Spring 5-31-2020

Comparing the protective effects of Resveratrol, Curcumin, and Sulforaphane against Doxorubicin-mediated inflammation in macrophages

Haidy Amr Abdelfatah Saleh
The American University in Cairo

THE AMERICAN UNIVERSITY IN CAIRO
الجامعة الأمريكية بالقاهرة

School of Sciences and Engineering

Comparing the protective effects of Resveratrol, Curcumin and Sulforaphane against Doxorubicin-mediated inflammation in macrophages

A Thesis Submitted to

The Chemistry Master's Program

In partial fulfillment of the requirements for

The degree of Master of Science

By:

Haidy Amr Abd Elfatah Saleh Hassan

Under the supervision of:

Dr. Anwar Abd Elnaser (Advisor)
Assistant Professor and Director of Global Public Health Graduate Program, Institute of Global Health and Human Ecology (IGHHE), The American University of Cairo (AUC)

Prof. Dr. Hassan Azzazy (Co-advisor)
Professor and Chairman, Chemistry Department, The American University of Cairo (AUC)

Dr. Eman Ramadan (Co-advisor)
Assistant Professor, Pharmacology Department, Faculty of Pharmacy,
The British University in Egypt (BUE)

Prof. Dr. Mohey Elmazar (Co-advisor)
Professor and Dean, Pharmacology and Toxicology Department, Faculty of Pharmacy,
The British University in Egypt (BUE)

Spring 2020

ACKNOWLEDGEMENTS

I would like to convey my immense gratitude to my advisor, Dr. Anwar Abd Elnaser. Indeed, I do thank him for his endless effort and support and for guiding me all the way throughout my lab work and giving me a true opportunity to learn new skills. He helped me know every single aspect in research till I completed my thesis successfully. Besides research, he taught me how to practice critical thinking and to analyze my outcome. I have really learned a lot from his dedication and persistence as an advisor. I am very proud to call myself his student and I will be in his debt forever.

I am very delighted to have worked with Dr. Eman Ramdan and to have been supervised and guided by her. I had a great chance to have hands on experience and to optimize part of my experiments at BUE under her supervision. I would not be able to plan my experiments and troubleshoot properly without her suggestions. I am grateful for her continuous care and consistent words of advice. I have learned a lot from her and I do really find myself in debt for her endless support and guidance.

I would also like to express my forever gratitude to Dr. Mohey Elmazar, my co-advisor and Dean of Pharmacy at BUE for his continuous support and supervision during my work. I am really lucky for his contribution in my thesis as he was always there any time to help me out in planning my experiments and writing my thesis. I am thankful for his support throughout my journey since day one of joining BUE and starting my Master Degree at AUC. I consider him a second father to me and a great support in my life.

I sincerely thank Prof. Dr. Hassan Azzazy, my co-advisor and Chairman of Chemistry Department for his encouragement and kind support. He was very keen to offer great help whenever asked. I feel blessed for his contribution in my thesis.

I am grateful from the heart to Prof. Hassan Elfawal, Dean of SSE at AUC for his motivation and his valuable comments during the seminar class and in my proposal defense which added to my research plan and opened my horizons to think differently.

I would also like to thank all my colleagues and friends at BUE and AUC. I consider myself very lucky to get a precious opportunity to join AUC and to obtain my Master Degree from such a highly reputable university.

Last but not least, I would love to dedicate this work to my father, my mother, my sister and all my family. I am forever grateful for their infinite support and encouragement and I admit that this work wouldn't have been possible without them. I am grateful from the heart to the many blessings of GOD throughout my journey.

ABSTRACT

Comparing the protective effects of Resveratrol, Curcumin and Sulforaphane against Doxorubicin-mediated inflammation in macrophages

Doxorubicin (DOX) anti-tumor activity is accompanied by severe side effects such as cardiotoxicity and cardiomyopathy. These side effects are hypothesized to be mediated through doxorubicin induced inflammation. The aim of the current study, therefore, was to compare the effect of three naturally derived compounds with anti-inflammatory effects namely, curcumin (CUR), resveratrol (RES) and sulforaphane (SFN) against doxorubicin-mediated inflammation through affecting TLR4 signaling pathway in the murine macrophage cell line RAW264.7 stimulated with Lipopolysaccharide/Interferon-gamma (LPS/IFN- γ).

In present study, RAW264.7 cells were stimulated with LPS/IFN-γ at a concentration of 10 ng/10 U/mL in the absence and presence of DOX (0.1 μM) plus increasing concentrations of CUR, RES or SFN (5 - 20 μM) for 24 h. Cell lysates of LPS/IFN- γ stimulated RAW264.7 macrophages were collected and assayed for the mRNA expression of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and toll like receptor 4 (TLR4) and miRNA expression of miR-146a, miR-155, and miR-21 levels using real-time PCR. Furthermore, TNF-α and IL-6 protein were characterized in culture supernatants *via* enzyme-linked immunosorbent assay (ELISA). Nitric oxide (NO) production was determined using the Griess assay and cell viability was assessed via 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Our results showed that LPS/IFN-γ with/out DOX significantly induced TNF-α, IL-6, and iNOS, and downregulated TLR-4 mRNA expression levels. Also, it upregulated miR-146a and miR-155, with no significant effect on miR-21. DOX alone was unable to induce any of these markers. Moreover, the LPS/IFN-γ mediated upregulation of TNF-α and IL-6 on the mRNA level was further translated to protein levels. Additionally, LPS/IFN- γ mediated induction of iNOS resulted in a significant increase in NO production that persists in the presence of DOX. Upon treating the LPS/IFN-γ stimulated macrophages with the different concentrations of the three naturally derived compounds, SFN was the only compound to show a significant decrease in LPS/IFN-γ-induced TNF-α, IL-6, and iNOS mRNA expression levels. This effect of SFN in response to LPS/IFN-γ stimulation was conserved at the TNF-α and IL- 6 protein levels and NO production in the absence and presence of DOX. Interestingly, SFN significantly inhibited the LPS/IFN-γ mediated induction of miR-146a and miR-155 miRNA levels in RAW 264.7 cells. Last but not least, the SFN inhibition of the inflammation markers and miRNAs was not due to any cytotoxic effects as evident by MTT assay.

