteins or RNA molecules **(Gerstein *et al* ., 2007).**

1.2.1. Gene Expression

Gene expression refers to the process by which information from a gene is used to synthesize a functional gene product, typically proteins or functional RNA molecules **(Shao *et al*., 2019).** It's crucial in various biological processes like development and response to stimuli (**Oliva *et al.*, 2020)**. Factors like copy number variations, gender, and regulatory variants can affect gene expression (**Liu *et al.*, 2020**). Gene co-expression networks help understand gene functionality and regulatory pathways. Understanding gene expression dynamics is essential for understanding biological processes and illnesses (**Hajieghrari *et al* ., 2022**).

1.4.2. Roll of Human Matrix Metalloproteinase 9 Gene and Tissue Inhibitor Metalloproteinase1Gene in Rheumatoid Arthritis

The MMP9 proenzyme has five conserved domains **(Augoff *et al.*, 2022).** MMP9, or gelatinase B, is crucial for extracellular matrix degradation (**Dai *et al.*, 2022).** Elevated levels of MMP9 are linked to rheumatoid arthritis (RA) and inflammatory processes. It regulates inflammatory cytokines and cell migration in RA pathogenesis, identified as a hub gene in RA synovial macrophages (**Li *et al.*,2023; Jiang *et al.*, 2024)**. MMP9 is implicated in various pathological conditions, such as cancer and cardiovascular diseases (**Luo *et al*., 2024).** The TIMP1 gene encodes a glycoprotein that inhibits MMPs and is secreted by fibroblasts (**Nikolov *et al.*,2020)**. TIMP1 regulates growth and apoptosis across different cell types **(Almuntashir *et al*., 2023).** The TIMP family, comprising TIMP1–4, contains proteins of approximately 21 kDa with about 40% amino acid sequence identity **(Korzen *et al.*, 2023).**

1.4.3. Effect of Chrysin Nanoparticles on Gene Expression

Chrysin and flavonoids affect gene expression of MMP9 and TIPM1 **(Augoff *et al.*, 2022)**. Chrysin modulates toll-like receptors, while flavonoids target MMP9 **(Kumari *et al.*, 2023).** This suggests chrysin and flavonoids may modulate MMP9 and TIPM1 expression through immunological and antioxidant pathways **(Zheng *et al.*, 2023)**. Chrysin nanoparticles significantly impact gene expression in cancer cells **(Mohammadian *et al.*, 2016).** Chrysin within PLGA-PEG nanoparticles enhances anticancer efficacy by increasing miR-22, miR-34a, and miR-126 levels in gastric cancer cells. Similarly, Bagheri et al. (2018) reported that nano-encapsulated chrysin and curcumin inhibit hTERT expression in colorectal cancer cells. These findings underscore the potential of chrysin nanoparticles in modulating gene expression to impede cancer proliferation. Furthermore, TIMP1 is essential for regulating matrix metalloproteinases and cell growth **(Bagheri *et al.*, 2018).** TIMP1 overexpression is linked to anti-metastatic properties in breast cancer **(Hassan *et al.*, 2012**).

1.3. Aims of the Study

The current study aims to:

1. Evaluate the serum level of inflammatory marker TNF-α, anti-inflammatory marker IL-10, and the levels of oxidative and antioxidant markers with total antioxidant capacity in the serum of obese and non-obese rheumatoid arthritis patients and compare them with the control group and Study the relationship between TNF, IL-10, NO, SOD, and total antioxidant capacity in serum with other biochemical markers such as lipids and investigate the diagnostic preferences of the study markers using ROC analysis.
2. Formulation, characterization, and evaluation of the effect of H-Nio-chrysin nanoparticles to target peripheral blood cells *in vitro* derived from rheumatoid arthritis patients and evaluation of different agents for these cells and study their applications as anti-inflammatory and antioxidant agents.
3. Detection of gene expressions including MMP9 and TIMB1 using reverse transcriptase polymerase chain reaction in niosomes after treatment with chrysin nanoparticles *in vitro*.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1. Study Design and Ethical Approval

A case-control study was conducted in the Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala. The subjects included were classified as shown in Figure (2-1) and the ethical approval was confirmed by the Kerbala College of Medicine, Kerbala University, and Kerbala Health Directorate(Appendice).

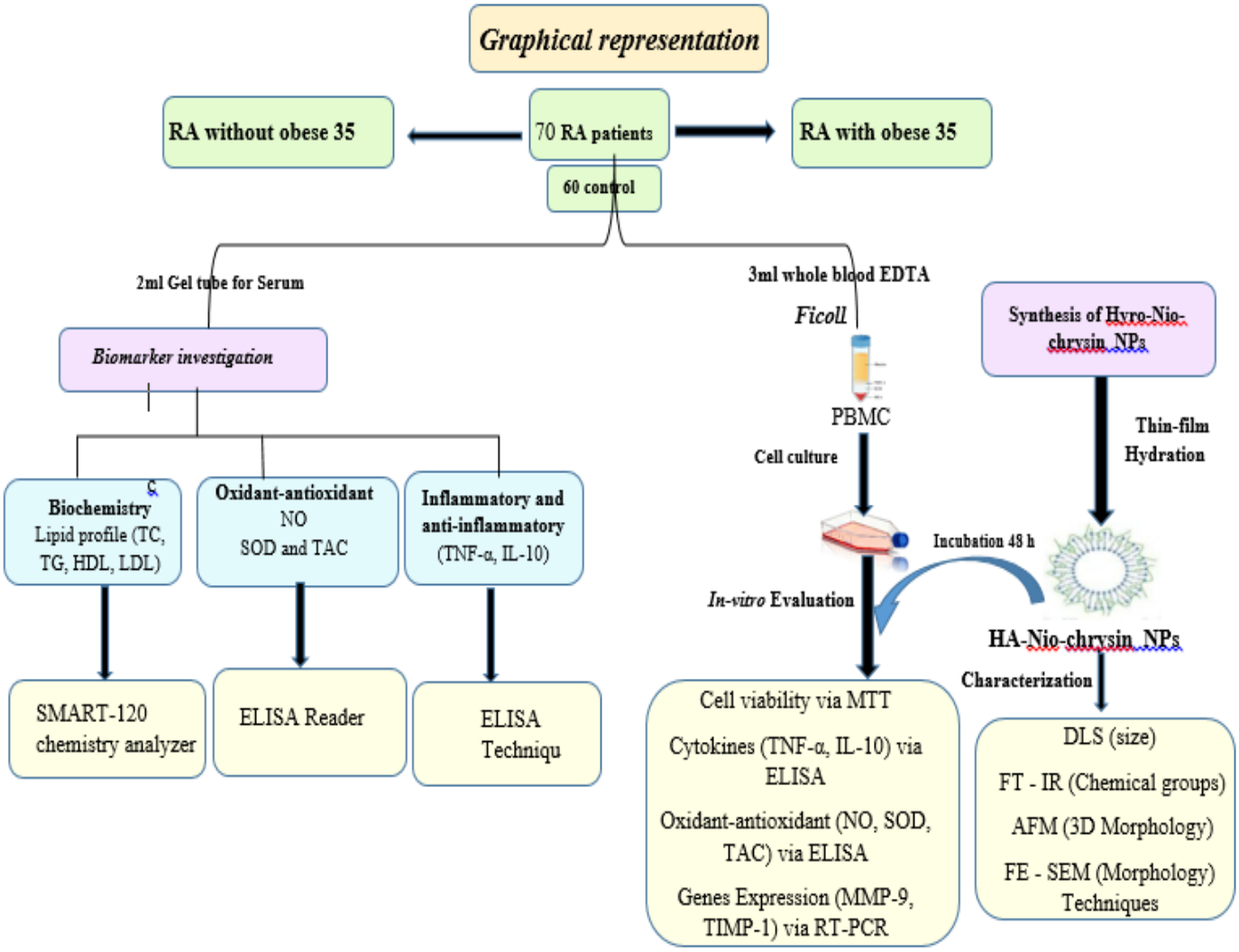

***Fig.* (2.1): Flow chart of study design.**

2.2. Subjects

2.2.1. Patients

This study included 70 female patients, and they were distributed into two groups. Their matched ages ranged between 35-65 years and patients were obtained from the Rheumatology Unit, Al-Hassan Teaching Hospital, Karbala Health Directorate; Marjan Teaching Hospital, Babylon Health Directorate - Iraq. Groups I and II each included 35 patients with non-obese RA and obese RA, respectively.

2.2.1.1. Patient Inclusion Criteria

The patient how was diagnosed with RA by a rheumatologist according to clinical examination and laboratory tests (anti-CCP, CRP, ESR, RF) to ensure inclusion in the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 (ACR/EULAR-2010) (**Aletaha D1 *et al*., 2010**). RA patients suffering from obesity were also selected according to the body mass index ≥30 kg/m2, and the ages of the patients ranged between (35-65) years. The severity of the disease was also determined by the rheumatologist by measuring DAS28. Cases whose DAS28 value was more than 3.2 were selected.

2.2.1.2. Patient Exclusion Criteria

Kidney disease, liver disease, cancer, and any acute or chronic inflammatory disease were excluded.

2.2.2. Control group

Sixty healthy females were recruited from hospital volunteers. Blood samples were collected, and no previous cases of rheumatoid arthritis or similar conditions were revealed. Participants were closely matched in BMI and age within the study

group. Demographic information was collected through self-reporting methodologies.

2.2.3. Blood Samples Collection

Five milliliters of fasting venous blood were obtained from each participant and they were divided into two distinct portions:

A. The first part included 2 ml of blood transferred into gel tubes, then separated by centrifuge for 10 minutes. The resulting serum was fractionated into 6 Eppendorf aliquots, and stored at −20°C until analysis to determine different biomarkers (TC, TG, LDL-C, HDL-C, TNF-α, IL-10, NO, SOD, FRAP) by SMART-120 analyzer and ELISA system.

B. Three ml of whole blood samples were used, after blood components were separated using Ficoll and peripheral blood cells were extracted, the structural properties of chrysin-loaded and hyaluronic acid-coated nanoparticles were investigated as anti-inflammatory and antioxidant agents and their effects on the study markers of peripheral blood cells of rheumatoid arthritis patients were demonstrated. The properties of the nanoparticles were measured using scanning electron microscopy (SEM), Fourier transform infrared (FTIR), AFM, MTT, and different DLS techniques. Then the effect of chrysin-loaded and hyaluronic acid-coated nanoparticles on the gene expression of different genes including MPP9 and TIMP1 was evaluated.

2.3. Materials

2.3.1 Chemicals and Diagnostic Kit

All chemicals and kits that were used in this study are listed in table (2-1)

Table (2-1): **Chemicals and diagnostic kits used with their origin.**

Chemicals and Diagnostic Kit	Company	Origin
DMSO	Merck	Germany
fetal bovine serum (FBS)	Biochrom	UK
HCL conc. (37%)	UKA	Germany
HDL-C Kit	AFLO	Germany
IL-10 ELISA kit	Karmania Pars Gene	Iran
MTT	Sigma	Germany
NO assay kit	Karmania Pars Gene	Iran
penicillin	Sigma	Germany
SOD activity assay kit	Karmania Pars Gene	Iran
streptomycin	Sigma	Germany
TAC assay activity	Karmania Pars Gene	Iran
TNF-α ELISA kit	Karmania Pars Gene	Iran
Total Cholesterol Kit	AFLO	Germany
Triglycerides Kit	AFLO	Germany

2.3.2. Instruments and Lab Equipment

Table (2-2): The instruments and laboratory tools used in this study were summarized in Table.

Table (2-2): **Instruments and Lab Equipment used in this study**

Apparatuses and Equipment	Company	Origin
Absorbance reader	Bio Tek	USA
Atomic force microscopy AFM,	Nanowizard II; JPK	Germany
Ccentrifuge	Hettich	Germany
Centrifugation over Histopaque	Sigma	Germany
Deep freezer	Cooltech	China
Dynamic light scattering system DLS	ZS 90, Malvern Instruments Ltd Malvern	UK
ELISA reader	Biotek	USA
FT- IR spectrophotometer	Shimadzu 8400 S Kyoto	Japan
Mechanical stirrer	Labtech	Korea
Micropipettes	Bioasic	Canada
Oven	Binder	USA
PH meter	Inolab	Germany
Refrigerator	Concord	Lebanon
Scanning electron microscopy SEM	PerkinElmer, Fremont, CA	USA
SMART-120 chemistry analyzer	AFLO	Germany
Spectrophotometry	Perkin Elmer, Fremont CA	USA
Ultra violet spectrophotometry	Perkin Elmer, Fremont CA	USA
Water bath	Memmert	Germany

2.4. Methods

2.4.1. Part A (Methods of Biochemical Studies)

2.4.1.1. Body Mass Index(BMI)

The BMI is a value derived from the mass (weight) and height of an individual. BMI was calculated by dividing the body weight (kg) by the square of height (m^2) according to the following equation **(Gray and Fujioka,1991):**

BMI, kg/ m^2 = Weight in kg /(Height in m)^2

Patients were classified into normal (18.5-24.9 kg/m^2), overweight (25.0 - 29.9 kg/m^2), and obese (≥ 30.0 kg/m^2) depending on reference ranges **(Mohseni, 2023).**

2.4.1.2. Determination of Lipid Profile in Rheumatoid Arthritis

2.4.1.2.1. Determination of Total Cholesterol Levels

A. Principle:

Cholesterol ester in the sample, under the existence of lipoprotein esterase, hydrolyzed into cholesterol and free fatty acid. Total cholesterol is oxidized by cholesterol oxidase to generate cholest-4-ene-3- ketone and hydrogen peroxide. The generated hydrogen peroxide, under the existence of peroxidase, reacts with hydroxybenzoic acid and 4-amino-antipyrine to produce H_2O and quinone imine pigments. The generated volume of quinone imine pigment is proportional to total cholesterol volume in the sample, by measuring the generated pigment volume at specific wavelength, total cholesterol concentration can be calculated **(Trinder, 1969; Bishop, 2020).** A schematic representation of the reaction is shown in following equations:

Cholesterol esterase

Cholesterol ester + H_2O ----------►Cholesterol + Free Fatty acids

Cholesterol oxidase

Cholesterol + O_2 ----------► Cholesterol-4-en-3-one + H_2O_2

Peroxidase

$2H_2O_2$ + phenol + 4-amino-antipyrine ----------►coloured complex +$4H_2O$

The quantity of the pink dye quinonimine is proportional to the total cholesterol concentration. The absorbance of quinonimine was measured at a 500 nm wavelength.

B. Reagents

Table 2-3: Reagents used for total cholesterol assay.

Reagent A (100 ml)	**Reagents**	**Concentration**
	4-AAP	1mmol/l
	CHE	300 U/l
	CHOD	300 U/l
	POD	1500 U/l
	Derivative of phenol	1mmol/l
Standard (5 ml)	Cholesterol	200 mg/dl

4-AAP, 4-aminoantipyrine; CHE, cholesterol esterase; CHOD, cholesterol oxidase; POD, peroxidase.

C. Procedure of Total Cholesterol Determination

Concentrations of total cholesterol were measured by using a SMART-120 chemistry analyzer and the procedure for blank, standard, and sample measurement is demonstrated. The mixture was prepared and incubated at 37°C for 5 minutes.

The sample-to-reagent ratio was 1:100, and the absorbance of the sample (Ax) and the standard (As) were read against a blank reagent at 510 nm.

Tale 2-4: The procedure of TC assessment.

Pipette	Blank (µl)	Sample (µl)	Standard (µl)
Reagent (A)	**1000**	**1000**	**1000**
Water	**10**	**-**	**-**
Sample	**-**	**10**	**-**
Standard	**-**	**-**	**10**

D. Calculation:

$$\text{Cholesterol mg/dL} = \frac{Ax}{As} \times \text{standard conc. (200) mg/dL}$$

Reference range: 150-220mg/dL.

2.4.1.2.2. Determination of Serum Triglycerides Concentration

A. Principle:

Methods for triglyceride determination generally involve enzymatic hydrolysis of triglycerides to glycerol and free fatty acids followed by enzymatic measurement of the glycerol released (**Kaplan *et al.*, 1989**). The glycerol participates in a series of coupled enzymatic reactions, in which glycerol kinase (GK) and glycerol phosphate oxidase (GPO) are involved, and hydrogen peroxide (H_2O_2) is generated. Produced H_2O_2 reacts with TOOS and 4-AAP to form a colored complex, whose absorbance is directly proportional to the concentration of triglycerides in the sample (**Fossati and Prencipe , 1982**).

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol} + \text{Free fatty acids}$$

GK

Glycerol + ATP ----------► Glycerol-3-phosphate + ADP

GPO

Glycerol-3-phosphate + O_2 ----------► Dihydroxyacetone phosphate + H_2O_2

POD

H_2O_2 + 4-AAP + TOOS ----------► colored complex + H_2O

B. Reagents

***Table* 2-5: Reagents used for TG assay.**

Reagent A (100 ml)	**Reagent**	**Concentration**	
	Magnesium chloride	15 mmol/l	
	ATP	4 mmol/l	
	4-AAP	1 mmol/l	
	TOOS	0.1 mmol/l	
	LPL	2500 U/I	
	POD	1800 U/I	
	G**K**	1000 U/I	
	GPO	5500 U/I	
Standard (10 ml)	Glycerol	200 mg/dl	

ATP, adenosine triphosphate; 4-AAP, 4-amino antipyrine; LPL, lipoprotein lipase; POD, peroxidase; GK, glycerol kinase; GPO, glycerol phosphate oxidase.

C. Procedure

Concentrations of triglycerides were measured by using a SMART120 chemistry analyzer and the procedure for blank, standard, and sample measurement. The mixture was prepared and incubated at 37°C for 5 minutes. The

sample-to-reagent ratio was 1:100, and the absorbance of the sample (Ax) and the standard (As) were read against a blank reagent at 510 nm.