Our findings suggest that SFN, compared to CUR or RES, significantly exerted an anti-inflammatory response in RAW264.7 cells stimulated with LPS/IFN-γ with/out DOX. This effect was confirmed on the transcriptional and post-transcriptional levels. Altogether, these findings provide an important rationale to utilizing SFN as a potential adjuvant for the prevention and/or treatment of DOX mediated cardiotoxicity that may be in part due to inflammation. The present study, therefore, can be implied in future clinical trials.

TABLE OF CONTENTS

List of Tables

List of Figures

LIST OF ABBREVIATIONS

AIC	Anthracycline-induced cardiotoxicity
AP-1	Activator protein-1
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CHF	Congestive heart failure
COX-2	Cyclooxygenase-2
CUR	Curcumin
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DAU	Daunorubicin
DCs	Dendritic cells
DIC	Doxorubicin-induced cardiotoxicity
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ELISA	Enzyme-linked immunosorbent assays
eNOS	Endothelial nitric oxide synthase
EPI	Epirubicin
ERK	Extracellular signal-regulated kinase
GSH	Glutathione
HDAC	Histone deacetylase
HF	Heart failure
hMDMs	Human monocyte-derived macrophages
IDA	Idarubicin
IFN-γ	Interferon gamma

Ig	Immunoglobulin
IKB-α	Inhibitor kappa B alpha
IKK	IκB kinase
IKK-ε	IκB kinase epsilon
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IP-10	IFN-inducible protein 10
IRAK	Interleukin-1 receptor-associated kinases
IRF-3	Interferon regulatory factor 3
IκB	Inhibitor of NF-κB
JNK	c-Jun NH2 terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LDL	Low-density lipoprotein
LOX	Lipooxygenase
LPS	Lipopolysaccharide
LTs	Leukotriens
MAPK	Mitogen activated protein kinases
MHC	Major histocompatibility complex
miRNA	Micro RNA
mRNA	Messenger RNA
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide
MyD-88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
Nrf-2	Nuclear factor erythroid-derived 2-like 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PDCD4	Programmed cell death protein 4 expression

PGE2	Prostaglandin E2
PGs	Prostaglandins
PPARs	Peroxisome proliferator-activator receptors
PRRs	Pattern recognition receptors
qRT-PCR	Real-time polymerase chain reaction
RES	Resveratrol
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Reverse transcription
SFN	Sulforaphane
SOD	Superoxide dismatuse
TAK1	Transforming growth factor beta-activated kinase 1
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TIR	Toll/IL-1R receptor
TIRAP	TRIF-related adaptor molecule
TLR	Toll like receptors
TNF-α	Tumor necrosis factor alpha
Top2	Topoisomerase 2
TRAF	Tumor necrosis factor receptor-associated family
TRIF	TIR domain containing adaptor protein-inducing IFN-β
UTR	Untranslated region

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Immunity

The immune system has been derived to protect the host against a population of constantly evolving pathogens and microbes (1). It also removes toxic or allergic substances that enter the bloodstream through mucosal surfaces, skin or intestinal microflora (1). Aside from its ability to respond to and destroy any invading pathogen, the immune system can distinguish between self and non-self-antigens, a property known as self-tolerance (1, 2). Any failure of self-tolerance, however, introduces the broad class of autoimmune diseases (2).

Immunity produces multiples of cells to keep the surveillance upon every part of our body (2). In adulthood, the majority of immune cells are produced from bone marrow, which includes different kinds of white blood cells, such as neutrophils, lymphocytes, monocytes, basophils and eosinophils (2, 3). Immune cells usually express receptors that enable cell-cell and cell-environment interactions; these receptors are primarily responsible for translating signals of danger, infection or abnormal cell death coming from the surrounding cell environment (2). In addition to extrinsic receptors, there are intrinsic receptors inside cytoplasm of the immune cell that can detect virus infection by binding to its nucleic acid (3). In response to infection, immune cells use a programmed system for detecting foreign substances and respond by producing cytokines that act as an alarm for surrounding tissues. This early response of the innate immune cells destroys infected cells leaving cell fragments that could further stimulate adaptive immune system directed to the inciting infection (2). The major inflammatory cytokines released during early immune response to infection include: tumor necrosis factor-alpha (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6) (3). These cytokines recruit other immune cells associated with local inflammation for pathogen elimination; and, therefore, any dysregulation of inflammatory cytokines production results in the development of inflammatory or autoimmune diseases (3). They could, therefore, be a promising therapeutic targets.

1.1.1 The Immune System

The immune system is based on two arms: the innate, non-specific immune system and the adaptive, specialized immune system **(3)**. Both systems use cellular and humoral defense

mechanisms, the first is defense cells in tissues and the latter is defense cells in blood and body fluids (3). The host uses both innate and adaptive mechanisms to detect and eliminate pathogenic microbes (1). In early immune response, the non-specific innate system initiates fast action and recruits immune cells rapidly to the site of infection or inflammation through cytokines and chemokines production (2, 3). The most important innate immune system cells are neutrophils and macrophages, which are effective phagocytes that act as the first line defense against foreign bodies (2, 4). They engulf foreign bodies and release enzymes that digest proteins, a process called phagocytosis (2). These cells are more efficient when the pathogen is tagged by either antibodies or proteins from the complement system, which explains the fast response observed in secondary infections (1). Phagocytes also help antigen-specific cells to easily detect microbes by digesting the marked pathogens to contain the infection, and then express the microbe antigen for easy detection (1). Thus, the innate immune response contributes to the activation of the antigen-specific cells and, therefore, the synergism between the two immune responses is a critical step for an effective immune response.