***Table* 2-6: The procedure of of TG assessment.**

Pipette	Blank (µL)	Sample (µL)	Standard (µL)
Reagent (A)	1000 µL	1000 µL	1000 µL
Water	10 µL	-	-
Sample	-	10 µL	-
Standard	-	-	10 µL

D. Calculation:

$$\text{Triglyceride (mg/dL)} = \frac{A_x}{A_s} \times \text{Standard conc. 200 mg/dL}$$

Female normal range: 35- 135mg / dL

2.4.1.2.3. Determination of HDL-Cholesterol Levels

A. Principle:

Low-density lipoprotein, very low-density lipoprotein, and chylomicron fractions were precipitated quantitatively by the addition of phosphotungstic acid which contains magnesium chloride at pH 6.2. After centrifugation of the supernatant containing HDL fraction, cholesterol concentration was measured in HDL fraction which is in turn determined by using a cholesterol kit and represented as HDL-C **(Trinder, 1969**).

A schematic representation of the reaction is shown in the following equations:

HDL-C + LDL-C + VLDL-C + Chylomicron (CM) → HDL-C + (LDL-C +VLDL-C +CM)

CHE, CHOD

HDL-C → Fatty acids $+H_2O_2$

H_2O_2 + 4-AAP + HDAOS → colored complex $+H_2O$

B. Reagents

Table 2-7: Reagents used for high-density lipoprotein cholesterol assay.

Reagent A (90 ml)	Reagents	Concentration
	Polyanions	1 mmol/l
	4-AAP	4 mmol/l
Reagent B (30 ml)	CHE	800 U/l
	CHOD	500 U/l
	Peroxidase	1500 U/l
	HDAOS	1 mmol/l
	Detergent	4 mmol/l

4-AAP, 4-aminoantipyrine; CHE, cholesterol esterase; CHOD, cholesterol oxidase.

C.Procedure

The mixture was prepared and incubated at 37 °C for 5 minutes, and the absorbance of the blank sample (Abx) was read against a blank reagent at 600 nm. In the next step, reagent B was added, mixed, and incubated at
37°C for 5 minutes and the absorbance of the sample (Ax) and the standard (As) were read against a blank reagent.

***Table 2-8*: The procedure of high-density lipoprotein cholesterol assessment.**

Pipette	Blank (µL)	Sample (µL)	Standard (µL)
Reagent (A)	300 µL	300 µL	300 µL
Water	4 µL	-	-
Sample	-	4 µL	-
Standard	-	-	4 µL
Reagent (B)	100 µL	100 µL	100 µL

D.Calculation:

$$\text{HDL-Cholesterol (mg/dL)} = \frac{Ax}{As} \times \text{standard conc (40mg).}$$

Female 48.762- 73.143mg / dL

2.4.1.2.4. Determination of LDL-Cholesterol Levels

A. Principle

LDL-C was calculated by the Friedwald formula since 1972 which had postulated a formula to calculate LDL-C value (**Friedewald *et al*., 1972)**

LDL-C (mg/dl) = Total Cholesterol – HDL-C – (Triglycerides/5)

When TG is less than 5 mmol/l (250 mg/dL).

2.4.1.3. Enzyme-Linked Immunosorbent Assays

2.4.1.3.1. Human TNF-α ELISA Test

A. Principle:

This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to HumanTNF-α. Samples (or Standards) are added to the micro-ELISA plate wells and combined with the specific antibody. The detection antibodies specific for Human TNF-α and Avidin Horseradish Peroxidase (HRP) conjugate are added successfully to each microplate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human TNF-α, detection antibody, and Avidin-HRP conjugate will appear blue. The enzyme-substrate reaction is terminated by the addition of a stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human TNF – α. You can calculate the concentration of Human TNF-α in the samples by comparing the OD of the samples to the standard curve.

B. Components of the kit used:

***Table (2-9)*: components of TNF-α kit**

Item	Catalogue Number	Volume
Human TNF-α coated plate	KPG-TNF-αP	96 vials
Standards	KPG-TNF-αSN1-4	200 µL
HRP-Avidin buffer	KPG-HA	5.5 mL
HRP(horseradish peroxidase)	KPG-HAA	540 µL
Substrate	KPG-SU	5.5 mL
Stopping	KPG-ST	3.5 mL
10X washing buffer	KPG-WB	40 mL
Detection Ab	KPG-TNFD	5.5mL

C. Reagent preparation

1. All reagents were brought to room temperature (18-25°C) before use.

2. Wash Buffer: The washing solution was prepared by diluting it with distilled water 10 times. 40 mL of concentrated washing solution was diluted with 360 ml of distilled water and 400 mL of washing solution was prepared.

3. Standard working solution: The available standards in the ready-to-use kit are as described in the following table:

Table (2.10): **Conc. of standards TNF -α**

OD	pg/ml	Standards
2- 1.8	200 pg/mL	S1
1.4-0.9	100 pg/mL	S2
0.6-0.3	50 pg /mL	S3
0.08-0.1	0 pg/mL	S4

4. HRP –Avidin buffer solution:

This solution was prepared in HRP spinning using a microfuge device. Then all its contents were added to the HRP-Avidin buffer vial

D. Procedure of TNF-α Test

1. Removed the plate from the foil pouch and the blank, standard, and sample wells were marked.

2. Fifty µL were added as standard (S1, S2, S3, and S4) and concentrations were (200, 100, 50, and 5 pg/mL) respectively to the first to fourth wells.

3. Fifty µL of the samples were added to the rest of the well and incubated for 60 minutes on a 200 RPM shaker at room temperature.

4. The ELISA plate wells were washed 3 times using the washing solution, incubated the plates for approximately 1 minute at room temperature and then drained.

5. Fifty µLof the conjugated antibody was added to all wells and incubated for 60 minutes on a 200 RPM shaker at 37 °C.

6. The ELISA plate wells were washed 3 times using the washing solution.

7. The HRP-Avidin solution was added 50 µL to all wells and incubated for 30 minutes on a shaker at least at RPM 200.

8. The ELISA plate wells were washed 5 times using the washing solution.

9. All wells received 50µL of substrate to all walls and incubated the plate at 37 °C for 15 minutes.

10. Stop Solution was added 25µL to all wells and the color of the wells changed from blue to yellow.

11. Absorbance was read at 450 nm using an ELISA reader.

E. Calculation of results

Make the concentration of the standards abscissa and the OD value the abscissa. The standard curve is plotted on the coordinate sheet. According to the OD value of the sample, its corresponding concentration (which is the sample concentration) is then determined; Or calculate the linear regression equation of the standard curve according to the standard concentration and OD value. Then replace the sample's OD value to calculate its concentration.

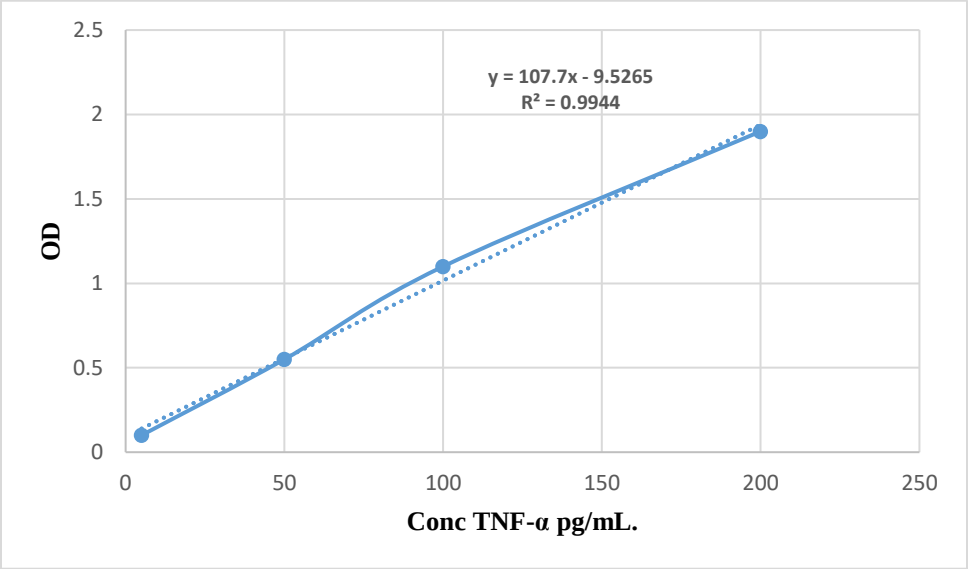

***Fig.* (2-2): Standard curve of TNF-α pg/mL**

2.4.1.3.2 Human IL-10 Level Determination

***** Principle, procedure, and Calculation of Results are mentioned in Human TNF-α ELISA Test section 2.4.1.3.1.

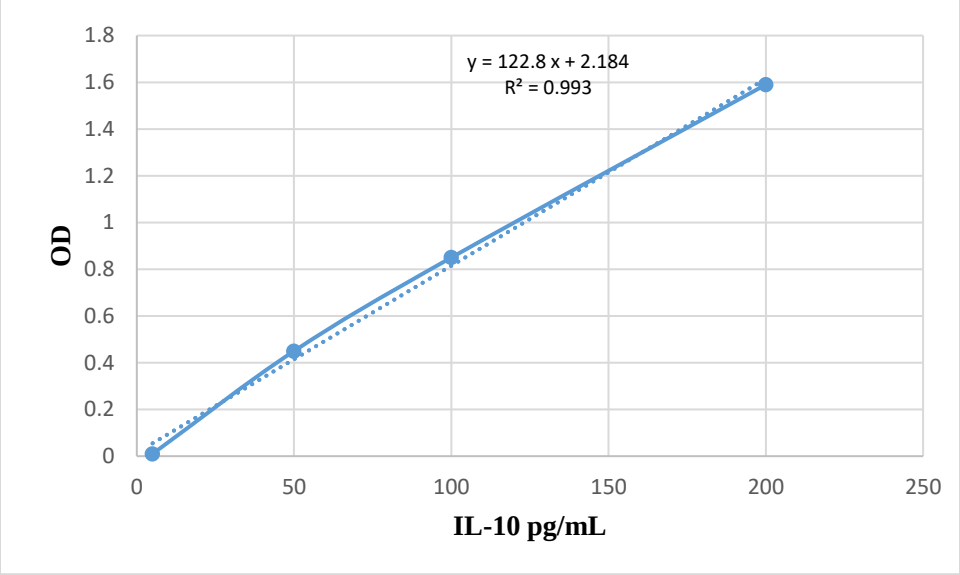

***Fig.* (2-3): Standard curve of IL-10 pg/mL**

2.4.1.3.3. Nitric oxide (NO) Assay

A. Principle:

Nitric Oxide was determined by the ELISA kit is designed for the specific detection of nitric oxide in biological samples. NO ELISA kits utilize plates coated with specific antibodies and conjugated detection enzymes. The assay will produce a detectable signal proportional to the amount of NO antigens found in the sample.

B. Components of the kit used:

***Table* (2-11): Reagents used for NO assay**

Item	Catalogue number
Solution A	CN.KPG-NO a
Solution B	CN.KPG-NO b
Solution C	CN.KPG-NO c
Solution D	CN.KPG-NO d
Solution E	CN.KPG-NO e

C. Reagent preparation:

1. All reagents were brought to room temperature (18-25°C) before use

2. **Preparation of solutions:**

*** Solution A** has been completely added to **Solution B.**

***Solution D** was prepared by adding 1 mL of distilled water to the vial and mixing it well.

3. Preparation of standard solution:

A standard solution was prepared. Mix D and C in a 1/1 ratio. Add 100 microliters of solution D and 100 microliters of solution C.

4. Preparation of standard solution with dilutions:

To prepare a standard with dilutions at concentrations of 100, 50, 25, 12.5, and 6.25 Micromole added 30, 15, 7.5, 3.75, and 1.85 µL of the preparation.

The mixture was transferred to standard microtubules 1 to 5, separately.

D. Procedure of NO:

1. One hundred microliters of the mixture of solutions A and B were added to all components microtubes, including standard, blank, and sample (as per the table below).
2. Thirty microliters of sample were added to the corresponding small tube samples and 30 µL of distilled water (DW) was added to the blank as shown in the table below.
3. Micro tubes were mixed vigorously and incubated for 10 minutes at room temperature in the dark.
4. One hundred fifty µL of solution E was added to all the microtubes and mixed.
5. One hundred fifty µL of standards, blanks, and samples were added to the ELISA wells and immediately read at 450 nm.

***Table* (2-12): procedure of NO**

	S1	S2	S3	S4	S5	Sample	DW
A and B mix	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
Prepared standard	30 µL	15 µL	7.5 µL	3.75µL	1.85 µL	-	-
Serum	-	-	-	-	-	30 µL	
Blank	-	-	-	-	-	-	30 µL

E. Calculation of results

Make the concentration of the standards abscissa and the OD value the abscissa. The standard curve is plotted on the coordinate sheet. According to the OD value of the sample, its corresponding concentration (which is the sample concentration) is then determined; Or calculate the linear regression equation of the standard curve according to the standard concentration and OD value. Then replace the sample's OD value to calculate its concentration.

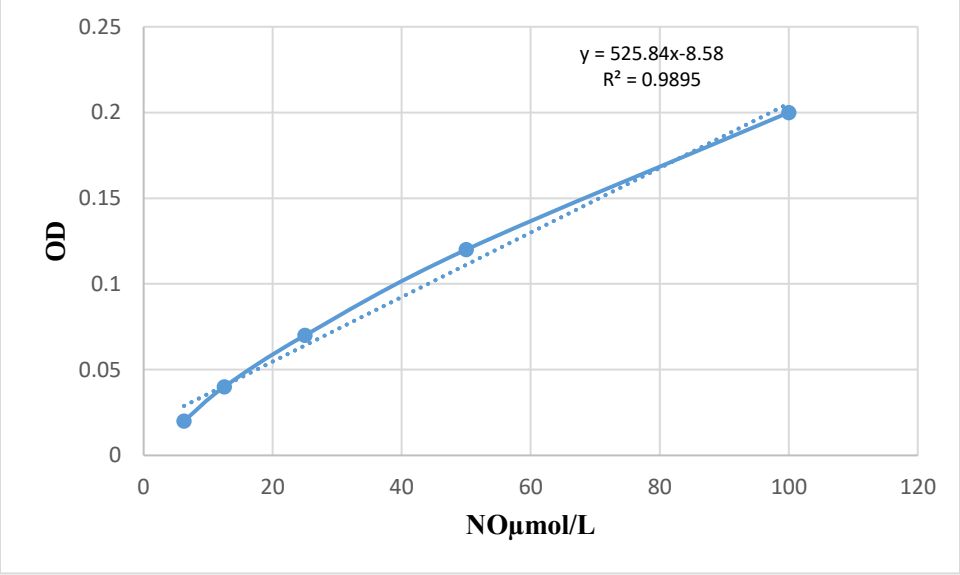

***Fig.* (2-4): Standard curve of NO µ mol /L**

2.4.1.3.4. Determination of Super Oxide Dismutase Activity Assay

A. Principle:

In the biochemical method, xanthine-xanthine oxidase is used to generate O_2•− and nitroblue tetrazolium (NBT) reduction is used as an indicator of O_2•− production. SOD will compete with NBT for O_2•−; the percent inhibition of NBT reduction is a measure of the amount of SOD present (**Weydert *et al*., 2010**).

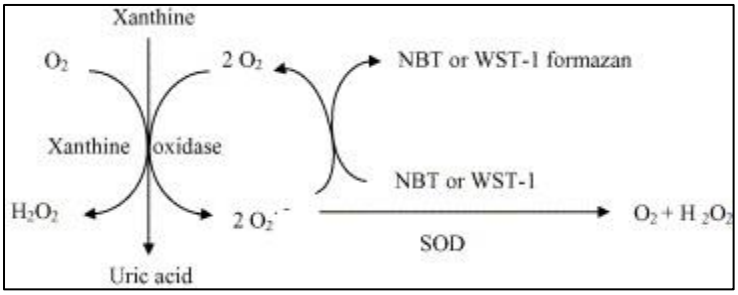

Figure (2-5) Principle of SOD activity (**Zhou *et al* ., 2006**)

B. Components of the kit used:

***Table (2-13)*: Reagents used for SOD activity**

Item	Catalog number
Solution A SOD enzyme	CN.KPG-SOD a
Solution B SOD buffer	CN.KPG-SOD b

C. Reagent preparation:

To prepare both solution A and solution B

- **Solution A** was dissolved in 300 mL of distilled water and mixed well.

- S**olution B** was prepared by dissolving 50 microliters of concentrated HCl (37%) in 15 mL of deionized distilled water, then all components of solution B were added to prepare this solution and mixed well.

D. Procedure of SOD Activity Determination

1. The plate, all reagents, and samples were left at room temperature for 20 minutes before starting the test.
2. Ten microliters of each sample were added with distilled water as a control to the ELISA plate.
3. Solution A was added 100 µL to all wells and immediately measured at 420 nm wavelength (A1).
4. Solution B was added 120 µL to all wells incubated for 7 minutes and then measured at 450 nm wavelength (A2).

E. Calculation of results:

The following formula was used to calculate SOD activity:

SOD Activity (U / mL) = [ΔA Test / ΔA CTD / 3] x [100 × 0.23 / 50] %

The meaning of **ΔA** Test is the difference between primary and secondary absorption in the examined samples. **ΔA of CTD** means the difference between primary and secondary absorption in the control.

2.4.1.3.5. Determination of Serum Antioxidant Status by FRAP Assay

A. Principle:

The FRAP assay is based on the reduction of ferric ions in an acidic medium, which leads to the formation of a colored ferrous-tripyridyltriazine complex. The intensity of the color produced is directly proportional to the antioxidant capacity of the sample being tested. The absorbance is typically measured at 593 nm **(Kubong, *et al.* 2020).**

B. Components of the kit used:

***Table (2-14)*: Reagents used for FRAP assay**

Item	Catalogue number
Solution A	CN KPG-Sa
Solution B	CN KPG-Sb
Solution C	CN KPG-Sc
Solution D	CN KPG-Sd
Solution E	CN KPG-Sd
Standard	CN KPG-STd FRAP

C. Reagent preparation:

2- Preparation of solutions:

- **Solution A** added 2.5 mL of solution E to the bottle of solution A and mixed them well.

- **Solution B** It was prepared 2.5 mL of DDW was added to the solution B bottle and mixed well.

- **Solution C** was also prepared by adding 15 mL of solution D to the solution C bottle and mixing it well.

3- Preparation of working solution:

Solutions A, B, and C were mixed immediately before testing in a 1/10 ratio to create a working solution for example each of two samples [300 µL, 25 µL, A, 25 µL B, and 250 µL C] were prepared. 19.5 mL for 130 samples (19500 µL) of working solution were prepared as follows: 1.95 mL (1950 µL) of solution A, 1.95 mL (1950 µL) of solution B and 15.6 mL (15.600 µL) of Solution C. The mixture

was stable for only 30 minutes and the solution was prepared immediately before testing.

4- Standard working solution:

Twenty-five mL of DDW was added to the standard bottle and mixed well where a 10 mM of Fe^{2+} solution was obtained. Then 2 mL of the standard containing 10 mM of Fe^{2+} was mixed with 8 mL of distilled water to create a 2 mM of Fe^{2+} standard. Then performed serial dilutions using the table below to create standards 1 through 6 using a 2 mM solution, as shown in Table (2-15) and

Table (2-15): **Serial dilutions of standard**

Std	**Concentrations Fe^{2+}**	**H_2O**	**Fe^{2+} , 2 mm**
1	1 mM (1000 µM)	500 µL	500 µL
2	0.7 mM (700 µM)	650 µL	350 µL
3	0.5 mM (500 µM)	750 µL	250 µL
4	0.3 mM (300 µM)	850 µL	150 µL
5	0.2mM(200µM)	900 µL	100 µL
6	0.1Mm(100µM)	950 µL	50 µL

D. Procedure:

1. Ten microliters of sample, standards, and distilled water were added respectively to the wells of the ELISA plate corresponding to the sample, standards, and blank.

2. One hundred forty µL of the working solution (A, B, C) was added to all wells and mixed for 30 seconds.

3. The plate was incubated at 37°C for 5 minutes and the OD of the samples with standards and blank was measured at a wavelength of 593 nm.

E. Calculation of results

The abscissa was designated for the concentration of standards, while the OD value was assigned to the ordinate. A standard curve was then plotted on the coordinate plane. The position of the sample concentration was identified based on its OD value, or the linear regression equation for the standard curve was determined using the standard concentration and absorption value. Subsequently, the concentration of the sample was calculated by substituting its OD value into the equation.

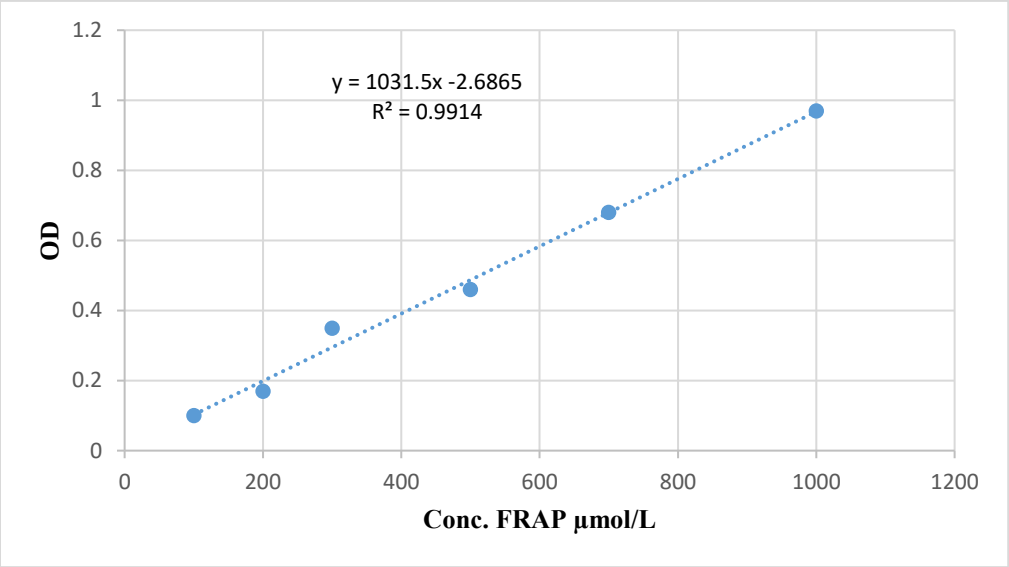

Fig. (2-6): **Standard curve of FRAP µmol /L**

2.4.2. Part II (Synthesis and Characterization of Nanoparticles)

2.4.2.1. Peripheral Blood Mononuclear Cells Isolation and Culture

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Histopaque density gradients (ficoll), washed three times with phosphate-buffered saline PBS, pH 7.4(1 liter of PBS with a pH of 7.4 was prepared by dissolving each of the following components in distilled water:

8 grams of sodium chloride

0.2 grams of potassium chloride

1.44 grams of sodium phosphate dibasic

0.245 grams of potassium phosphate monobasic

The pH was adjusted using hydrochloric acid (HCl) and the volume was completed with distilled water to reach 1 liter), and re-suspended (1×10^6 cells/mL) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 U/mLof penicillin, and 10 µg/mL of streptomycin at 37 °C with 5% CO_2.

2.4.2.2. Synthesis of Niosome Nanoparticles

Span-60 (36 mg) and cholesterol (6 mg) were dissolved in chloroform (3 mL) and methanol (6 mL) and evaporated with a rotary evaporator at 120 RPM at 60 °C for 1 hour to synthesize blank niosomal nanoparticles (blank Nio NPs). After the formation of the lipid-formed film, the temperature of the mixture was cooled to 24 °C. The thin film was hydrated with 10 mL of PBS for 1 hour at 60 °C like the above. The final solution was mixed thoroughly by ultrasonication over an ice bath for 30 min to reduce the size of the synthesized nanoparticles and stored at 4 °C for future use.

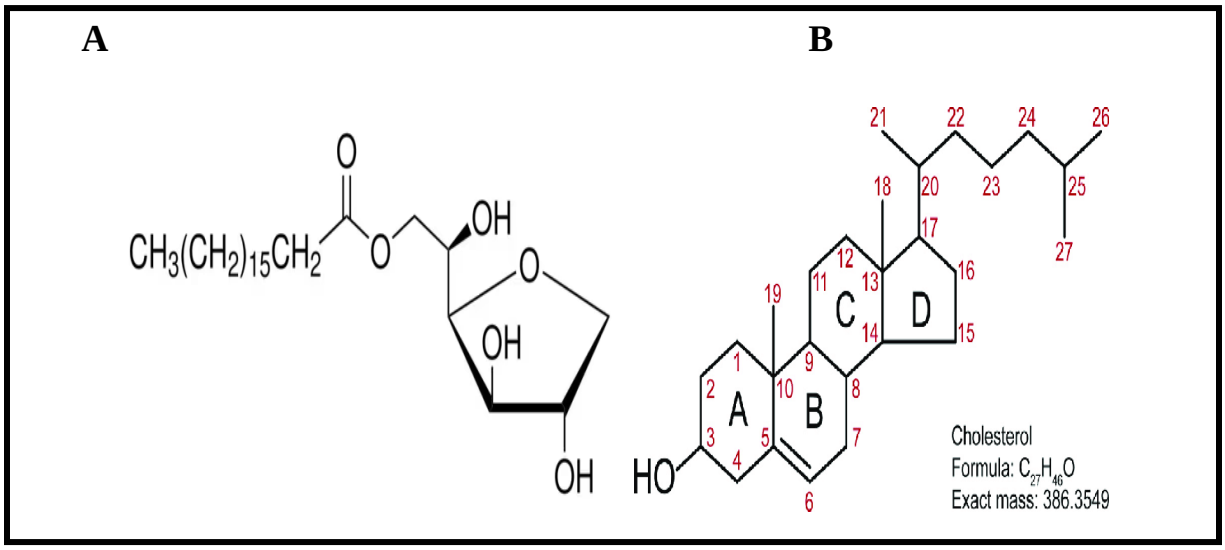

Fig. (2.7): **A. span 60 B. cholesterol**

2.4.2.3. Synthesis of Chrysin Loaded Niosomal Nanoparticles

The chrysin-loaded niosomal NPs (Nio-chrysin NPs) were synthesized with the same method as above with the addition of 2.54 mg chrysin to chloroform and methanol along with span 60 and cholesterol.

2.4.2.4. Synthesis of Chrysin Loaded Hyaluronic Acid Coated with Niosomal Nanoparticles

To synthesize chrysin-loaded hyaluronic acid-coated niosomal NPs (H-Nio-chrysin NPs), 10 mL of normal saline containing 0.1% (w/v) hyaluronic acid solution was added dropwise to blank Nio-chr NPs, while the mixtures were stirring at ambient temperature for one hour to reform the NPs and coating the hyaluronic acid onto the NPs surface

2.4.2.5. Physiochemical Properties of Synthesize Nanoparticles

The size, polydispersity index (PDI), and zeta potential of the synthesized niosomal NPs were analyzed by Zeta sizer dynamic light scattering system DLS. The surface morphological properties of the synthesized niosomal NPs were recorded with scanning electron microscopy (SEM). Spectral analysis of the compounds before and after NP preparation was analyzed by using an FT-IR spectrophotometer in the region of 4000-400 cm^{1} with a spectra resolution of 4 cm^{-}

2.4.2.6. Chrysin Release from Niosomal Nanoparticles

To determine the *in vitro* drug release profile of niosomal NPs, a dialysis membrane tube (12 kDa) was used. Briefly, 10 mL of H- Nio-chrysin NPs was transferred into a dialysis bag and placed in phosphate buffer saline (PBS) (pH = 7.4) at 37 °C with gentle shaking at 100 RPM. At specific time intervals, 5 mL of immersing buffer solution was analyzed with ultraviolet spectrophotometry and

replaced with fresh PBS. The absorbance of the immersed chrysin was measured at the maximum wavelength of chrysin which is 348 nm (λ_{max} of chrysin).

2.4.2.7. Peripheral Blood Mononuclear Cells Proliferation

The effect of varying doses of free chrysin, blank Nio NPs, Nio-chrysin NPs, and H- Nio-chrysin NPs on the viability of PBMCs was determined by performing an MTT reduction assay. An MTT assay is a colorimetric assay that detects the color change from yellow of the tetrazolium dye to purple due to the formation of formazan in the presence of viable cells with active metabolism (**Oh *et al.*, 2022**). An MTT reduction assay determined the effect of various doses of free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs on the viability of PBMCs.

1.Firstly, 5×10^3 cells were seeded in each well of 96-well plates and incubated for 24 hours at 37 °C with 5% CO_2.

2.The cells were treated with free chrysin (2.5-20 μM), Nio-chrysin NPs (2.5-20 μM), and H- Nio-chrysin NPs (2.5-20 μM) at 37 °C with 5% CO_2.

3.After 48 hours, the medium containing treatment substances was replaced with 200 μL of MTT solution and incubated for 4 hours at 37 °C and in dark conditions.

4.The MTT solution was excluded from wells, and 200 μL of dimethyl sulfoxide (DMSO) which is a chemical that dissolves many organic and inorganic substances was added to each well, followed by shaking on a plate shaker for 20 minutes.

5.The optical density of wells was measured at 570 nm using Microplate Absorbance Reader, and the cell viability effects of free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs were calculated using GraphPad Prism 8.4 software.

2.4.2.8. Anti-inflammatory and Pro-Inflammatory Cytokine Measurement

1. Peripheral blood mononuclear cells (1×10^5) were seeded in a 6-well plate and incubated for 24 at 37 °C with 5% CO_2 to attach the plates.

2. The PBMCs cells were treated with IC50 concentration of pure chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs for 48 hours at 37 °C with 5% CO_2. A group of cells remained untreated as a control.

3. The TNF-α and IL-10 levels in treated and untreated PBMCs were measured by an enzyme immunoassay using the human ELISA Kit

2.4.2.9. Nitric oxide Estimation

The concentration of NO in treated and untreated PBMC supernatant was determined using the measurement of residual nitrites by Griess's method. PBMCs were seeded in 6-well plates (1×10^5 cells), then incubated for 24 hours and treated with free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs for 48 hours at 37 °C with 5% CO_2. Afterward, 100 µLof supernatants of PBMCs culture were incubated with the same amount of Griess reagent for 20 minutes at 24 °C in darkness. The absorbance at 450 nm was determined with a microplate absorbance reader, and the concentration of nitrite was calculated from a standard sodium nitrite ($NaNO_2$) standard curve.

2.4.2.10. Determination of TAC, SOD

Total antioxidant capacity (FRAP)and SOD levels in both treated and untreated peripheral blood mononuclear cells were performed using the methodologies outlined by Erel (**Erel, *et al* .2004**), Marklund **(Marklund, *et al.*1974)**, and Flohe Gunzle (**Gunzler, *et al* . 2018**), respectively.

2.4.3. Part III (Method of gene expression)

2.4.3.1. Real-Time Polymerase Cain Reaction

Quantitative PCR analysis was conducted utilizing a LightCycler instrument. The amplification protocol involved an initial denaturation step at 95.8 °C for 10 minutes, followed by 40 cycles with the following conditions for the detection of MMP9 and TIMP1, 95.8 °C for 5 seconds, primer annealing at 58.8 °C for 10 seconds, and primer extension at 72.8 °C for 20 seconds. Also, GAPDH expression was selected to normalize the expression levels of the intended mRNAs. Table (2 - 16) lists the primer sequences for quantitative PCR. Fluorescence emitted by SYBR Green I was detected after each amplification cycle to assess the accumulation of PCR products throughout the cycling process. Following each run, melting curve profiles were generated to validate the specificity of transcript amplification. The monitoring and quantification of fluorescence emission readings from cycle to cycle were conducted utilizing the second derivative maximum method through Light-Cycler Software. Standard curves for GAPDH and other primers were established by serially diluting total complementary cDNA. All determined concentrations are expressed relative to the concentration of the respective standards.

***Table (2-16)*: Primer sequence utilized for quantitative PCR**

Gene	Forward Primer	Reverse Primer
MMP9	CCACTACTGTGCCTTTGAGTCC	AGAGAATCGCCAGTACTTCCC
TIMP1	CCTTCTGCAATTCCGACCTC	CATCTTGATCTCATAACGCTGGT
GAPDH	ATCCTGGGCTACACTGAGCAC	CCTGTTGCTGTAGCCAAATTCGT

2.4.3.2. RNA extraction

RNase-free microtubes, tubes, and falcons were used for RNA extraction. First, the flask cells were separated by trypsin and counted. About $1*10^6$ cells along with 2 mL of RPMI medium containing 10% FBS were transferred to each well of a 6-well plate and the plate was incubated for 24 hours at 37°C and 5% carbon dioxide for the cells to adhere to the wells. After 24 hours, the medium of the wells was emptied and each well was washed twice with PBS. Then the plates were incubated for 48 hours in an incubator at 37°C and 5% carbon dioxide. After 48 hours, the contents of the wells were emptied and 500 microliters of Trizol was added to each well. After 10 minutes, the trizol was collected from the wells and transferred to a microtube, and 200 µL of chloroform was added to each microtube. Next, the microtubes were transferred to a refrigerated centrifuge and centrifuged for 10 minutes at 12,000 rpm and 4°C. After centrifugation, 3 phases were formed inside the microtubes: the upper phase (transparent color) containing RNA, the middle phase (dark and white color) containing DNA, and the lower phase (pink color) containing protein. The supernatant phase of each microtube was collected and transferred to other microtubes. 200 µL of isopropanol were added to each microtube and the microtubes were incubated for 10 minutes at 4°C and centrifuged for 10 minutes at 12000 RPM with a refrigerated centrifuge at 4°C. Next, the supernatant was removed and 1 mL of 75% alcohol was added to each microtube. After centrifugation at 7500 rpm for 5 minutes at 4°C and adding 10 µL of DEPC water to the samples using NanoDrop device in the OD260/OD280 ratio, the quality of the extracted RNA samples was measured.

2.4.3.3. cDNA synthesis

cDNA is synthesized from an mRNA template by the enzyme reverse transcriptase. Reverse transcriptase enzymes require primers to synthesize RNA.

Since then, two types of random hexamers and oligo dt primers have been used to make RNA.. The difference between these two types of primers is that oligo dt is attached to the polyadenine tail of mRNA, while random hexamers are randomly attached to different parts of mRNA. After connecting the primers, the reverse transcription enzyme synthesizes cDNA from template RNA.

2.4.3.3.1 Method

cDNA synthesis was done according to the method mentioned by the kit manufacturer. In this way, 5 µL of extracted RNA were mixed with 1 µL of random primer and 14 µL of cDNA master mix, and the samples were incubated for 30 minutes at 50°C. To inactivate the reverse transcriptase enzyme, the samples were incubated at 90°C for 5 minutes. After cDNA synthesis, the samples were transferred to a -20-degree refrigerator until use.