1.1.1.1 Innate Immune System

The innate immune system is the non-specific, inherited immune defense mechanism encoded in the germ-line genes of the host (1). Innate immune system includes: I) Physical barriers such as, skin epithelial layer, epithelial mucus-lining, and cilia to clean the mucus layer (1). II) Physiologic barriers such as, temperature, low pH, and chemical mediators, including complement system, which is a group of soluble proteins in biological fluids that tag pathogens for phagocytes, lysozyme, which cleaves bacterial cell wall, and interferon, which induces antiviral defense in neighboring non-infected cells (3). III) Inflammation is mainly due to leakage of vascular fluid from damaged or infected tissues that help in phagocytes chemotaxis to the area of infection (3). VI) Innate immune cells, which are white blood cells either found in tissues, such as macrophages, fibroblasts, mast cells, and dendritic cells or circulating in blood, such as monocytes and neutrophils (4). In addition, Natural killer cells are a type of cytotoxic lymphocyte that used to recognize virally infected or cancerous cells through detecting changes in cell surfaces (2). Collectively, the majority of these cells are known as antigen-presenting cells (APCs) that are used to detect microbe invasion or tissue damage with intracellular or extracellular expressed receptors,

known as pattern recognition receptors (PRRs) that bind to molecular patterns expressed on the surfaces of invading microbes (5). APCs are heterogeneous, specialized immune cells which mediate cellular immune response by presenting environmental antigens, such as ligands for TLR and other PRRs for recognition by T lymphocytes (6, 7). They are found in large numbers in the skin and mucosal sites, where pathogen encounter is most likely, and include dendritic cells, macrophages, Langerhans cells and B cells (7) (Fig. 1.1).

1.1.1.2 Adaptive Immune System

In contrast to the innate immune system, adaptive immune response is an antigen-specific system that deals with manipulative infections which is not resolved by the innate immune system mechanisms (1). Different from innate PRRs, the antigen-specific receptors of the adaptive immune cells are encoded by germ-line genes that undergo somatic rearrangement to form vast number of specific T cell receptor (TCR) and immunoglobulin, B cell antigen receptor (Ig) genes (1, 6). Adaptive immune system is activated at a late stage and recruits specialized lymphocytes that possess the property of adaptation and memory, which further enhances fast response against second-time infection (2). Generally, adaptive immune system includes**:** Antigen-specific T-lymphocytes, which are activated to proliferate in response to antigen presenting cells (APCs) (3). T-cells are specialized into T-helper cells (CD4 T cells), T-killer or cytotoxic-T cells (CD8 T cells), and memory-T cells (3). Through their TCR receptors, T-cells interact with antigenic peptides complexed with major histocompatibility complex (MHC), which is an immune cell surface protein expressed on APCs (8). Subsequently, subtype CD8 T cells interact with peptides expressed on MHC class I, expressed on nucleated cells. Antigens presented to MHC class I are intracellular either from the host genome of cells or from replicating virus. While, CD4 T cells react with MHC class II found on APCs in response to mainly bacterial antigen ligands, such as Toll-like receptors (TLRs) ligands (6). The other cellular tool for adaptive immune response is B-lymphocytes, which differentiate either into memory cell that is used later in the future, or into plasma cells to produce antibodies (3). Antibodies are the integral humoral part in adaptive immunity, which are produced in response to signals received from T cells or other cells, such as dendritic cells (6) (Fig. 1.1).

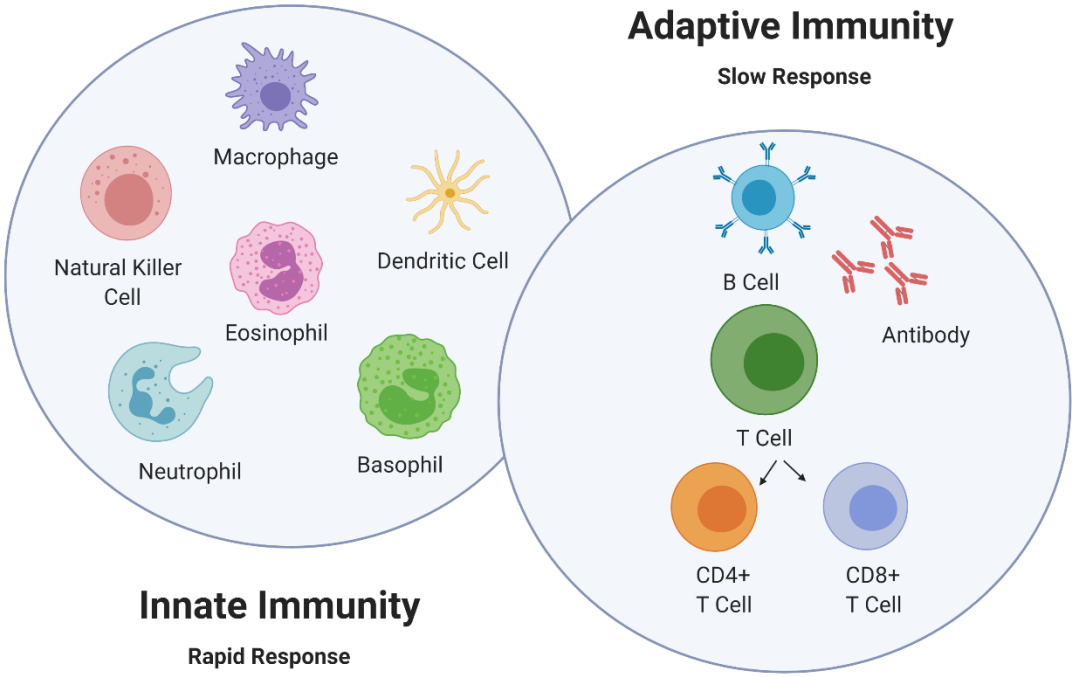


Fig. 1.1. Schematic diagram of innate and adaptive immune systems (9).

(Created with Biorender.com)

1.1.2 Immune Response

The response of innate immunity begins when pattern recognition receptors (PRRs) expressed in immune cells detect either pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins, and carbohydrates, or damage-associated molecular patterns (DAMPs) released from damaged cells (5, 10). Then oligomerization of the receptor followed by assembly of the activated PRRs subunits occurs, which initiates signaling cascades leading to activation of mediators that promote attraction of leukocytes to the site of infection or injury (5, 10). Then these leukocytes, including macrophages, neutrophils, and dendritic cells phagocytose microbial

elements and release more pro-inflammatory cytokines including TNF-α, IL-6, IL-12, and type I and II interferons (IFNs), which collaboratively attempt to contain the pathogen until highly specific, activated cells of the adaptive immune response are recruited to completely eliminate the infection (10, 11). Upon engulfment of a pathogen, antigen presenting macrophages express antigen peptides derived from the engulfed pathogen on the immune cell surface protein MHC class II in order to recruit CD4 T cells, one of the adaptive immune cells (6, 11). This connection between phagocytic immune cells and T-lymphocytes, therefore, shapes the link between innate and adaptive immunity.