2.4.3.4. Real Time PCR

The mRNA expression level of the aforementioned genes was investigated by RT-PCR test. SYBER Green qPCR Master Mix kit was used to perform the RT-PCR (MIC) test. All the steps of the test were done on ice and to prevent contamination, all the work was done under the laminar hood. The principles of performing the Real-Time PCR method are based on the fluorescence property of SYBER Green dye is the most widely used RT-PCR method. The SYBER Green dye binds to the small groove of double-stranded DNA and emits fluorescence light, and the more the reaction proceeds and the more product is produced, the more this dye binds to DNA and produces more fluorescence. To normalize the level of gene expression in the comparison of the control groups and the treated group, genes whose expression levels are constant in the cell are used. In this project, ß-actin and GAPDH genes were used to normalize the results. The melting curve was used to evaluate the quality of RT-PCR. With this analysis, expression intensity, product

concentration, and the degree of purity of the amplified product can be obtained in each sample. If the peak of the graph is seen at low temperatures (about 65 to 75 degrees Celsius). It indicates the presence of a primer dimer because SYBER Green color non-specifically binds to the primer dimer in the reaction vessel, which causes false positive results. Also, the dimer sequence length of the primers is small and around 20 bp, which separate at low temperature and cause false positive results. The device draws the curve after completing cycle number 40. In this stage, the temperature of the device gradually increases from 60 to 95 degrees Celsius, during this temperature increase, the two-stranded products are separated from each other based on the length of the organic bases and the percentage of CG, which causes the intensity of the SYBER Green fluorescence color to decrease. The melting curve shows this reduction and determines the presence of primer dimer or the presence of any non-specific product by creating different peaks, which confirms the accuracy and validity of the reaction and the obtained product. To perform the RT-PCR test, 7 µL of SYBR Green qPCR Master Mix 0.3 µL of a mixture of forward and reverse primers, 2 µL of synthesized cDNA, and 4.7 µL of DEPC water were combined and according to the List of primers used in table 2-17 is mentioned.

***Table (2-17)*: Program implemented by Real-time PCR**

Cycle	Time	Temperature	Level
1	5 min	95	Primary denaturation
36	10 sec	95	Denaturation
36	35 sec	55	Primer binding
36	20 sec	72	Lengthening
1	5 sec for each degree	65-95	Melting

2.4.4. Programs and Software:

- Microsoft Office version 2016.
- ELISA reader software.
- SPSS version-21 for statistics analysis.
- Excel 2016
- Online DAS- calculators

2.4.5. Statistical Analyses

The data analysis for this work was generated using the Statistical Package for the Social Sciences software, version 22.0 (IBM, SPSS, Chicago, Illinois, USA). Values were illustrated by n (%) for categorical, Scale variables were presented by mean ± standard deviation for normal data. The distribution of the data was checked using the Shapiro-Wilk test as a numerical means of assessing normality. Biomarkers were compared using the one-way analysis of variance (ANOVA) was done to compare the means of different groups. Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p-values ≤ 0.05 (two-tail) were considered to be statistically significant. The optimal threshold with high specificity and sensitivity for critical cases was detected using receiver operating characteristic (ROC) analysis. It was found that all the values of P were two-sided, and a $P \leq 0.05$ was considered to be statistically significant.

Chapter Three

Results

3. Results

3.1. Part I (Biochemical and clinical result)

3.1.1. Demographic Characteristics

The demographic characteristics of the patients and control groups are outlined in Table (3-1), where the highest proportion of patients in both groups was found within the age bracket of (45-54), with percentages of (48.57%) and (45.70%) for the (non-obese RA and obese RA) groups, respectively. The patients' medical histories revealed that the largest proportion had a familial history of RA (85.70% for non-obese RA and 65.7% for obese RA) as opposed to the control group.

Table (3-1): **Demographics characteristic of the study groups.**

Factors		Patient				Control N = 60	
		Non-obese RA N =35		Obese RA N = 35			
		N	(%)	N	(%)	N	(%)
Age group	(35-44)	7	20	12	34.3	25	41.60
	(45-54)	17	48.57	16	45.70	20	33.4
	(55-64)	11	31.43	7	20.0	15	25
Family history	positive	30	85.70	23	65.7	0	0
	negative	5	14.3	12	34.3	60	100
BMI, kg/m^2	Normal	35	100	0	0	60	100
	Obese	0	0	35	100	0	0

RA: Rheumatoid arthritis, BMI: body mass index, N: Number

3.1.2. Distribution of Anthropometric Parameters

Table 3-2 showed no statistically significant age differences between the rheumatoid arthritis group and the control group (P = 0.163), indicating a homogeneous age distribution in the sample. Statistically significant differences

were observed in BMI between the patient group and the control group (P ≤ 0.001).***Table (3-2):*** **Comparison between study groups in Age, BMI and family history**

Groups	**RA N =60**	**Control N=60**	***P* value**
Age (year)	**45.0 ±6.88**	**46.07 ± 7.99**	**0.163**
BMI (kg/m^2)	**32.35 ± 2.20**	**20.0 ± 1.57**	**≤0.001**

T- test was significant at 0.05, BMI: body mass index; SD: standard deviation

3.1.3 Distribution of Lipid Profile with Groups

Table (3-3) shows that there was a significant difference in the mean ± standard deviation of the lipid profile level among the studied groups. The value of TC, TG and LDL were significantly higher among rheumatoid arthritis patients (232.31 ± 16.85, 166.08 ± 22.14 and 160.57 ± 29.59) mg/dL compared to the control group (146.18 ± 25.61, 94.97 ± 19.1 and 99.0 ± 15.54 mg/dL respectively (P = 0.01) (P = 0.05) (P = 0.01). In addition, the mean ± standard deviation of HDL-C was significantly lower among rheumatoid arthritis patients (34.44 ± 2.94 mg/dL) compared to healthy controls. More than (58.35 ± 8.02 mg/dL) (P value = 0.05)

Table (3-3): **The Comparison of the lipid profile for RA Patients (with or without obesity) compared to control groups**

Groups	**RA N=70**	**Control N=60**	***P* value**
TC (mg/dL)	**232.31 ±16.85**	**146.18 ±25.61**	**<0.01 ****
TG (mg/dL)	**166.08 ±22.14**	**94.97 ±19.10**	**<0.05***
HDL-C (mg/dL)	**34.44 ± 2.94**	**58.35 ± 8.02**	**<0.05***
LDL-C (mg/dL)	**160.57 ± 29.59**	**99.0 ± 15.54**	**<0.01****

T- test was significant at 0.05; SD: standard deviation, * (P≤0.05), ** (P≤0.01)

TC: Total Cholesterol, TG: Triglycerides, HDL-C: High-Density Lipoprotein, LDL-C: Low-Density Lipoprotein

3.1.4. Distribution of TNF-α, NO, IL-10, FRAP and SOD among Various Groups

The table below indicates that there was a significant difference in the mean ± standard deviation of the levels of the studied markers between the study groups. The mean ± standard deviation of both TNF-α and IL-10 levels were significantly higher among RA patients (71.77 ± 13.04 pg/ml) and (25.31 ± 5.25 pg/ml) compared to the apparently healthy control group (15.59 ± 1.24 pg/ml) and (10.37 ± 1.33 pg/ml), P < 0.001 and 0.05, while the mean ± standard deviation of NO was significantly higher among RA patients (90.13 ± 20.24 μmol/L) than that in the healthy control group (22.93 ± 2.96 μmol/L), respectively, P < 0.01. Also, the mean total antioxidant capacity as a marker of FRAP and SOD activity levels was significantly lower among RA patients (158.44 ± 26.45 μmol/L) (9.59 ± 1.22 U/mL) compared to healthy controls (709.30 ± 141.32 μmol/L) (50.16 ± 0.74 U/mL).

Table (3-4): **The comparison of the study markers for RA Patients (with or without obesity) compared to control groups**

Groups	RA N=70	Control N=60	*P* value
TNF-α (pg/mL)	71.77 ± 13.04	15.59 ± 1.24	<0.01
IL-10 (pg/mL)	25.31 ± 5.25	10.37 ± 1.33	<0.05
NO (μmol/L)	90.13 ± 20.24	22.93 ± 2.96	<0.01
FRAP (μmol/L)	158.44 ± 26.45	709.30 ±41.32	<0.05
SODU/mL	9.59± 1.22	50.16±0.74	<0.01

T- test was significant at 0.05; SD: standard deviation, Tumors necrosis factors- alpha (TNF-α), Interleukin – 10(IL-10), Nitric oxide (NO), Ferric reducing ability of plasma (FRAP), Superoxide dismutase(SOD).

3.1.5. Correlation between Biomarkers and studied parameters in patient groups.

3.1.5.1. Correlation between TNF-α and studied parameters:

In non-obese RA patients, TNF-α showed a positive correlation with TC level (r = 0.45), LDL–C (r = 0.37), and a negative correlation with HDL-C (r = -0.29), FRAP (r = -0.28), while other variables showed a non-significant association with TNF. In obese RA patients, TNF-α presented a positive correlation with LDL-C level (r = 0.40), NO marker (r = 0.32), and IL-10 marker (r = 0.40) had a positive correlation with TC level (r = 0.50). While TNF-α presented a negative correlation with HDL-C level (r=-0.31), Table 3-5.

Table (3-5): **Correlation coefficient of TNF-α with other biomarkers among Patients group.**

Parameters	Non-obese RA	Obese RA
	r	*r*
TC	**0.45****	**0.50****
TG	0.08	0.08
HDL-C	**-0.29***	**-0.31****
LDL-C	**0.37****	**0.40****
SOD	-0.009	-0.20
FRPA	**-0.28***	-0.01
NO	0.06	**0.32****
IL-10	0.05	**0.40****
****Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: Pearson correlation coefficients**		

3.1.5.2. Correlation between IL-10 and studied parameters:

In non-obese RA IL-10 showed a positive correlation with TG, and NO respectively (r=0.30) (r=0.31) additionally was a negative correlation with HDL-C and FRAP respectively (r=-0.42) (r=-0.29).

In obese RA patients, there was a positive correlation between IL-10, TC, and LDL-C respectively (r = 0.30) (r = 0.30).

Table (3-6): **Correlation coefficient of IL-10 with other biomarkers among Patients group.**

Parameters	Non-obese RA	Obese RA
	r	***r***
TC	0.03	**0.30***
TG	**0.30***	0.06
HDL-C	**-0.42***	-0.12
LDL-C	-0.01	**0.30***
SOD	-0.09	0.17
FRPA	**-0.29***	-0.04
NO	**0.31***	0.03
****Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: Pearson correlation coefficients**		

3.1.5.3. Correlation between Nitric Oxide and studied parameters:

Among non-obese RA patients, the NO marker presented a positive significant correlation with each of TC (r=0.42), TG (r=0.30), and a negative correlation with FRAP (r=-0.33) whereas, in obese RA patients, the NO marker showed a correlation with TC (r=0.30), Table 3-7.

Table (3-7): **Correlation coefficient of NO with other biomarkers among Patients group.**

Parameters	Non-obese RA	Obese RA
	r	***r***
TC	**0.42****	**0.30****
TG	**0.30****	0.06
HDL-C	-0.16	-0.19
LDL-C	0.12	0.18
FRAP	**-0.33****	-0.02
SOD	-0.05	-0.09
****Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: Pearson correlation coefficients**		

3.1.5.4. Correlation between FRAP and studied parameters:

In obese RA patients, the (FRPA) or total antioxidant capacity (TAC) marker had a negative correlation with TG (r= -0.35). Other studied variables showed no significant correlation with the FRPA marker as shown in Table (3-8).

Table (3-8): **Correlation coefficient of FRAP with other biomarkers among Patients group.**

Parameters	Non-obese RA	Obese RA
	r	***r***
TC	-0.05	-0.14
TG	-0.15	**-0.35****
HDL-C	0.06	0.09
LDL-C	-0.51	-0.11
SOD	0.07	0.18
****Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: Pearson correlation coefficients**		

3.1.5.5. Correlation between SOD and studied parameters:

Regarding SOD, the correlation studies with other parameters indicate a non-significant correlation between SOD markers with studied markers among non-obese RA patients. Whereas, in obese RA patients, there was a negative correlation between SOD and TG (r=-0.31), Table 3-9.

Table (3-9): **Correlation coefficient of SOD with other biomarkers among Patients group.**

Parameters	Non-obese RA	Obese RA
	r	***r***
TC	-0.10	-0.17
TG	-0.11	**-0.31***
HDL-C	0.15	0.10
LDL-C	-0.11	-0.04
****Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level, - = negative; r: Pearson correlation coefficients**		

3.1.6. Receiver Operating Characteristic

The Receiver Operating Characteristic (ROC) curve and area under the curve (AUC) analysis for the FRAP, TNF-α, SOD, NO, and IL-10 for the non-obese RA and obese RA groups were determined. The results of the ROC and AUC analysis as a diagnostic parameter indicated that all the above parameters have a good performance, data was presented in Table (3-10) (3-11) and Figure (3-3) (3-4).

Table (3-10): **ROC curve for the studied markers in non-obese RA group**

Parameters	Cut-off	Sensitivity	Specificity	AUC	P-value	CI(95%)
TNF-α	**≥54.5**	**89%**	**75%**	**0.883**	**0.0001**	**0.806-0.961**
IL-10	**≥18.02**	**82%**	**60%**	**0.685**	**0.006**	**0.505-0.805**
NO	**≥34.22**	**85%**	**70%**	**0.885**	**0.0001**	**0.814-0.956**
FRPA	**≤403.5**	**71%**	**93%**	**0.841**	**0.0001**	**0.912-0.994**
SOD	**≤15.17**	**89%**	**88%**	**0.953**	**0.0001**	**0.840-0.970**
AUC: Area under the curve, ROC: Receiver Operating Characteristic Curve, CI: Confidence interval						

UC: Area under the curve, ROC: Receiver Operating Characteristic Curve, CI: Confidence interval

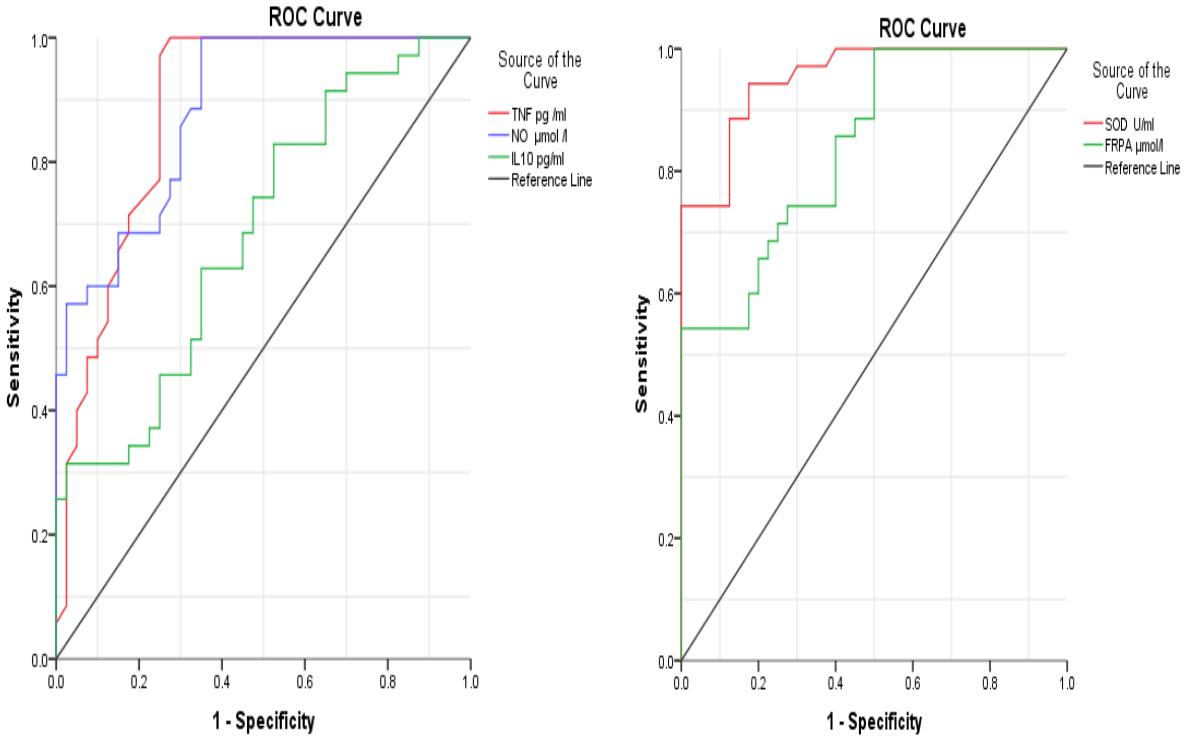

Fig. (3-1): **Receiver Operating Characteristic curve of serum TNF-α, NO, IL-10 FRAP and SOD levels as discriminators of patients with non–obese RA.**

Table (3-11): ROC curve for the studied markers in Obese RA group

Parameters	Cut-off	Sensitivity	Specificity	AUC	P-value	CI(95%)
TNF-α	≥58.5	77%	83%	0.900	0.0001	0.832-0.968
IL-10	≥19.82	69%	75%	0.895	0.0001	0.828-0.936
NO	≥66.44	86%	90%	0.966	0.0001	0.931-1.000
FRPA	≤185.54	87%	79%	0.948	0.0001	0.904-0.991
SOD	≤12.76	89%	83%	0.905	0.0001	0.756-0.927
AUC: Area under the curve, ROC: Receiver Operating Characteristic Curve, CI: Confidence interval						

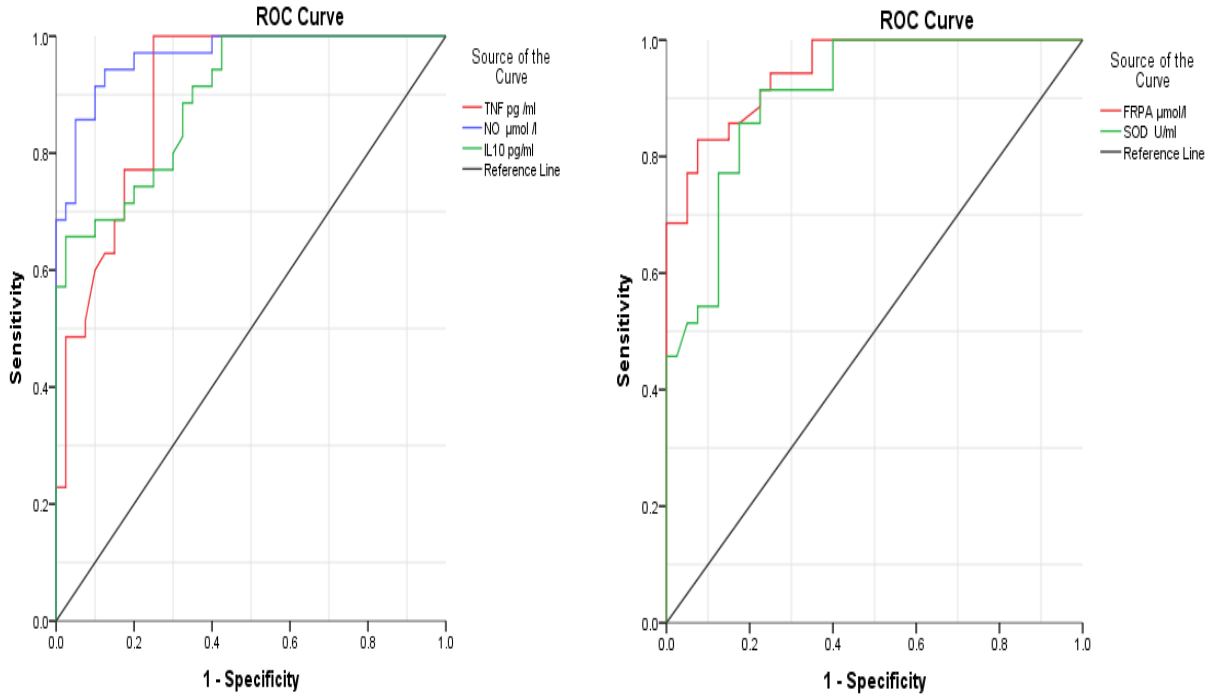

Fig (3-2): Receiver Operating Characteristic curve of serum TNF-α, NO, IL-10, FRAP, and SOD levels as discriminators of patients with obese RA.

3.2.Part II (Nano Studies Result)

3.2.1.Preparation and Characterization of Chrysin Nanoparticles Coaded with Hyaluronic Acid

The nanoparticles were produced by the thin film hydration method technique, allowing for large-scale production. The mean diameter of blank Nio NPs, Nio-chrysinNPs, and H- Nio-chrysinNPs are estimated as 138±14.1, 172±8.4, and 199±10.7, separately.The H- Nio-chrysinNPs have the largest size compared to other NPs shown in figure (3-3).

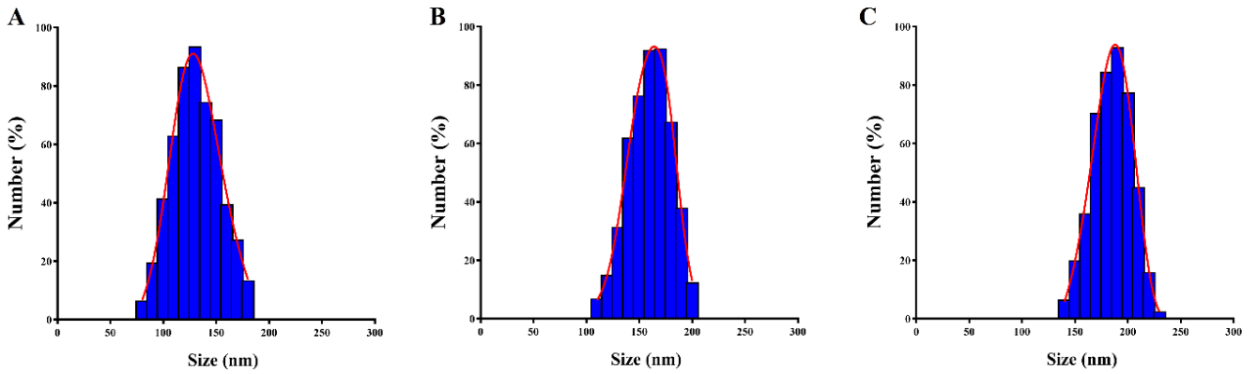

Fig (3.3): The DLS (dynamic light scattering) results of niosomal NPs A.Blank niosomal nanoparticles (blank Nio NPs), B. Chrysin loaded niosomal(Nio-chrysinNPs), C.Chrysin loaded hyaluronic acid coated niosomal (H- Nio-chrysinNPs).

Table (3.12) shows the zeta and Poly Disparity Index PDI values in addition to the size of the niosomal NPs, where the zeta values ranged from (-12.74 - 15.38mV). Chrysin-loaded niosomal recorded the highest zeta value and PDI (0.729).

Table (3-12): The size, zeta potential and PDI values for fabricated NPs.

Nanoparticles	Size (nm)	Zeta potential (mV)	PDI
Blank Nio	**138±14.1**	**−12.74±5.3**	**0.372**
Nio-chrysin	**172±8.4**	**−18.76±4.1**	**0.729**
H-Nio-chrysin	**199±10.7**	**−15.38±2.8**	**0.653**

The nanostructures image of synthesis NPs were investigated by SEM, as can be seen in Fig(3-4). Chrysin NPs structures within the particle sizes around (138-199) nm.Scanning Electron Microscopy (SEM)analysis of prepared Chrysin NPs revealed the distribution and NP size. The results of SEM images show that synthesized H- Nio-chrysinNPs were smooth, spherical particles, singular or in aggregates with particle sizes in the range of (114.23), (149.43), and (155.01 nm).

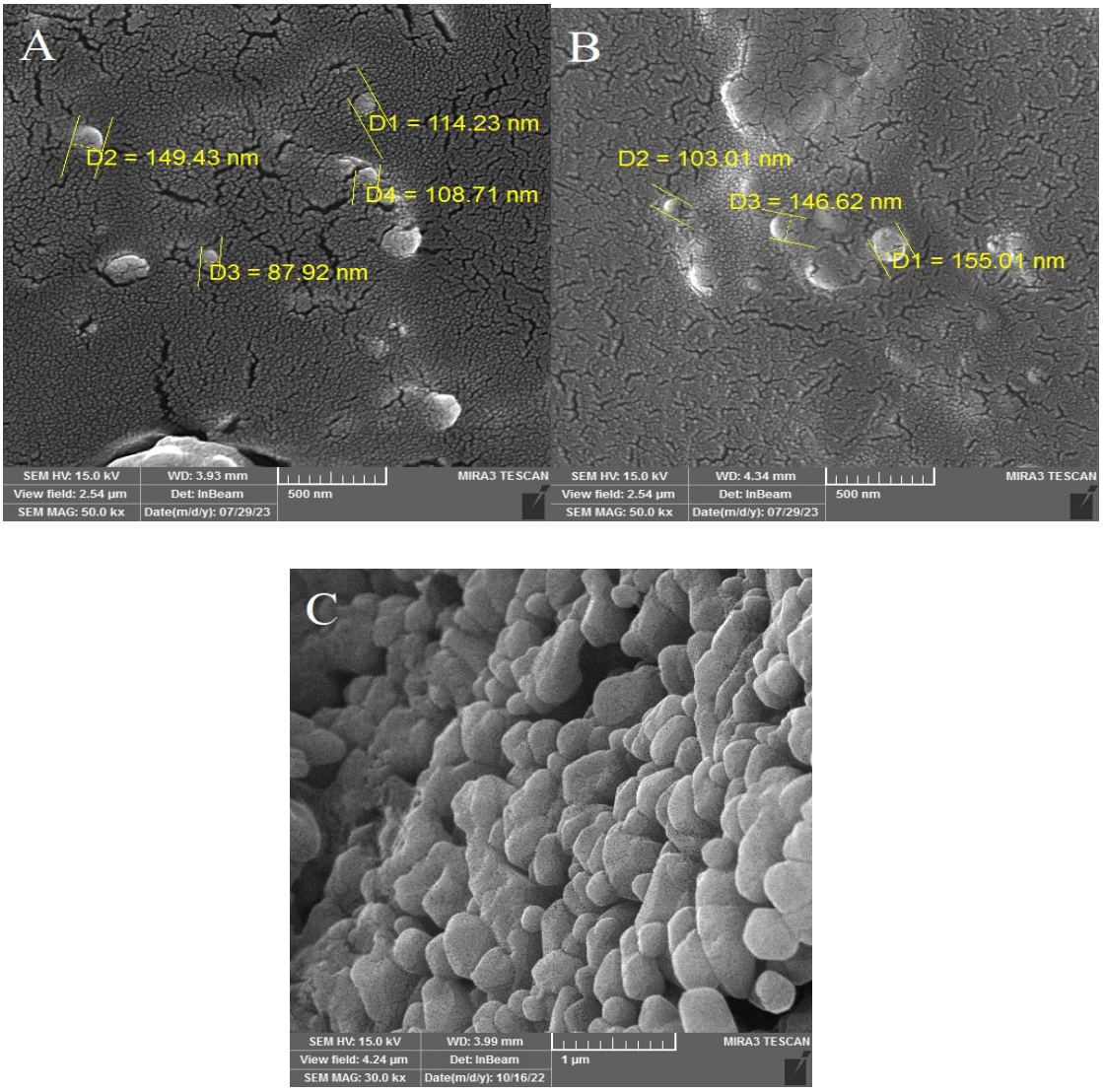

Fig. (3-4): SEM images of niosomal NPs revealed their spherical morphology. A) blank Nio NPs, B) Nio-chrysinNPs, C) H- Nio-chrysinNPs.

The Atomic Force Microscopy (AFM) results revealed particles with a maximum size of 129 nm, demonstrating uniform dispersion of NPs without any aggregation, as depicted in Fig (3-5).

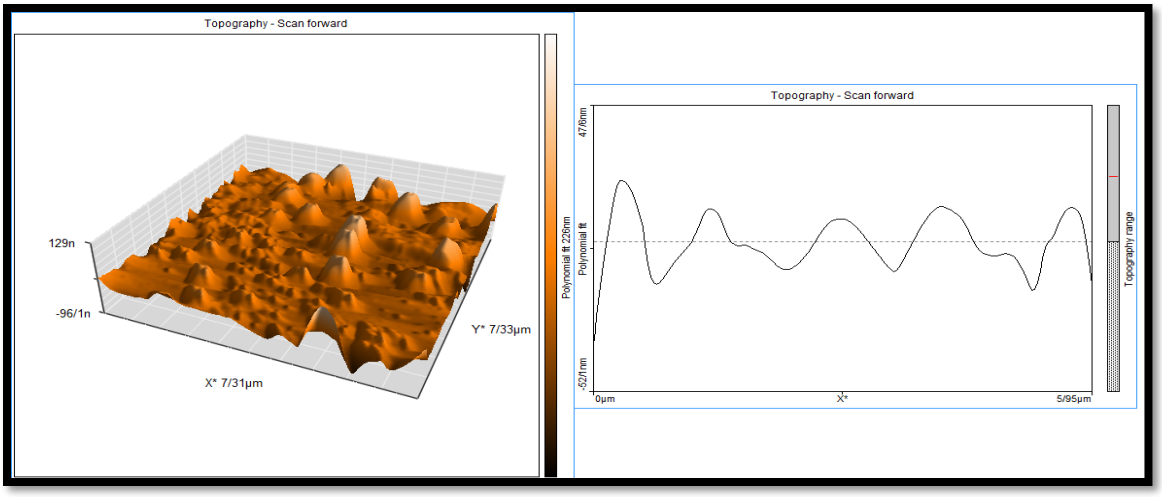

Fig. (3-5): **AFM image of Nio-chrysinNPs agrees with DLS and SEM results of Hyalo-Nio-chrysin**

3.2.1.1. Analysis of Characteristic Bands of Chrysin by Fourier transform infrared (FTIR) of Blank Nio NPs, Hyaluronic Acid

The reactivity and structural changes of functional groups used to prepare NPs were investigated by FTIR spectroscopy and the results are shown in Figure (3-6). The FTIR spectrum of chrysin NPs and H-chrysin NPs nanocomposite indicates that H-chrysin NPs were synthesized purely.

The FT-IR spectrum **Figure (3-6)** indicated that:

1. These FTIR results delineate peaks associated with functional groups inherent to the niosomal compounds, including the 1096 cm^{-1} peak linked to the stretching C–O alcohol bond in the structures of cholesterol and Span 60 **As** shown in band A.
2. The presence of a band at 1048.92 cm^{-1} is attributed to the C–O–C stretching vibration of HA As shown in band B.

3. Chrysin further revealed absorptions at 3012.79 cm^{-1} (OH), 2929.87 cm^{-1}, 2713.84 cm^{-1}, and 2630.91 cm^{-1} (aromatic C–H stretching), and 1653.00 cm^{-1} (α, β-unsaturated carbonyl, C═O) As shown in band C.

4. Upon the integration of hyaluronic acid into the drug-loaded niosome, a discernible peak at 1655 cm^{-1} corresponding to the amide group emerged. The empty niosome displayed stretching peaks for C-O, C=O, and C-H at 1125 cm^{-1}, 1747 cm^{-1}, and 2900 cm^{-1}, respectively. Furthermore, it manifested a carbonyl bond at 1625 cm^{-1} and a -NH stretching vibration at 3100–3400 cm^{-1}, As shown in band D and E.

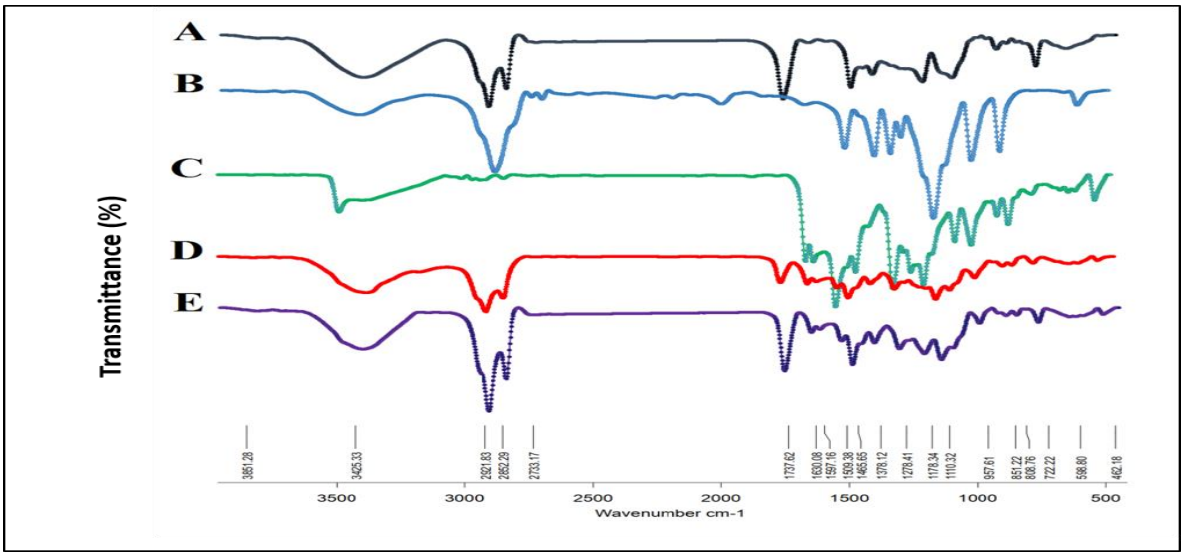

Fig (3.-6): **FTIR (Fourier-Transform Infrared Spectroscopy) analysis of (A) blank Nio NPs, (B) hyaluronic acid, and (C) chrysin show their characteristic bands in FTIR of (D) Nio-chrysin NPs, and (E) H- Nio- chrysin NPs**

3.2.1.2. Determination of the Proliferation of Free Chrysin NPs and Chrysin Coaded with Hyaluronic Acid H- Nio-chrysin NPs by MMT

The MTT reduction assay is a widely used method to measure cell viability and cytotoxicity. It is based on the conversion of MTT into formazan crystals by living cells, which is then quantified by measuring the absorption at specific

wavelengths (**Constante *et al.*, 2022**). Figure (3-7) shows the inhibitory effect of pure chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs on PBMCs with various doses. The free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs have negligible and insignificant proliferation effects on PBMCs at 2.5, 5, 15, and 20 µM concentrations. The only significant result ($P < 0.01$) is demonstrated in the H-Nio-chrysin NPs treated group at 10 µM concentration, based on these results 10 µM of free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs have been used for other experiments of this study.

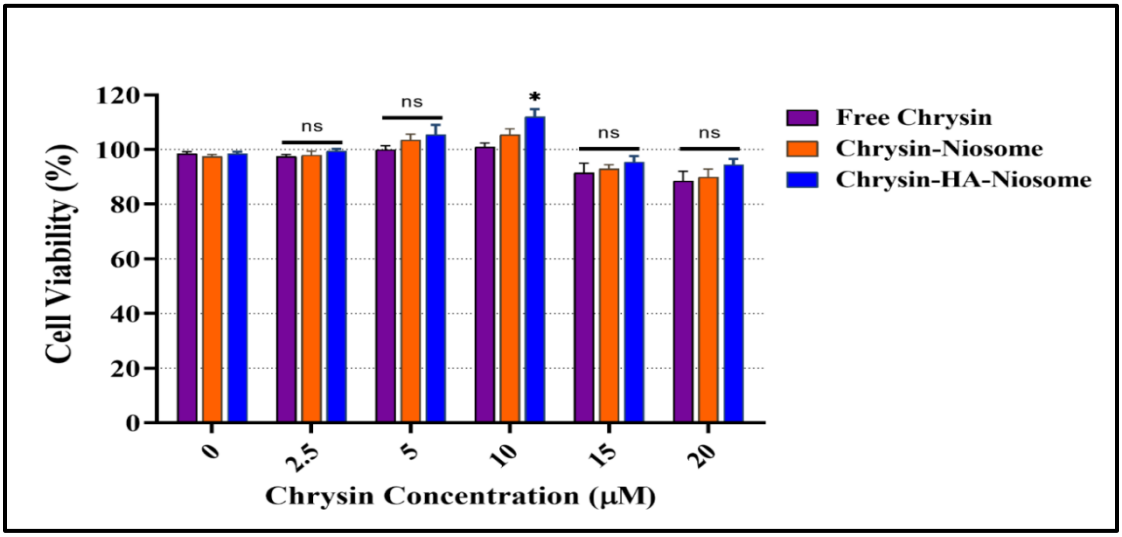

Fig. (3-7): **The proliferation effects of pure chrysin, Nio-chrysinNPs, and H- Nio-chrysinNPs on PBMCs.**

3.2.1.3. Determination of Chrysin Release Pattern

In Figure (3-8) shown that at 120 h chrysin release from Nio-chrysin NPs and H- Nio-chrysin NPs at 37°C. After 120 h, 64% and 76% of loaded chrysin were released from H- Nio-chrysin and Nio-chrysin NPs at pH 7.4, respectively. The maximum release rate was 37% and 43% within 12 h of the experiment.