1.1.2.1 Inflammation

Inflammation is a complex set of defense mechanisms acting complementary to restore homeostasis in body systems after injuries or infections (12). Inflammation is one of the mechanisms of the innate immunity and consecutively define the adaptive immune response against certain pathogens (10). Inflammation is triggered first when innate immune cells identify infection or tissue injury, then inflammatory response is activated, expressed in the form of redness, swelling, heat and pain (13). These inflammatory signs are mainly a result of the vasodilation due to the release of histamine, prostaglandins, and nitric oxide that leads to a noticeable increase in blood flow and accumulation of circulating leukocytes (10). Hand in hand, secreted cytokines from activated immune cells, such as TNF-α and IL-1 enhance the vascular permeability of leukocytes through rising the levels of leukocyte adhesion molecules on endothelial cells (5, 14). Also, inflammation is induced by allergens, which form antibody complexes that stimulate Fc receptors on mast cells, releasing histamine (10).

1.1.2.2 Inflammatory Mediators

A plethora of inflammatory mediators are secreted during different inflammatory responses and they are categorized to pro- and anti-inflammatory mediators (15). From the inflammatory mediators commonly associated with several inflammatory conditions are the cytokines (e.g., interferons, interleukins and tumor necrosis factor α), the chemokines (e.g., monocyte

chemoattractant protein 1), the eicosanoids (e.g., prostaglandins and leukotrienes), the Nitric oxide, and the transcription factor nuclear factor-κ B (NF- κB) (15).

Cytokines are small proteins released from activated cells that mediate the innate immune response and regulate the function of other cells through cell-cell communication (1, 16). Cytokines can be considered as "hormones" of inflammatory responses, but unlike a hormone which is a primary product of a specialized cell, cytokines can be produced by many different cell types including those of the immune system and they are more potent than hormones (17). Cytokines are categorized into lymphokines, which are cytokines from lymphocytes, monokine, which are cytokines from monocytes, chemokine, which are cytokines with chemotactic activities, and interleukin, which are cytokines released by one leukocyte to act on another (18). Cytokines have autocrine and paracrine action, because they act on their secretory cells, and nearby or distant cells (18). Cytokines are grouped into pro-inflammatory, such as TNF-α, IL-1 β, IL-6, and anti-inflammatory, such as IL-10, IL-4, IL-11, and IL-13 (18, 19).

An important pro-inflammatory cytokine, Tumor necrosis factor (TNF)-α is associated with multiple inflammatory diseases and cancers (15, 20, 21). Similarly, Interleukins also possess pro-inflammatory activity, including IL-1α, which stimulates the secretion of pro-inflammatory cytokines such as TNF-α and IL-1β, yet has been also associated with anti-inflammatory activity (22, 23). IL-6 and IL-12 family of cytokines mainly act as pro-inflammatory cytokines but can be also involved in some anti-inflammatory reactions (24-26). In contrast, IL-10 is a potent anti-inflammatory cytokine, which hinders the action of many pro-inflammatory mediators maintaining tissue homeostasis and alleviating the damage from inflammatory exaggerated response (27-29). *Chemokines*, a subgroup of cytokines also contribute to the process of inflammation; they are chemo-attractants that drive the migration of inflammatory leukocytes, inflammatory lipid mediators, reactive free radical species, which are unstable and reactive molecules with unpaired electrons in the outer orbital, and bioactive amines and enzymes (1, 16, 30). They also affect angiogenesis and the activity of myeloid cells (17)

Second to cytokines and chemokines, Eicosanoids, including Prostaglandin (PG) and leukotrienes (LTs) are involved in inflammatory response (15). Prostaglandin (PG) E2 is the most common PG in human physiology and pathology (15). Physiologically, it plays a role in regulating normal body temperature, gastric mucosal integrity, renal blood flow and the function of female

reproductive system; however, alterations in PGE2 activity results in pathological conditions, such as inflammatory diseases, abnormal changes in body temperature, and colorectal cancer (15, 31). PGs are synthesized from arachidonic acid, coming from cell membrane phospholipids, by the inducible enzyme COX-2 enzyme (31). While Leukotrienes (LTs), such as LTB4, are synthesized from arachidonic acid by 5-lipooxygenase (5-LOX) enzyme and are also linked to human inflammatory illness states and asthma (32-34).

Indeed, it is known that immune cells liberate a number of reactive species at the site of inflammation leading to oxidative stress, which establishes the link between oxidative stress and inflammation (35). Nitric oxide (NO) is a physiological mediator that acts as a vasodilator and increases circulatory flow in the body (36). Although NO is a common inflammatory mediator that is important in host immune defense against infection, its overexpression is responsible for many inflammatory diseases (36). NO is generated by nitric oxide synthase (NOS) enzyme, which mainly exists in inducible NOS (iNOS) and endothelial nitric oxide synthase (eNOS) isoforms (15, 37). This reactive nitrogen species can upregulate the expression of the pro-inflammatory genes through initiating an intracellular signaling cascade (38).

Besides the aforementioned enzymes associated with inflammatory responses, the transcription factor NF-κB is highly involved in the pathophysiology of inflammation and cancer, and it is a fundamental regulator of immune responses induced by LPS or pro-inflammatory cytokines (15, 39-41). The NF-κB machinery in humans includes p50 and p65, which regulate both physiological and pathological processes (15, 39, 40, 42). NF-κB proteins are localized in the cell cytoplasm in a quiescent form bound to a family of inhibitory proteins, known as inhibitor of κB (I-κB), which blocks the nuclear translocation of NFκB (41, 43-46). When NF-κB is provoked with an inflammatory stimuli, the I-κB inhibitory proteins are phosphorylated and degraded, then NF-κB is translocated to the nucleus and activates the transcription of inflammatory-associated genes, such as those encoding iNOS, COX-2, and pro-inflammatory cytokines through binding to a consensus sequence in the promoter region of the target genes (40-42, 47). It has to be mentioned that the phosphorylation of I-κB in the resting state is directly inhibited by I-κB kinase (IKK), and that the inhibition of NF-κB activity has consistently been effective for controlling inflammatory diseases in animal models (41, 48-50).

1.1.3 Role of Macrophages and Phagocytosis in Inflammation

Phagocytosis is an important defense mechanism against microbial pathogens; it depends on pathogen lysis followed by pathogen-antigen presentation (3). Macrophages is one of the phagocytic leukocytes that are distributed as a network among tissues, and accelerate the immediate detection of both invading pathogens and associated tissue damage (3, 5).