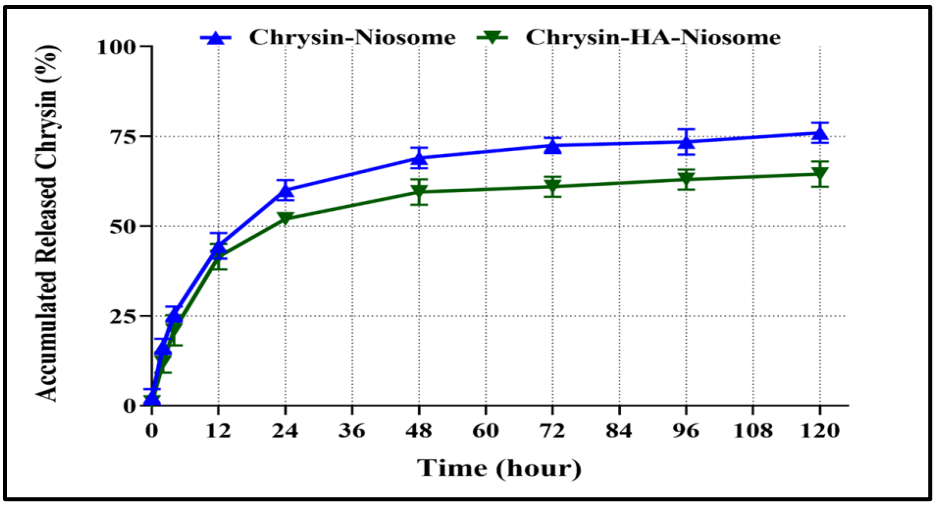

Fig. (3-8): **The 120 h *in vitro* release experiment of chrysin from Nio-chrysinNPs, and H-Nio-chrysinNPs at 37°C and pH 7.4 show a biphasic release pattern.**

3.2.1.4. Determination of TNF-α and IL-10 Level in Pure Untreated Chrysin, Nio-chrysin NPs, and H Nio-chrysin treated PBMCs.

The changes in TNF-α levels in untreated and free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs treated PBMCs of patients are shown in **Figure (3-9).** The highest reduction in TNF-α levels compared to the control group was achieved with H- Nio-chrysin NPs treatment. Figure (**3-9**) demonstrates these cytokines changes in rheumatoid and healthy cells. Treatment of healthy and RA cells with chrysin, Nio-chrysin NPs, and H-Nio-chrysin NPs resulted in increasing the level of IL-10.

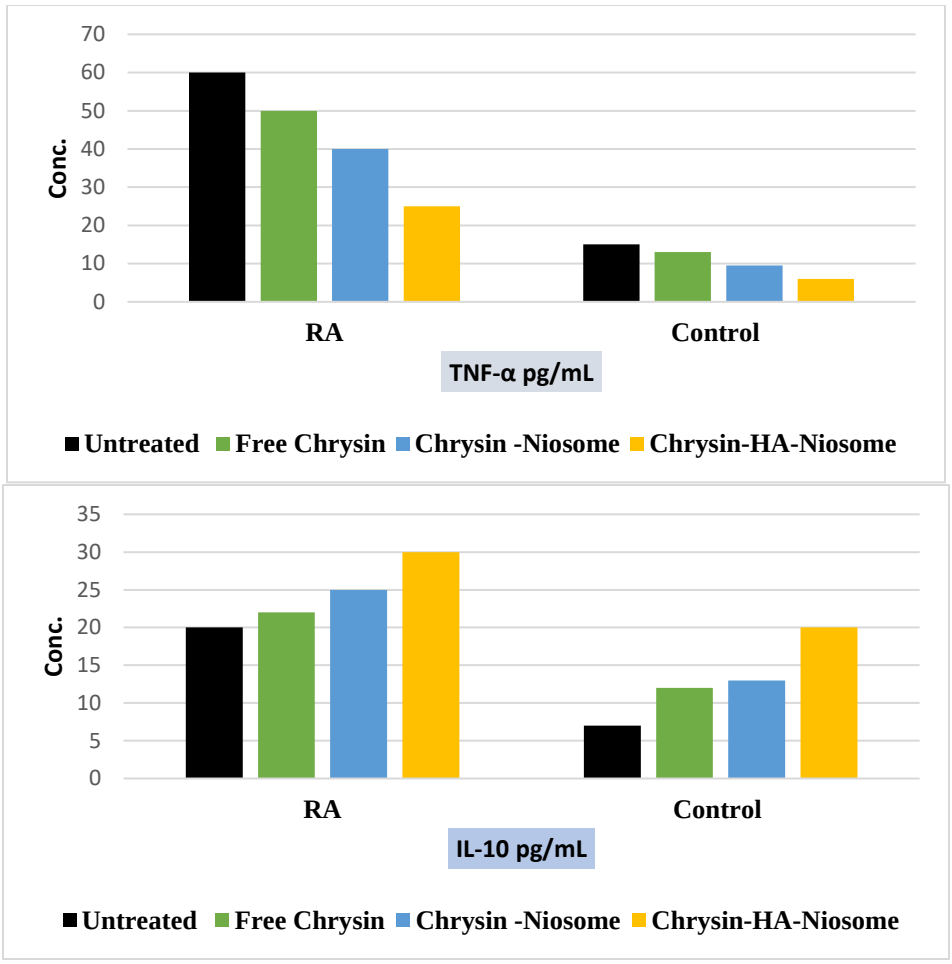

Fig. (3-9): **Comparison of C. IL-10 and A. TNF- α levels in untreated and treated (pure chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs) PBMC**

3.2.1.5. Determination of Nitric Oxide Level in Pure Untreated Chrysin, Nio-chrysinNPs and H Nio-chrysin treated PBMCs.

Figure (3-10) shows the nitric oxide level of control (untreated) and pure chrysin, Nio-chrysinNPs, and H- Nio-chrysinNPs treated PBMCs. After 48 hours of treatment, the nitric oxide levels in the patient's group with free chrysin, Nio-chrysinNPs, and H- Nio-chrysinNPs were 39µmol/L, 37µmol/L, and 22µmol/L, respectively

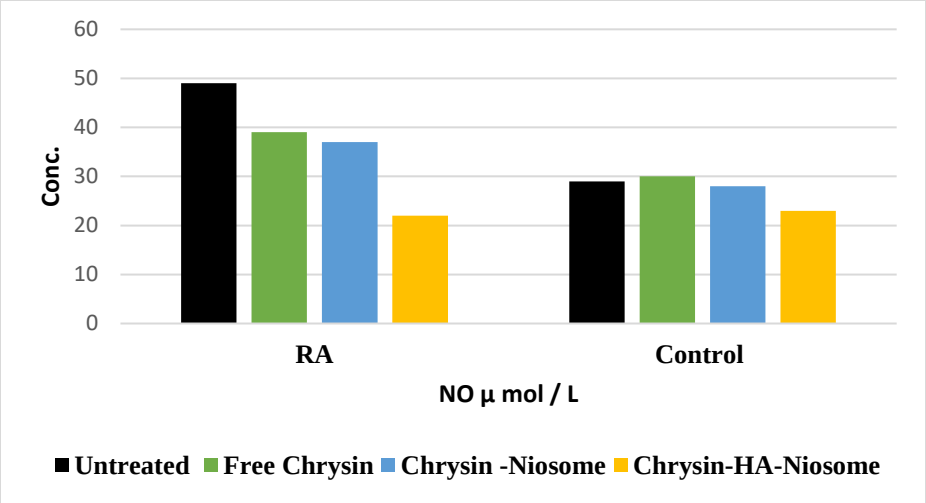

Fig. (3-10): **Nitric oxide level changes in untreated and pure chrysin, Nio-chrysinNPs, and H- Nio-chrysinNPs treated PBMCs**

3.2.1.6. Determination of TAC(FRAP) and SOD Level in the Control Group and Pure Untreated Chrysin, Nio-chrysin NPs, and H Nio-chrysin in treated PBMCs

As shown in Figure (**3-11a,b**), the activity of TAC (FRPA) and SOD was found to increase in the treated peripheral blood cells compared to the control peripheral blood cells. SOD activity was measured in RA patients and healthy cells treated with free chrysin, Nio-chrysin, and H-Nio-chrysin NPs nanoparticles. H-Nio-chrysin nanoparticles could significantly increase the activity of SOD and FRPA in both RA patients and healthy cells. From the figure below, it was observed that the highest increase in both SOD, and FRAP was in the H-Nio-chrysin NPs group, where the increase in SOD level in the patient's group with free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs were 29 U/mL, 30U/mL, and 40 U/mLwhile the increase in FRAP level in the patient's group with free chrysin, Nio-chrysin NPs, and H-Nio-chrysin NPs were 330,350,450 µmol/L .

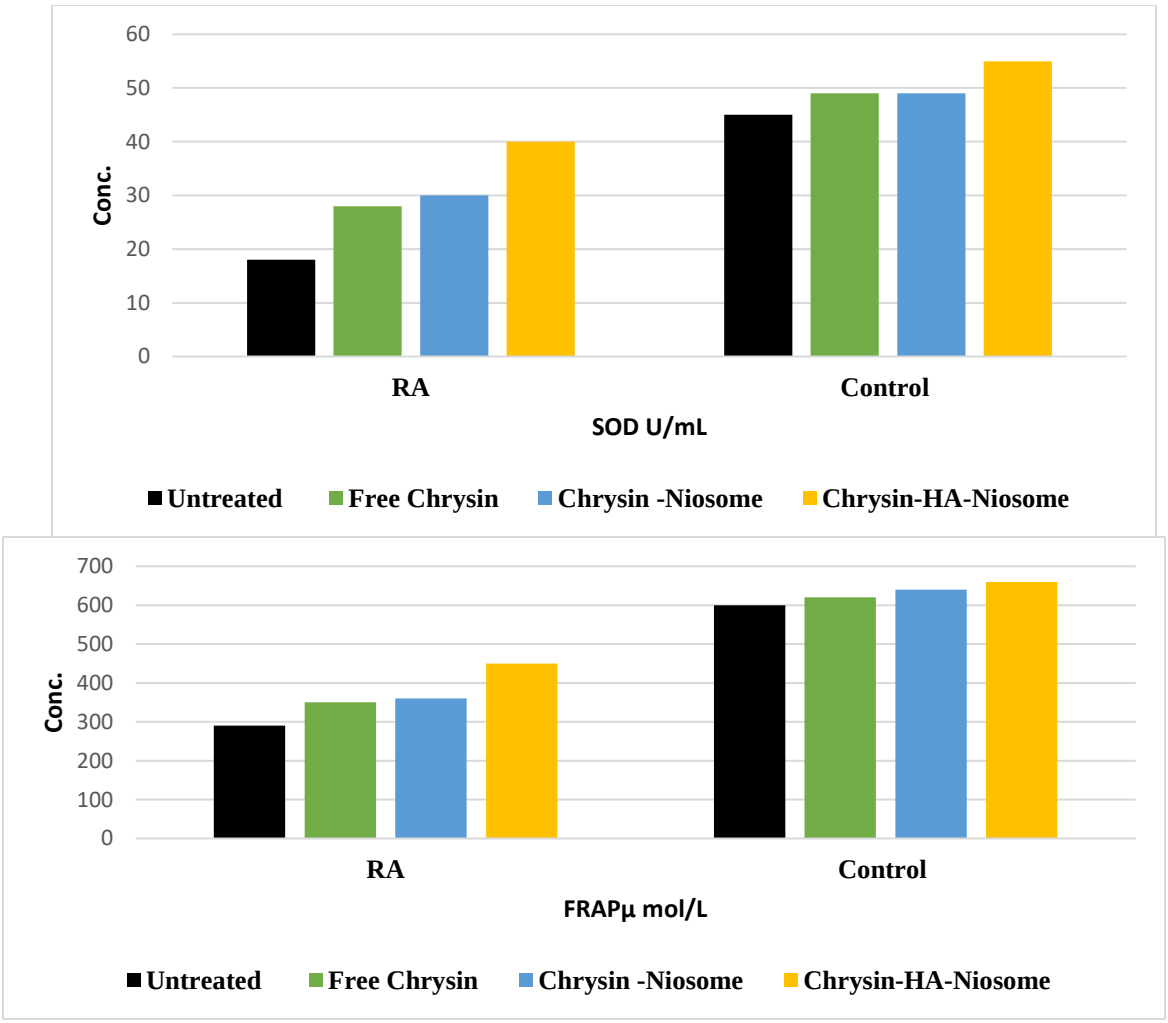

Fig. (3-11): **Comparison of changes in activity of total antioxidant capacity FRAP, superoxide dismutase SOD, in control and treated PBMCs.**

3.3. Part III (Molecular Studies and Result)

3.3.1. Molecular Studies and Gene Expression:

Figure (3-12) shows the expression level of these genes in PBMCs before and after treatment with pure chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs. As we note from the figure the level of the MMP9 gene decreases with the increase in the level of TIMP1.

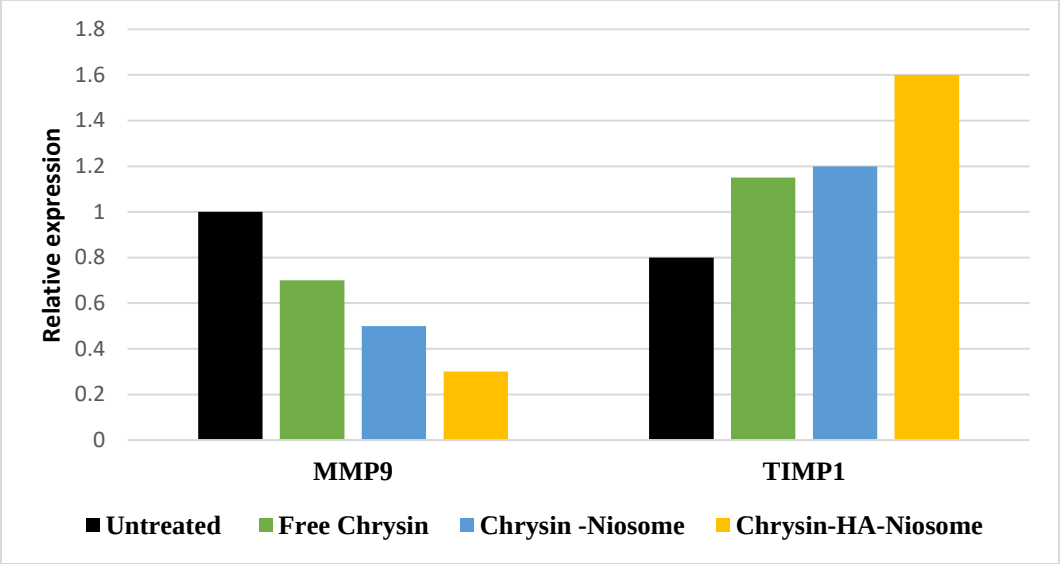

Fig. (3-12): **Expression level of these genes in PBMCs before and after treatment with pure chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs**

Chapter Four

Discussion

4.Discussions

4.1. Part I (Discussion of Biochemical Results)

4.1.1. Distribution of Demographic Characters of Studied Groups

In the current study, there were no statistically significant differences in the mean age between RA patients, obese RA patients, and control groups, due to proper matching between groups. This study is similar to (**Al-Atabi and Zanki, 2023**). It also agrees with the views of Svensson et al. in Sweden (**Svensson *et al*., 2020)**, who suggest that in case-control studies, age and sex should be identical or nearly identical, and Hussein in Iraq (**Hussein, 2019**) who found no statistically significant differences between the patient and control group. As for the mean age in the current study of RA patients, the largest proportion was within the middle age group (45-54) years, and this mean is consistent with (**Aboud, 2019**) in Iraq.

The positive correlation between BMI and RA is important. In patients with RA, a statistically significant relationship exists between body mass index and disease activity ($P < 0.0001$), especially in females. High BMI values increase the risk of developing an inflammatory condition due to high levels of C-reactive protein in the blood. High BMI levels are linked to increased inflammation and joint swelling in RA patients **(Dar *et al*., 2018)**.

The study focuses on females due to higher prevalence of RA compared to males, a trend consistent with previous research indicating hormonal influences and higher susceptibility to the disease **(Oweis *et al.*, 2020, Osman *et al.*, 2021, Zen *et al.*, 2023)**. The higher representation of females compared to males can be elucidated by their augmented immune response and the hormonal differences typically linked to estrogen, making them more susceptible to autoimmune conditions than their male counterparts (**Bagherpour-Kalo *et al*., 2023).**

4.1.2. Comparison of lipid profile between non-obese RA, Obese RA, and Control Groups

Research shows RA patients have higher lipid indicators (TC, TG, LDL) and lower HDL levels, indicating a significant association between dyslipidemia and RA **(Lei *et al*., 2023, Chen *et al*.,2021)**. Elevated TG and TC levels are linked to factors like obesity and insulin resistance, with a strong association between high-fat content, obesity, and female RA patients **(Turesson *et al.*, 2015)**. The elevated inflammation results in an increase in the liberation of free radicals, initiating the oxidation process of LDL-C. This oxidation leads to the creation of oxidized LDL-C, which induces the accumulation of LDL-C **(Tsoupras *et al.*, 2018)**.

Research indicates abnormal HDL anti-inflammatory and antioxidant functions in RA patients, possibly due to protein composition changes **(Zimetti *et al.*, 2021; Yan *et al.*, 2023)** and prolonged treatment periods causing increased oxidative stress and decreased antioxidant potential (**Nawaz *et al*., 2021**).