Originally, macrophages are differentiated monocytes that are involved in host immune response (51). They release inflammatory signaling molecules, such as cytokines and chemokines, which in turn activate other immune cell types and chemotactically recruit other immune cells to the site of infection (52, 53). Physiologically, macrophages differentiate from hematopoietic stem cells in bone marrow, and exhibit heterogeneity that originates from their differentiation pathways from monocytes and also from different tissue microenvironments in the body (54, 55). Such heterogeneity in morphology, signaling, phenotype and response to stimuli is mainly due to the different functions required by macrophages in different tissues (56). They can adapt quickly to new stimuli, and have the ability to polarize and differentiate to osteoclasts, Kuppfer cells or dendritic cells depending on the physiological state (57-60).

The polarization state of macrophages is dependent on the type of stimulation (4). Macrophages are classified as M1 or M2 subgroups, in terms of their surface markers and the types of secreted cytokines (61). The M1 macrophages (iNOS+, CD80+, MHCII+) are pro-inflammatory cells that are classically activated *via* TLR stimulation by LPS or IFN-γ, and usually secrete IFN-γ and TNF-α (61, 62). On the other hand, M2 macrophages (Arg-1+, CD163+,CD206+) are anti-inflammatory cells that are triggered by IL-4 or IL-10 (63, 64). Macrophages can also be activated innately *via* a humoral route through antibody or opsonins binding to cell Fc or complement receptors (65, 66).

Activated macrophages are characterized by their great capacity to produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, IL-23, and IFN- γ, and they release NO through iNOS overexpression (1, 67). Subsequently, M1 macrophages possess the ability to eliminate infections caused by bacteria, viruses or fungi (68, 69).

On the contrary, M2 polarized macrophages via alternative activation can diminish the inflammatory process, which is caused by M1 polarized macrophages, by producing anti-

inflammatory factors, such as IL-10 and transforming growth factor beta (TGF-β) (70). The M2 polarized macrophages are thought to stimulate tissue repairing mechanisms post inflammation (71).

1.1.3.1 Pattern Recognition Receptors (PRRs)

The innate immune system, including macrophages and other innate immune cells are activated through the previously mentioned germ-line encoded PRRs which recognize PAMPs and DAMPs (10, 11). DAMPs are endogenous molecules in cells that are released in cases of cell damage or stress, and they include heat shock proteins, ATP, IL1a cytokine, uric acid, serum amyloid A, cytoplasmic proteins (S100A8 and S100A9), and the DNA-binding nuclear protein HMGB1 (5, 10). While PAMPs are pathogen-derived molecules important for microbial survival, such as lipopolysaccharide (LPS), flagellin, and bacterial and viral nucleic acids (5, 10). PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (retinoic acid inducible), pentraxins and C-type lectin receptors (CLRs) (16).

One of the essential and first-identified members of PRRs are TLRs (5, 16). They are expressed on various immune cells, including macrophages, dendritic cells (DCs), B cells, specific types of T cells, and even on nonimmune cells such as cardiac cells, fibroblasts and epithelial cells (1). TLRs are type I transmembrane proteins characterized by the extracellular leucine-rich repeats (LRRs) that recognize different microbial epitopes and has a cytoplasmic signaling domain similar to that of the interleukin 1 receptor (IL-1R), called Toll/Interleukin 1 receptor (TIR) domain, which is responsible for signal transduction (1, 4, 5, 72). TLR family was first identified in Drosophila flies, and now twelve members of the TLR family have been identified in mammals (5, 72) . Grouped as subfamilies, TLR1, TLR2, and TLR6 recognize lipids, TLR7, TLR8, while TLR9 recognize nucleic acids (10, 11). Some receptor/ligand pairs are commonly known, such as TLR4 and LPS, TLR5 and flagellin, TLR 1, 2, and 6 with lipoproteins (72). In addition, TLRs are either expressed on the cell-surface, e.g. TLRs 1, 2, 4, 5, and 6, or internalized to the endosome, e.g. TLRs 3, 7, 8, and 9 (5, 11, 72). Together with phagocytic-antigen presentation, the activation of TLRs lead to inflammatory cytokines expression, which further recruits antigen-specific cells (72). On this account, TLRs have a strong bond with the phagocytic process of bacteria, confirmed by

the phagocytic impairment in the absence of either TLR4/TLR2 or TLR signaling adaptor (MyD88) (72, 73).

1.1.3.2 TLR4 signaling pathway during inflammation

Activation of signal transduction pathways by TLRs leads to the induction of various genes that function in host defense, including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules (72). Mammalian TLRs also induce multiple effector molecules, such as iNOS and antimicrobial peptides that can directly destroy microbial pathogens (10). Both TLRs depend on TIR domains to activate NF-κB and MAPK (mitogen activated protein kinases) and induce target genes, yet there are multiple differences in pathways induced by individual TLRs (5, 72). Upon ligand binding (e.g. LPS), TLR4 dimerizes and induce the recruitment of intracellular adaptor proteins that triggers two standard models of signaling cascades: myeloid differentiation primary response gene 88 (MyD88)-dependent and Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-beta (TRIF) - dependent pathways (10, 72).

First, MyD88-dependent pathway originates from cytoplasmic TIR domain. The activation of MyD88 causes autophosphorylation of interleukin-1 receptor-associated kinases (IRAK), namely IRAK1, IRAK2, and IRAK4, which associate temporarily with TNF receptor-associated factor 6 (TRAF6) (10, 72). This autophosphorylation and oligomerization for IRAK and TRAF6, respectively, will finally lead to the activation of IκB kinase (IKK) (in response to TAK1/TAB complex activation) and mitogen-activated protein kinase (MAPK), namely ERK, JNK, p38 (14, 72). Then, succeeding signal dissemination results in the activation and the translocation of nuclear factor kappa B (NF-κB) to the nucleus and the subsequent activation of the activator protein-1 (AP-1) transcriptional program, respectively (10). And both NF-κB and AP-1 control inflammatory responses through the induction of inflammatory cytokines, such as TNF-α, IL-12, and others (10, 72). Second, TRIF - dependent pathway, which mediates the late phase activation of NF-κB, primarily recruits TRIF, and this results in the ubiquitination of TNF receptor-associated factor 3 (TRAF3) which will induce TANK-binding kinase 1 (TBK1) binding to IκB (inhibitor of NF-κB) kinase epsilon (IKKε)) (10, 74-78). Thenceforth, the TBK1-IKKε complex phosphorylates the transcription factor interferon regulatory factor 3 (IRF3), ultimately driving the

expression of interferon-beta (IFN-β), which induces STAT1-dependent genes encoding monocyte chemoattractant protein 5 (MCP-5), IFN-inducible protein 10 (IP-10) and iNOS (79, 80) (Fig. 1.2).

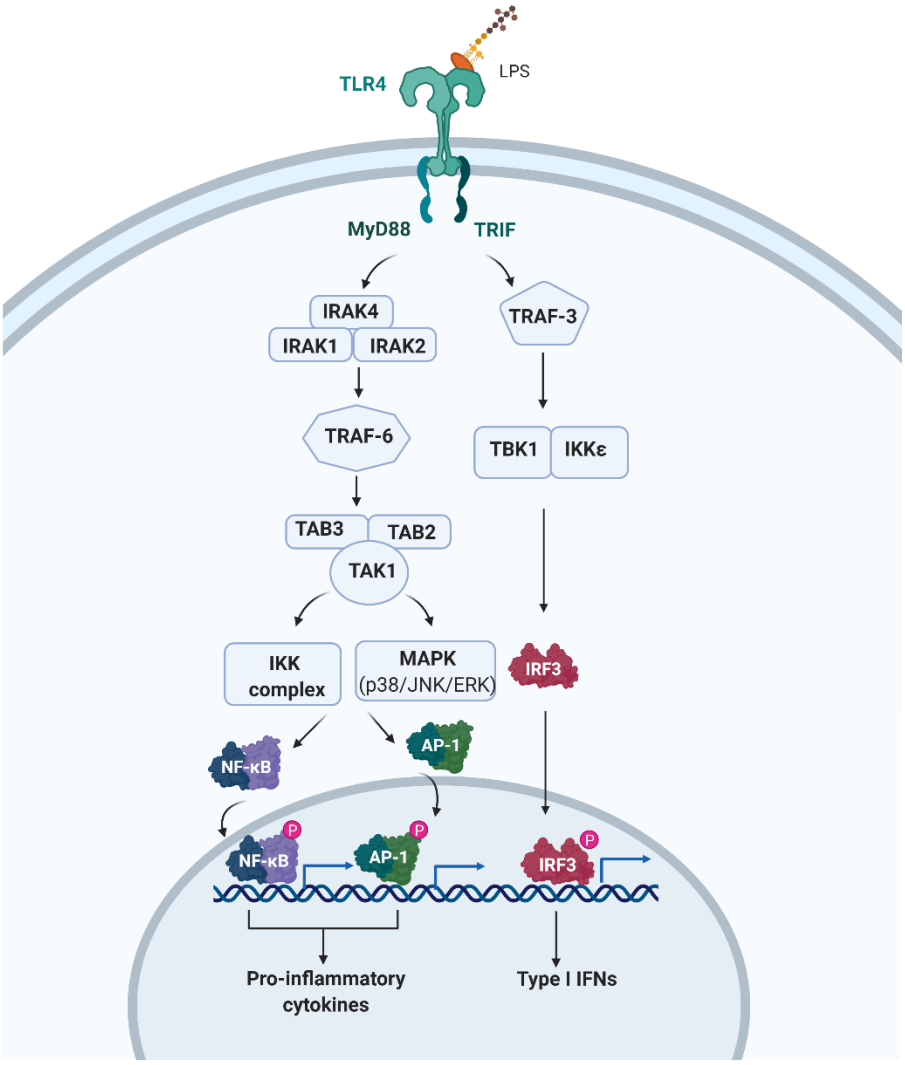


Fig. 1.2. TLR4 signaling pathway (81).

(Created with Biorender.com)

1.1.4 Role of miRNAs in TLR4 Signaling during inflammation

miRNAs are short double-stranded, small, non-coding RNA molecules comprised of ~22 nucleotides in length (82). They bind imperfectly to the 3` untranslated region (UTR) of a target mRNA sequence causing its down-regulation at the post-transcriptional level (83). UTR is found 3′ to the coding region, and is considered the non-protein coding section of mRNA (84). miRNAs are currently viewed as essential regulators in key immune responses, for example regulation of maturation, proliferation, differentiation and activation of both innate and adaptive immune cells (85). TLR-responsive miRNAs are either up-regulated or downregulated after LPS treatment (85). In inflammatory reactions, the expression of the most miRNAs is triggered in an NF-κB-dependent manner after TLR stimulus (82). Thus, miRNAs expression is dependent on TLR stimulation, which is confirmed by the results of Baltimore lab demonstrating the relationship between miR-146a and miR-155 upregulation in human monocytes with LPS stimulation (86-90). In this context, miRNAs are classified as either "early response miRNAs", expressed rapidly after LPS stimulation such as miR-146 and miR-155 or "late response miRNAs", expressed in macrophages at a later time after LPS treatment such as miR-21 (86-90). Not only expressed in response to TLR activation, miR-146a-5p, miR-155-5p, and miR-21 are also involved in the regulation of TLR downstream signaling through TLR-induced transcriptional factors (91, 92). In addition, miRNAs also target cytokines such as type I IFNs, TNF-α, IL-6, IL-12, and IL-10 and this has been indicated by the presence of binding sites for miRNAs on the mRNAs encoding for these cytokines and chemokines (93-95). The miRNAs: miR-21, miR-146a, and miR-155 are predominant in much miRNA research because of their expression levels succeeding TLR stimulation especially in macrophages (85) (Fig. 1.3).