4.1.3. Comparison of Inflammatory Marker (TNF-α) in Studied Groups

The study revealed elevated serum TNF-α levels in RA patients, potentially due to synovial inflammation causing osteoclast differentiation, which contributes to joint deterioration (**Yokota *et al.*, 2021).**

The study confirms Lee et al.'s 2021 findings that obese individuals have higher TNF-α levels, a key cytokine in inflammatory pathways, particularly in RA **(Li *et al*., 2021).**

Older adults have higher plasma TNF-α concentrations, linked to increased risk of atherosclerosis and rheumatoid arthritis, consistent with bruunsgaard's findings **(Bruunsgaard *et al*., 2000).**

The study supports Rodriguez-Carrio's association between lipid profile and inflammatory mediators in RA (**Rodriguez-Carrio *et al* 2017**), with TNF-a inhibiting HDL production (**Yan *et al*,.2023**). However, inconsistent studies due to factors like age, obesity, and treatment effects (**Kronzer *et al*., 2021).**

Elevated TNF-α levels can lead to increased NO levels, causing cytotoxic consequences **(Lind *et al.*, 2017).** This induction is linked to inflammatory processes and nitric oxide synthesis, potentially influencing disease progression. **(Karki *et al*.,2021; volova *et al.*, 2020).**

IL-10, a key inflammatory cytokine, enhances immune regulatory functions in monocytes, potentially enhancing their responsiveness to immune complexes, despite its unclear efficacy in RA joint inflammation **(Chen *et al.*, 2021; Pašková, 2019)**. RA and obesity-related inflammation are linked to a shift in cytokine response, with pro-inflammatory cytokines being more prevalent (**Dissanayake *et al*., 2021**). This was observed in a study by Schmidt et al, where it was found that IL-10 was elevated in people suffering from obesity, as inflammatory cytokines were elevated **(Schmidt *et al*., 2015).**

The study found a negative correlation between TAC (FRPA) and TNF-α, suggesting overproduction of inflammatory markers contributes to increased ROS release in RA patients and decreases antioxidants **(García-González *et al*., 2015).**

4.1.4. Anti-inflammatory marker (IL-10) with other studied groups

RA patients exhibit higher IL-10 levels, indicating a compensatory response to counteract excessive inflammatory signals (**Vasilev *et al*., 2024**)), with IL-10 being the most powerful anti-inflammatory cytokine, playing a dual role. **(Hernández-Bello *et al.*, 2017; Kaya *et al.*, 2021)**.

This investigation demonstrated a noteworthy positive correlation between IL-10 and elderly individuals diagnosed with RA, as illustrated in Table (3-6).

Likewise, the identical investigation identified the correlation as positive but lacking statistical significance between age and IL10 **(Eltahir *et al*., 2020).**

The study found a significant positive correlation between IL-10 and various markers, except for HDL and FRAP, suggesting a controversial role of IL-10 in human health (**Mühl 2013; Moraitis *et al*., 2015**). IL-10 has also been linked to increased cholesterol production by hydroxy-3-methylglutaryl-coenzyme A reductase activity. This is what was found in the study of Shahbaz *et al* **(Shahbaz *et al.,* 2019).**

The results of the study were similar to those of a previous study in which serum IL-10 levels were higher in obese individuals than in non-obese individuals. Liu et al.'s study showed higher serum IL-10 levels in obese individuals with elevated TG levels **(Liu *et al*., 2018).**

This study also showed a negative correlation between the level of total antioxidants FRPA and IL10, these are consistent with the study of Garcia and McGrath (**Garcia Gonzalez *et al.,* 2015; Mcgrath and Young.,2015).** The study found a positive correlation between NO and IL-10, which is linked to obesity, oxidative stress, inflammation, metabolic biomarkers, and inflammatory cytokines in various conditions **(Arbizu *et al*., 2023; Saleem *et al*., 2023**).

4.1.5. Oxidant (NO) / Antioxidant levels (SOD, FRAP) with other studied groups.

Obesity-related oxidative stress in RA patients leads to mitochondrial dysfunction and increased ROS levels, potentially causing skeletal muscle damage and oxidative stress in tissues **(Eid *et al*., 2019; Jing *et al*., 2023, López-Acosta *et al.,* 2023).**

Nitric oxide production in RA patients is higher than the control group, contributing to tissue damage and escalating in inflammatory arthritis **(Khojah *et***

***al*., 2016).** Experimental and clinical studies provide evidence that dysfunction of the NO pathway is associated with hyperlipidaemia. This was demonstrated by Kajikawa *et al* study **(Kajikawa *et al.*, 2016)**. Triglycerides may impair endothelial function, with high cholesterol affecting nitric oxide production. Endothelial toxicity by cholesterol and LDL-C does not alter activity, and nitric oxide synthase acts as a negative regulator **(Darwin *et al*., 2020).** The study reveals a significant negative correlation between NO and total antioxidants, indicating an imbalance between reactive oxygen species and antioxidants **(Eid *et al*.,2019; López-Acosta *et al*., 2023)**. Low anti-oxidants in blood are linked to higher RA risk (**Sczepanik *et al.*, 2020**). ROS production is crucial for bone resorption during inflammation, impacting RA. Disease-generated ROS and lipid peroxides reduce antioxidant levels, resulting in low antioxidant levels (**Sczepanik *et al*.,2020; Epsley *et al*., 2021)**.

The study found that serum FRAP levels were significantly lower in individuals with RA and obese RA compared to healthy subjects, indicating a decreased antioxidant response **(Malik *et al.*, 2017).**

Djordjevic and Rondanelli found antioxidants are important in autoimmune disorders, especially RA, and low levels can cause the condition. Researchers tried to boost antioxidants to reduce oxidative stress and free radicals, with results consistent with their peers (**Rondanelli *et al.*, 2021, Djordjevic *et al*., 2023).**

Obese RA patients show a negative correlation between FRAP and BMI, suggesting reduced antioxidant capacity due to obesity, potentially negating the difference in oxidative stress burden **(Najafizadeh *et al.*, 2021).**

LPO impacts antioxidants, with FRPA inversely correlated with lipid profile, linked to hyperlipidemia. Research by Rivera and Rafiei supports this inverse relationship (**Rivera-Mancía *et al.*, 2018; Rafiei *et al*., 2022)**.

In our current study, a decrease in the SOD value was observed in patients with RA and obese RA compared to the control group according to Table 3-4, This is consistent with many studies (**Ponist *et al.*, 2019; Al-Jawadi and Jankeer, 2021).** In Table (3-9), Studies show decreasing SOD activity with increased TG and LDLC, with correlations highlighting TG. Low antioxidants and high blood lipids are linked to dyslipidaemia. **(Ma *et al*., 2020),** while others did not do so, as Mansego denied the existence of a relationship between them **(Mansego *et al*., 2015).**

SOD is negatively associated with TG, suggesting a relationship between oxidative stress and lipid metabolism as pointed out by Liu in 2020 (**Liu *et al*., 2020).** Moreover, there is a modest relationship between SOD activity and TG levels **(Jakubiak *et al*., 2021).** Overall, research suggests that SOD levels may play a role in lipid metabolism and oxidative stress, with implications for conditions such as dyslipidaemia **(Lewandowski *et al*., 2020; Guevara-Cruz *et al*., 2021).**

4.2. Part II (Discussion of Nano studies results)

4.2.1 Characterization of hyaluronic acid loaded with chrysin and decorative nanoparticles, and the effect of chrysin on the parameter of the studies

The study highlights chrysin's potential for therapeutic interventions due to its anti-inflammatory and antioxidant properties. However, challenges like poor solubility, low stability, rapid metabolism (its half-life is only 13 min in human hepatocytes) **(Smith *et al.*, 2005)**, and low cellular uptake hinder its clinical applications (**Deldar *et al.*, 2018; Gao *et al*., 2021**). Nanotechnology offers sustained release methods to address these issues, enhancing therapeutic delivery and mitigating inflammation **(Syam Kumar *et al*., 2023;Ding *et al*., 2023).**

In this study, niosomes are chosen for advanced drug delivery due to their biodegradability, non-toxicity, stability, and cost-effectiveness, making them ideal for chronic inflammatory diseases like RA **(Kumar *et al.*, 2022).** Furthermore, their nonpolar characteristics and lipid composition are analogous to that of human cells.

Chrysin nanoparticles were coated with hyaluronic acid (HA) to the enhances biocompatibility and allows for specific targeting of CD44 receptors, which are often overexpressed in activated macrophages involved in RA pathology (**Gorantla *et al*., 2023**). Chrysin's encapsulation with HA enhances its efficacy in treating inflammatory conditions, providing pain relief, and demonstrating biocompatibility and biodegradability, targeting high CD44 levels in RA tissues **(Marinho *et al*., 2021**). Conversely, drug encapsulation in engineered nanoparticles can enhance drug delivery safety and target specificity, minimizing damage to healthy tissues (**Jahanban *et al.*, 2018**).

Nanoparticle size is a critical parameter that determines their cellular interactions, biodistribution, mechanical properties, and quantum effects **(Hoshyar *et al* ., 2016;NT al Rashid et al ., 2020)**. Careful control of size is essential for optimizing nanoparticles for biomedical applications like drug delivery, imaging, and therapeutics. The nanoparticle (NP) size influences treatment distribution and absorption, impacting tissue penetration efficacy **(Islam *et al*., 2017).** Dynamic light scattering (DLS) revealed an average diameter of 138 ± 14.1 nm for empty Nio NPs . Encapsulation of chrysin increases the diameter to 172 ± 8.4 nm, with H-Nio-ch NPs exhibiting the largest size at 199 ± 10.7 nm due to hyaluronic acid coating and chrysin loading.

DLS indicates a larger average diameter than SEM, as shown in Figure 3-4 This difference arises because SEM measures particles in a dry state, while DLS assesses particles in solution. The wetting of particles increases their dimensions,

corroborated by Khan *et al* who demonstrated that nanoparticles exhibit a larger size in solutions (**Khan *et al.*, 2019**).

The corresponding zeta potential of the as-prepared sample was evaluated, as shown in Table 3-12, and after loading chrysin inside the Nio-NPs, the surface charges of the Nio-NPs shifted from -12 mV to -18 mV. A negative shift indicates the presence of a negatively charged carboxyl group of the chrysin. The change to -15 mV indicates a decrease in negative charge after coating with HA due to the amide groups. On the other hand, the negative zeta values decreased with increasing nanoparticle size, which resulted in enhanced stability. The results of the study closely matched those of Tavakoli *et al* where the negative charge decreased from 15 to 18 mV as the nanoparticle size increased from 172 to 199 nm. This result provides further evidence for the successful formation of Nio-chrysin NP nanoparticles (**Tavakoli *et al*., 2018**).

Zeta potentials have been used to measure and estimate the surface charge and stability of nanomaterials, as changes in these properties directly affect the biological activity of NPs (**Balika, 2022**).

Zeta potential value is crucial for nanoparticle stability, preventing particle aggregation and enhancing electrostatic force, and controlling it can lead to more effective formulations (**Umbarkar *et al*., 2020**).

The Polydispersity Index (PDI) is a crucial parameter in nanoparticle characterization, revealing uniform particle size distribution, vital for drug delivery, diagnostics, and biomedical applications **(Clayton *et al*., 2016) .**

As per Table 3-12, the particle size distribution (PDI) is recognized as a key factor in the assessment of the stability and functionality of NPs designed for drug delivery applications was proven by Wilhelm Romero in 2021 (**Wilhelm Romero *et al*., 2021).** The coating of chrysin nanoparticles with HA reduces the PDI from 0.7 to 0.6, enhancing homogeneity and stability **(Siddhardha *et al.*, 2020**), and is

crucial for nanoparticle attributes like stability, release kinetics, and drug efficacy (**Mato *et al*.,2015**). Low PDI is crucial for nanoparticles' consistent behavior, affecting pharmacokinetics, biodistribution, and therapeutic efficacy. Narrow size distribution enhances targeting to specific tissues while minimizing side effects (**Kunasekaran and Krishnamoorthy 2015; Danaei *et al*., 2018**). As per the Dynamic Light Scattering (DLS) findings presented in Table 3-12, the blank Nio, Nio-ch, and H-Nio-ch NPs all fall within the acceptable range concerning size, zeta potential, and PDI values.

The shape of nanoparticles can affect their circulation, distribution, extravasation, cellular uptake, and therapeutic performance **(Zhu *et al*., 2019).** The study found that the nanoparticles were properly prepared, with smooth spherical shapes, as shown in Figure (3-4). The nanoparticles were either single or aggregated, with particle sizes ranging from 114.23 to 155.01 nm, and had a porous structure for loading the therapeutic. This is consistent with previous studies, suggesting that the size of the nanoparticles is the only difference **(Hajizadeh *et al*., 2019; Barani *et al*., 2021).**

AFM images of the NPs are shown in Figure 3-5, and these images agree with previous DLS results. The AFM results also showed particles with a maximum size of 129 nm, which indicates a uniform dispersion of NPs without any aggregation or fusion and agreed with the study of Koley et al (**Koley *et al.*, 2011**).

FT-IR is an effective analytical method used to detect functional chemical groups and characterize covalent bonding information. Nio NP formation was confirmed by Fourier transform infrared (FTIR) techniques. The active groups in Chrysin showed distinct bands at 2625 cm^{-1} and 2343 cm^{-1}, which indicates O-H stretching vibration and intramolecular hydrogen bonding, and this was revealed by Siddhardha et al study **(Siddhardha *et al*., 2020).**

The FT-IR spectrum in Figure (3-6) of chrysin also revealed absorptions at 3012.79 cm-^{1} (OH), 2929.87 cm^{-1}, 2713.84 cm^{-1}, and 2630.91 cm $^{-1}$ (C–H stretch), and 1653 00 cm^{-1} (α, β-unsaturated carbonyl) and the results of the study were similar to the results of Jasim *et al* in his study (**Jasim *et al*., 2022).** FTIR results identified these peaks associated with functional groups inherent in niosomes, including the peak at 1096 cm $^{-1}$ associated with the extended C–O alcohol bond in cholesterol and Span 60 structures **(Haddadian *et al.,* 2022).** The reason for the presence of a band at 1048.92 cm^{-1} is attributed to the C-O-C stretching vibration of HA **(Niu *et al.,* 2022).** Hyaluronic acid successfully incorporated into drug-loaded niosomes, resulting in a noticeable amide group peak. Empty Nio exhibited extended peaks, carbonyl bonds, and -NH stretching vibrations. **(Rezaei *et al.,* 2022).**

The MTT reduction assay is a widely used method to measure cytotoxicity, cell viability, optimal dose of treatment and its effect on blood cells **(Constanta *et al*., 2022)**. Figure 3-7 shows the effect of chrysin and chrysin- loaded NPs on the proliferation of PBMCs by MTT. Chrysin has been shown to have a cytotoxic effect on cancer cells without affecting normal cells **(Wu *et al*., 2022).**

The results are consistent with those obtained in another study by Chen et al **(Chen *et al*., 2021**). H- Nio-chrysin NPs consist of Span 60, cholesterol, chrysin, and hyaluronic acid. Hyaluronic acid and cholesterol are natural components found in the human body, and studies have confirmed their safety for normal cells **(Wu *et al.,* 2022).** The treatment with H-Nio-chrysinNPs at 10 μM significantly enhanced PBMC survival the inclusion of hyaluronic acid on their surface facilitates results in the localization of chrysin in the periphery of PBMCs, thus enhancing the potential for cellular uptake and therapeutic effect.

The drug release mechanism from NPs is affected by various factors such as particle size, surface properties, and porous structure of NPs **(Slowing et al., 2007;**

Odeniyi et al., 2018; M, Ways et al, 2020). Sustained release of drugs from NPs is desirable for medical applications **(Javaid et al., 2021).** Niosomes consist of biodegradable and non-immunogenic components that can carry lipophilic and amphiphilic drugs, making them attractive for drug delivery **(Rao et al., 2018; Hazira et al., 2023).** Niosomes have been reported to exhibit sustained release patterns for several drugs, and this has been demonstrated in studies by Basiri and Mavaddati et al **(Mavaddati et al., 2015; Basiri et al., 2017).**

Figure (3-8) shows that after 120 hours, 64% and 76% of the chrysin loaded from H- Nio-chrysin and Nio-chrysin NPs were released at pH 7.4, separately, and the results agreed with the results of Lee et al.'s study where chrysin NPs showed that It is more soluble at pH 7.4 because it is similar to the body's pH (**Lee *et al*., 2018**). The observed release profile showed two distinct phases, with peak release rates of 37% and 43% in the first 12 h of the experiment, followed by a subsequent decline. This rapid initial release (first stage) can be attributed to the surface attachment of drugs to the niosomal NPs through weak linkers rather than encapsulation (**Varma *et al*., 202**0). The sustained, controlled release (second stage) is due to the dissolution, diffusion and transfer of drugs from the core of the nanoparticle to its surface, and the demonstration of a moderate and sustained release behavior (**Tavakoli *et al*., 2018**).

Figure 3-9 shows the changes in TNF-α, in untreated, free chrysin Nio-chrysin NPs, and H- Nio-chrysin NPs treated for PBMCs of confirmed patients. The highest reduction in TNF-α levels compared to the control group was achieved using H- Nio-chrysin NPs treatment. This is a result of the fact that the HA encapsulating the nanoparticle makes the cellular access of chrysin and its anti-inflammatory effect easier and keeps it close to the PBMCs. These results were close to the results of Prasanna et al who demonstrated the effectiveness of chrysin-NPs in reducing inflammatory factors such as TNF-α (**Prasanna *et al*.,**

2021). In their study, Kumar et al also demonstrated the role of chrysin nanoparticles in downregulating the expressions of IL-6, IL-1β, and TNF-α (**Kumar *et al*., 2017**).