1.1.4.1 miR-146a

First, miR-146a, one of the miR-146 family that is present in chromosome 5 and 10, is a significant key molecule in inflammatory response (85, 96-98). Upregulation of miR-146a has been reported in inflammatory diseases such as osteoarthritis and rheumatoid arthritis (99). A recent study illustrated that miR-146a play a role in the regulation of oxidized low-density lipoprotein (oxLDL) accumulation and inflammatory response in macrophages through negatively regulating TLR4 and inhibiting the activation of TLR4-dependent signaling pathways (100-102). Another study illustrated the role of miR-146a as a negative regulator of type1 interferon response

in human peripheral blood mononuclear cells (PBMCs) (103). Furthermore, previous reports demonstrated that miR-146a negatively regulated TLR system through targeting TRAF6 and IRAK1 (components of the MyD88-dependent pathway for TLR-mediated signaling) (88, 104-107). This mechanism of miR-146a has been also reported to be involved in regulating cytokines release and apoptosis in human dendritic cells (88, 108). IRAK2 and IRAK4 has been addressed as targets of miR-146a, which results in decreasing inflammatory cytokines (88, 104, 109). Moreover, increased expression of TRAF6 has been observed with 5q chromosomal deficient models, leading to impaired innate immune signaling causing leukemia and bone marrow failure (110). Also, miR-146a-5p has been shown to degrade IRF3 transcript in LPS-treated human monocytes (111). From this angle, a recent study showed that by targeting IRAK1, IRAK2, and TRAF6 in LPS stimulated macrophages, miR-146a sequentially suppresses the production of type I IFNs, TNF-α, IL-1β, and IL6 (104, 106) (Fig. 1.3).

1.1.4.2 miR-21

Second, miR-21 is a cancer associated miRNA that induced by NF-κB activation, and acts as a negative regulator of TLR signaling inflammatory responses (85, 112). miR-21 inhibits MyD88 and IRAK1 expression during hepatitis C viral infection in PBMCs (113). The role of miR-21 in LPS-induced RAW 264.7 macrophages has been reported in a Sheedy et al. study, in which miR-21 downregulated the expression of programmed cell death protein 4 expression (PDCD4) (114). PDCD4 acts as a tumor suppressor protein that activates the pro-inflammatory mediators NF-κB and IL-6, and suppresses the anti-inflammatory cytokine IL-10 which inhibits induced levels of miR-155 (114, 115). Thus, miR-21 positively induced IL-10 production, and downregulated NF-κB activity, controlling lethal LPS response (85, 114) (Fig. 1.3).

1.1.4.3 miR-155

Similar to miR-21, miR-155 is another tumor-associated miRNA that has a significant role in the TLR-mediated immune response and can target related signaling proteins, which are components of the NF-κB pathway (82). miR-155 is most commonly known to enhance inflammatory reactions, evidenced by Baltimore et al. study which linked miR-155 upregulation

with mammalian inflammatory reactions (88, 116). Other studies, such as that of Tili et al. showed that miR-155 expression can have both positive and negative effects on NF-κB signaling proteins, however they supported the positive feedback of miR-155 on NF-κB pathway by increased serum TNF-α in miR-155 transgenic mice (117). In further support of the positive role of miR-155 in inflammation, miR-155 drives inflammatory cytokine production by targeting SH2 (Src homology 2)-containing inositol phosphatise-1 (SHIP-1), which is a negative regulator of TLR2- and TLR4-induced functions (118, 119). This results in a rise in the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3), acting on PI3K/Akt, and subsequently boosting MAPK activity (120) (Fig. 1.3).

However, Ceppi et al. identified the anti-inflammatory effect of miR-155 through targeting TAB2, inhibiting its activation of TAK1, and further NF-κB and MAPK in human monocyte-derived DCs, thus acting as an anti-inflammatory agent (121). Therefore, miR-155 is an interesting and significant player in downstream inflammatory pathways (120) (Fig. 1.3).

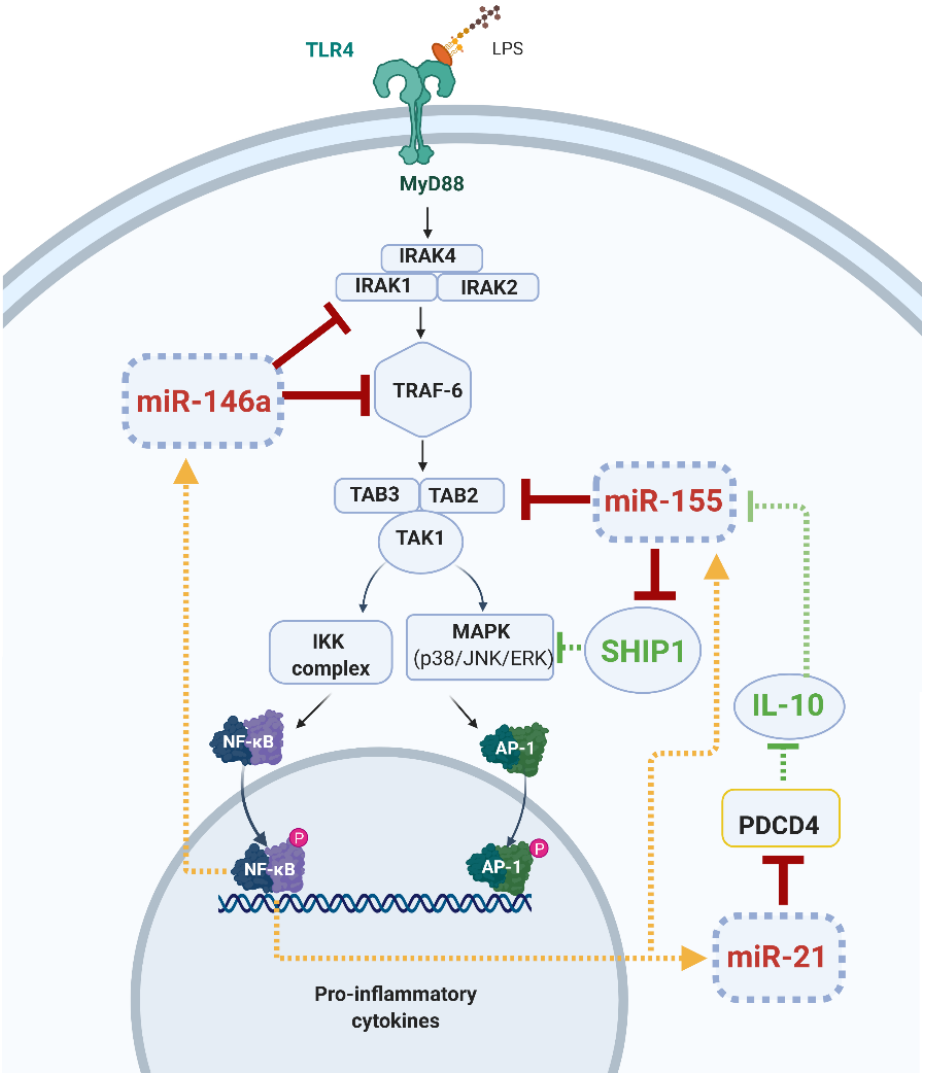


Fig. 1.3. Role of miRNAs in TLR4 signaling. miR-155, miR-21 and miR-146a involvement in TLR4-induced immune response (120).