Research suggests that IL 10 is involved in the development of RA with evidence suggesting its influence on the development of RA **(Degboe *et al*., 2019)**. Figure (3-9) shows Chrysin, Nio-chrysin NPs, and H-Nio-chrysin NPs increase IL-10 levels in rheumatoid cells and healthy cells, similar to Pang et al.'s study on flavonoids **(Pang *et al.*, 2019)**.

Figure 3-10 illustrates the methodology for regulating the concentration of nitric oxide (unprocessed), pure chrysin, Nio-chrysin NPs, as well as H- Nio-chrysin NPs exposed to PBMCs. Following a 48-hour duration of exposure, the levels of NO registered in the cohort treated with free chrysin, Nio-chrysin NPs, and H- Nio-chrysin NPs were reported as 39µmol/L, 37 µmol/L, and 22 µmol/L, correspondingly, as per the investigation conducted by Adangale et al. According to their research, nano-chrysin exhibits notable efficacy in scavenging free radicals and mitigating oxidative stress, thereby ameliorating the imbalance in oxidation-reduction processes **(Adangale *et al*., 2022).**

Nio-chrysin NPs enhance chrysin bioavailability, while H-Nio-chrysin NPs improve bioavailability through targeted delivery with hyaluronic acid, a key factor in RA pathogenesis **(Huang *et al*., 2023).** Excessive production of nitric oxide can lead to inflammation and contribute to the development of chronic inflammatory diseases, including RA, NO levels were found to be elevated in the serum of patients with RA compared to the control group **(Weitoft *et al*., 2022).** The anti-inflammatory activity of chrysin has been reported in several studies. It inhibits the inducible iNOS and release of NO and pro-inflammatory cytokines such as TNF- α and IL-1β **(Lee *et al*., 2015; Lateef *et al* ., 2014).**

In Figure, (3-11 a, b) it was found that TAC and SOD activities increased in PBMCs treated with H- Nio-chrysin NPs compared to control PBMCs. TAC refers to the total antioxidant capacity represented by FRAP, which is a measure of the ability of antioxidants to counteract oxidative stress and maintain redox homeostasis in biological systems **(Silverstrini *et al*., 2023).** There is an inverse relationship between TAC and the risk of developing RA, and this has been demonstrated by Vasilev et al **(Vasilev *et al.*, 2024).** Superoxide dismutase is an antioxidant enzyme that neutralizes superoxide radicals and protects against oxidative stress **(Rosa *et al.*, 2021).** The results of the current study were similar to the results of Mohammed et al, who found that antioxidant levels increased more after treatment with nano-chrysin compared to free chrysin **(Mohammed *et al*., 2023).**

4.3. Part III (Discussion of Molecular and Gene Expression Results)

Elevated MMP9 gene expression is observed in conditions marked by significant bone resorption, including RA **(Szumilas *et al*., 2020).** Treatment with pure Chrysin caused decreased expression of MMP-9 genes compared with the control group. Also, MMP-9 expression levels were significantly decreased in the H- Nio-chrysin NPs group compared to the control ($p < 0.01$) (**Tavakoli *et al*., 2018)**.

Figure (3-12) illustrates the expression levels of the examined genes in PBMCs both pre and post-treatment with pure chrysin, Nio-chrysin NPs, and H-Nio-chrysin NPs, resulting in a statistically significant outcome ($p \leq 0.01$). The administration of Nio-chrysin NPs was found to decrease MMP9 expression while elevating TIMP1 expression **(Talebi, M.,2021; Ciceu *et al*., 2021).** The regulatory role of tissue inhibitors metalloproteinase 1 (TIMP1) gene in MMP9 activity has been well documented **(Moghadam et al ., 2020).** The study found no significant

changes in TIMP1 expression in treated groups of pure chrysin and Nio-chrysin NPs due to improved bioavailability and PBMC interaction with hyaluronic acid **(Kim *et al*., 2020 ; Talebi *et al*., 2020)**.

Chrysin and its nanoparticles appear promising as potential treatments for inflammatory conditions such as rheumatoid arthritis, by reducing oxidative stress, suppressing pro-inflammatory cytokines, and modulating cellular pathways and gene expression of disease-related genes.

Nanoparticles in medicine face safety and toxicity challenges due to interaction with biological systems, requiring extensive clinical trials and sterile manufacturing for widespread clinical use **(Murthy and shashlik,2007; Dobson *et al* 2024**).

Conclusions and Recommendations

Conclusions

1. RA is characterized by elevated inflammatory markers such as TNF-α, and anti-inflammatory markers such as IL-10 which correlate with disease activity. RA patients experience increased oxidative stress and reduced antioxidant defences, contributing to the disease's pathogenesis and progression.

2. Obesity promotes a state of chronic inflammation and oxidative stress, with increased levels of inflammatory markers (TNF-α) and ROS (NO) coupled with reduced antioxidant defenses (SOD, FRAP).

3. Chrysin-loaded HA-coated nanoparticles showed a significant reduction in oxidant levels and suppression of inflammatory cytokine activity, as well as suppression of MMP9 gene expression and increased TIMP1 gene expression in patients. Taken together, these results suggest the potential therapeutic efficacy of chrysin-loaded HA-coated nanoparticles in alleviating inflammation and modulating immune response in RA patients.

Recommendations & Future Work

The current research project contains the following recommendations for the future:

1. The significant impact of hyperlipidaemia on patients with RA is very clear, so the research recommends making lipid analysis an approved method for those with a family history of the disease and monitoring the lipid profile of those with the disease to reduce the progress and development of RA.

2. There exists a necessity for additional research focused on a particular age of women, with a confirmation on the examination of hormonal variation occurring before and after to the onset of menopause.

3. Further prospective studies with larger sample sizes are needed to confirm our findings.

4. More research is necessary to understand the precise and complete effect of chrysin on markers of inflammation and oxidation in patients with RA. And also to explore its effects on people with obesity as a risk factor for the disease. Proposing it as a new therapeutic approach and improving its therapeutic potential by taking advantage of nano-based development.

5. More research and experiments are needed to study the effect of nano-chrysin on the gene expression of genes associated with RA more broadly and to use blood serum in the analyses instead of PBMCs.

6. Since the study was conducted ***in vitro***, further studies are needed to determine the effect of chrysin ***in vivo*** and its ability to adapt to body conditions.

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Appendix

Medical Journal of Babylon

Open access and peer reviewed international journal

The journal is indexed in DOAJ, EBSCO, Scimago Journal Ranking, SCOPUS

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Editor-in-Chief: Mufeed J. Ewadh, **Publisher:** Wolters Kluwer Medknow, **EISSN:** 2312-6760

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Acceptance of article for publication in Medical Journal of Babylon

Dear Dr.

Date: 2-07-2024

Sara Nadhim Sahib*, Fadhil Jawad Al-Tu'ma and Atheer Hameid Odda

I am pleased to inform you that your manuscript (**MJBL_534_24**) titled as:

Role of Inflammatory Status in Sera of Rheumatoid Arthritis in Obese/Non-Obese Iraqi Women and their Relations with Oxidative Status

has been accepted for publication in Medical Journal of Babylon.

We have received the payment for publication. So, you will receive the galley proof within 4-5 weeks. You must have to solve the query if we point out any in the galley proof.

After correction of galley proof, your article will be published online at https://www.medjbabylon.org

Best Regards

Prof. Mufeed J. Ewadh
Editor-in-Chief-MJBL

Corresponding author: Sara Sahib. Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala / Kerbala – Iraq.
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Ministry of Higher Education & Scientific Research
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جمهـــورية العـــراق
وزارة التعليم العالي والبحث العلمي
جامـــعة كربلاء
كلـــية الصيـــدلـــة
مجلة كربلاء للعلوم الصيدلانية

Number: 497
Date: 29.6.2024

COLLEGE OF PHARMACY UNIVERSITY OF KERBALA

Acceptance Letter

Dear Sir/Madam,

I am pleased to inform you that your manuscript titled " **Evaluation of Serum Antioxidants in Rheumatoid Arthritis of Iraqi Women** " authored by " **Sara Nadhim Sahib ; Fadhil Jawad Al-Tu'ma and Atheer Hameed Odda** " has been accepted for publication in the Kerbala Journal of Pharmaceutical Sciences. After careful consideration and peer review, we believe that your work makes a significant contribution to the field and will be of great interest to our readers.

Your manuscript will be an excellent addition to our upcoming issue.

Additionally, please ensure that you have completed and returned all necessary copyright forms and any other documentation required for publication.

Once again, congratulations on your acceptance, and thank you for submitting your outstanding work to Kerbala Journal of Pharmaceutical Sciences. We look forward to publishing your article and hope to receive more of your valuable research in the future.

Thanks,

Prof. Dr. Ahmed Salih Sahib

Editor in Chief

Email:kerbala.jps@uokerbala.edu.iq

العراق- محافظة كربلاء- مكتب بريد كربلاء- ص ب 1125

Certificate

3rd International Conference

For Pathological Analyses

26-27 February 2024
Basrah-Iraq

This Certificate is Awarded to
Sara Nadhim Sahib ; Fadhil Jawad Al-Tu'ma and

Atheer Hameed Odda

In the recognition of his/her participation in the Third International Scientific Conference for Pathological Analyses as a (Participant)

Prof. Dr. Mohanad Jawad Kadhim
Chancellor of Basra University

Asst. Prof. Dr. Majid Noori Humoud
Dean of Science College

The 12th International Scientific Conference of Al-Nahrain University College of Medicine

Certificate of Participation

This is to certify that **Dr. Sarah Nadhim Sahib** has Participated as a speaker in the 12th International Scientific Conference of Al-Nahrain University College of Medicine, held on **15-16th April 2024, Baghdad, Iraq.**

Prof. Dr. Anees K. Nile
Dean of College of Medicine/ AL-Nahrain University
President of Conference

University of Kerbala
College of Medicine
Medical Research Bioethical Committee
No: 23-43
Date: 21/12/2022

ETHICAL APPROVAL LETTER

Sara Nadhim Sahib
Biochemistry department\ College of Medicine \ University of Kerbala

Title of Project:
"Evaluating the Effects of Nano-Formulated Chrysin on the Inflammatory and Oxidative Markers Levels in Peripheral Blood of Rheumatoid Arthritis Patients of Iraqi Women"

This is to certify that proposal provided have satisfactorily addressed the research bioethical guidelines..
Please consider the following requirements of approval:

1. Approval will be valid for one year. By the end of this period, if the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to announce to the Committee. And you should inform the committee if the study extends over one year.
2. Please remember the Committee must be notified of any alteration to the project.
3. You must notify the Committee immediately in the event of any adverse effects on participants or of any unforeseen events that might affect continued ethical acceptability of the project.
4. Always consider the confidentiality of participants/ patients' information and/or opinions. And they must never be obligated to participate in the study and can withdraw at any time.
5. At all times you are responsible for the ethical conduct of your research in accordance with the standard bioethical guidelines.
6. The Committee should be notified if you will be applying for or have applied for internal or external funding for the above project.
7. This document does not compensate administrative or ethical approval might be required from directorate of Education in Karbala or other bodies.
8. All participants must be informed about the research issue and objectives prior to taking blood samples.
9. No cost or extra money for investigation should be charged on patients

Professor Dr. Ali A. Abutiheen
Chair, Medical Research Bioethical Committee
College of Medicine – University of Kerbala

Appendix

University of Kerbala – College of Medicine

Department of Chemistry and Biochemistry
Student Name: **Sarah Nadhim Sahib**
Supervisors:
Prof. Dr. Fadhil Jawad Al-Tu'ma - College of Medicine – University of Kerbala
Dr.Atheer Al-Ghanimi – College of Medicine – University of Karbala

((Experimental Data))

Evaluating the Effects of Nano-Formulated Chrysin on the Inflammatory and Oxidative Markers Levels in Peripheral Blood of Rheumatoid Arthritis Patients of Iraqi Women

Sample NO:	
Type of study: a case-control	
Inclusion Criteria: Rheumatoid arthritis RA, and rheumatoid arthritis with obesity	
Exclusion Criteria: Kidney disease, liver disease, cancer, and any acute or chronic inflammatory disease. Diabetes, all other autoimmune disorders, and infectious diseases.	
Rheumatoid arthritis description : severe(chronic) , moderate , slightly	
Age:	
Family History:	
Type of Treatment:	
Clinical Biochemical Markers :	
Total Antioxidant Capacity(FRAP)	
Superoxide dismutase SOD	
Tumor Necrosis factor –α TNF-α	
Nitric oxide NO	
Interleukin 10 IL-10	
Lipid Profile	
Total Cholesterol:	
HDL-Cholesterol:	
Triglycerides:	
LDL-Cholesterol:	
Height:	**Weight:**
BMI:	
Nanoparticles Studies	
Synthesis and characterization of hyaluronic acid-decorated loaded NPs and evaluation of MMP9 and TIMP1 gene expression in rheumatoid arthritis patients.	

الملخص

التهاب المفاصل الروماتويدي هو مرض مناعي ذاتي مزمن، مع انتشار أعلى بين الإناث. يتأثر تطوره بالإجهاد التأكسدي، وزيادة أنواع الأكسجين التفاعلية، والسمنة. يعاني مرضى التهاب المفاصل الروماتويدي من مستويات مرتفعة من الكوليسترول، بما في ذلك الكوليسترول الكلي، والكوليسترول الضار، والكوليسترول الثلاثي، وانخفاض الكوليسترول الحميد. يواجه الكريسين، وهو مركب فلافونويدي له العديد من الأنشطة البيولوجية، تحديات مثل ضعف قابلية ذوبانه في الماء، وانخفاض الاستقرار، والتمثيل الغذائي السريع، وانخفاض الامتصاص. يمكن لتكنولوجيا النانو التغلب على هذه العقبات من خلال السماح بالإطلاق البطيء والمتحكم فيه لجسيمات الكريسين النانوية، مما قد يحسن من توصيل العلاج ويقلل من الإفراط في التعبير عن الحالة الالتهابية. تلعب الجينات المهمة، مثل MMP9 وTIMP1، دورًا مهمًا في تقدم المرض وتطوره.

الأهداف يركز جهد البحث على صياغة وتقييم تأثير جسيمات الكريسين النانوية على الالتهاب والإجهاد التأكسدي ومستويات التعبير الجيني لدى المرضى المصابين بالتهاب المفاصل الروماتويدي. كما أنها تتضمن تقييم السمنة ومستوى الدهون وأهميتها في هذه الحالة المرضية وكذلك فحص ارتباطها بالمرض.

طرق العمل أجريت دراسة حالة وشاهد في مستشفى الإمام الحسن المجتبى ومستشفى مرجان الطبي من منتصف أكتوبر 2023 إلى مارس 2024، والتي شملت 130 مريضًا مقسمين إلى 70 حالة التهاب مفاصل روماتويدي و60 حالة ضوابط صحية ظاهريًا. تم تقسيم مجموعة التهاب المفاصل الروماتويدي إلى 35 غير مصابين بالسمنة و35 مصابين بالسمنة. تمت مطابقة الدراسة حسب الفئات العمرية وتم جمع 5 مل من الدم الوريدي من مرضى التهاب المفاصل الروماتويدي. تم تقسيم الدراسة إلى ثلاثة أجزاء. قام الجزء الأول بتقييم مستويات TNF-α و IL-10و NOو SODو FRAPوالدهون في التهاب المفاصل الروماتويدي. تم فحص وقياس مستوى الدهون في الدم باستخدام جهاز التحليل الكيميائي SMART-120 ونظام ELISA للكشف عن مستوى TNF-α و NOو IL-10و SODو .FRAPالجزء الثاني، بعد فصل مكونات الدم باستخدام Ficollواستخراج خلايا الدم المحيطية، تم التحقيق في المختبر في السمات البنيوية للجسيمات النانوية المحملة بالكريسين والمغلفة بحمض الهيالورونيك كعوامل مضادة للالتهابات ومضادات الأكسدة وإظهار آثارها على علامات الدراسة لخلايا الدم المحيطية لمرضى التهاب المفاصل الروماتويدي. تم قياس خصائص الجسيمات النانوية باستخدام المجهر الإلكتروني الماسح (SEM) والأشعة تحت الحمراء لتحويل فورييه

(FTIR)وتقنيات AFM و MTTو DLSالمختلفة. كان الجزء الثالث لتقييم تأثير الجسيمات النانوية المحملة بالكريسين والمغلفة بحمض الهيالورونيك على التعبير الجيني لمختلف الجينات بما في ذلك MPP9 و .TIMP1تم إجراء التحليل الإحصائي باستخدام الحزمة الإحصائية للعلوم الاجتماعية الإصدار 21.

النتيجة أظهرت الدراسة زيادة في مستويات ملف الدهون، وخاصة في المجموعة البدينين المصابين بالتهاب المفاصل الروماتويدي، وكذلك زيادة في جينات TNF-α و IL-10و NOو .MMP9بالإضافة إلى ذلك، أظهر المرضى انخفاضًا في مستويات HDL-C ومضادات الأكسدة وجين .TIMP1 كان تأثير جسيمات الكريسين النانوية المغطاة بحمض الهيالورونيك واضحًا على علامات الدراسة وكذلك التعبير الجيني، حيث قللت جسيمات الكريسين النانوية من الإجهاد الالتهابي والأكسدي، وزادت من التعبير عن جينTIMP1 ، وقللت من التعبير عن جينMMP9.

الخلاصة تبحث الدراسة الحالية في المؤشرات الحيوية كأدوات قوية وفعالة في تشخيص التهاب المفاصل الروماتويدي، جنبًا إلى جنب مع تقييم الفعالية في تقليل العلامات الالتهابية والأكسدة مع تعزيز علامات مضادات الأكسدة من خلال استخدام الكريسين النانوي المغلف بحمض الهيالورونيك.

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تقييم تأثير الكريسين النانوي على مستويات العلامات الالتهابية والأكسدة لدى النساء العراقيات المصابات بالتهاب المفاصل الروماتويدي (دراسة مخبرية)

رسالة ماجستير مقدمة

إلى مجلس كلية الطب/ فرع الكيمياء والكيمياء الحياتية/ جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في الكيمياء السريرية

من قبل

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