(Created with Biorender.com)

1.1.4 *In vitro* models of macrophages role in inflammation

Optimizing a model system that reliably and accurately reiterate significant cellular and molecular characteristics of inflammatory signaling in macrophages is crucial step for confident *in vitro* testing of anti-inflammatory drugs (56). In highlight of this, isolated primary macrophages and macrophage cell lines are commonly used *in vitro* as inflammation models; however, some differences are reported between the two models, in terms of differentially expressed genes and magnitude of induction/repression (122).

Primary macrophages can either be human monocyte-derived macrophages (hMDMs) or mouse bone marrow-derived macrophages (BMDMs) (122). BMDM model is recommended for its easy culturing and propagation into large numbers (122). Also, isolated BMDMs from knockout mice facilitate studying the role of specific genes and pathways (122). While, hMDMs model is more physiologically-related because it comes from the natural host, and it is easily isolated from human blood monocytes and differentiated *in vitro*; however, it has an ethical drawback due to human sampling and exhibits genetic variations which affect results interpretation (122). Generally, primary cells have difficulty obtaining a true homogenous population and sensitivity, and they require additional nutrients and macrophage colony stimulating factor (M-CSF), which is normally secreted by RAW 264.7 (123, 124).

Macrophage cell lines can either be human, such as THP-1 and U937, or murine, such as J774 and RAW 264.7 (125, 126). The use of cell lines, on the other hand, has many advantages that is mainly related to their homogeneity, easy propagation in the laboratory, and no animal involvement; however, they may have some genetic and phenotypic differences because they are derived from transformed or immortalized cells (122, 127-129).

The human monocytic cell lines (e.g. U937 and THP-1) require differentiation *via* addition of 12-O-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester stimulant that affects cell behavior through activation of protein kinase C and release of calcium, which might reflect on research outcome (56, 130-132). While, murine macrophage cell lines (e.g. RAW 264.7, J774A.1, and IC-21) are used as models of macrophage activation in a plethora of studies because they are stable, immortal, mature, and have adherent phenotype (56, 133). They exhibit F4/80 and Macrophage-1 antigen (Mac-1), such as CD11b maturity markers for macrophage-like phenotype (134, 135).

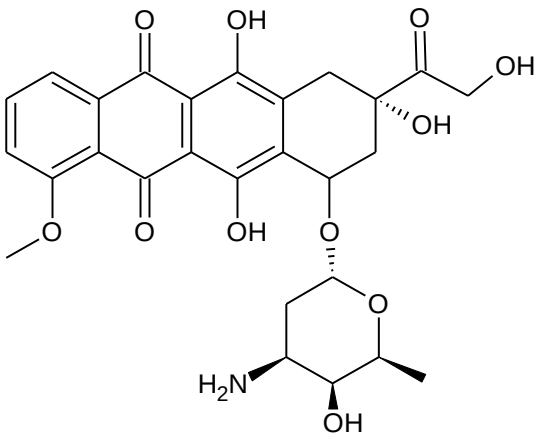
Besides, RAW 264.7 cell line is commonly used as inflammation models in different pharmaceutical screening research (56). The RAW 264.7 cells are monocyte/macrophage-like cells, originating from Abelson leukemia virus transformed cell line derived from BALB/c mice (61). These cells are being described as an appropriate model of macrophages (61). They are capable of performing pinocytosis and phagocytosis (61). Upon LPS stimulation RAW 264.7 cells increase NO production and enhance phagocytosis (61). Furthermore, these cells are able to kill target cells by antibody dependent cytotoxicity (136). Murine cells for *in vitro* use are usually characterized in respect to standard macrophage markers (F4/80, CD14, CD11b, Fc receptor) and reliable cytokine response to chemical stimuli (56). RAW 264.7 cells are phenotypically similar to BMDM primary cells, expressing closely upregulated levels of CD11b and F4/80 expression, and TLR4 and CD14 receptor expression profiles (56, 137). It is worth mentioning that CD14 is a preferentially expressed co-receptor on macrophages that binds to LPS together with TLR4 and initiates classical inflammatory responses (52, 55, 56). However, RAW 264.7 cells express lower levels of TNF-α and IL-6 than BMDMs, which have greater sensitivity to LPS-induced TNF-α compared with immortalized cell lines (56). Such differences warrant justification for the use of IFN- γ as an augmentation adjuvant stimulant with LPS for RAW 264.7 cell line enabling the host to respond quickly to relatively low doses of LPS and thereby activating antibacterial defenses (138).

1.2 Anticancer chemotherapy

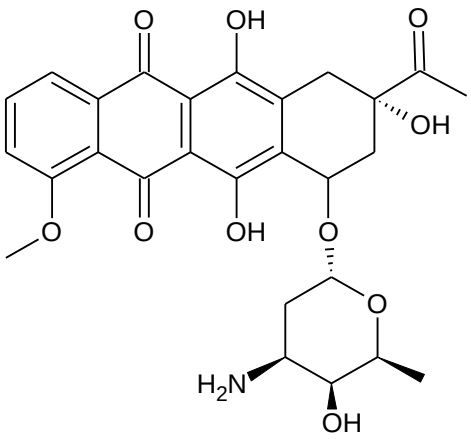
Cancer is the second leading cause of death worldwide, a disease that has severe effects on human population (139, 140). A continuous investigation for new therapies become a critical demand (139). The last years have witnessed great developments in anticancer drugs, which are classified as either chemotherapy, hormonal therapy or immunotherapy (141). Most commonly used, chemotherapy includes a number of families that are defined by both their chemical structure and mechanism of action such as: alkylating agents, antibiotics, antimetabolites, topoisomerase I and II inhibitors, mitosis inhibitors, platinum compounds and others (141).

1.2.1 Anthracyclines Use in Chemotherapy: Doxorubicin and its family

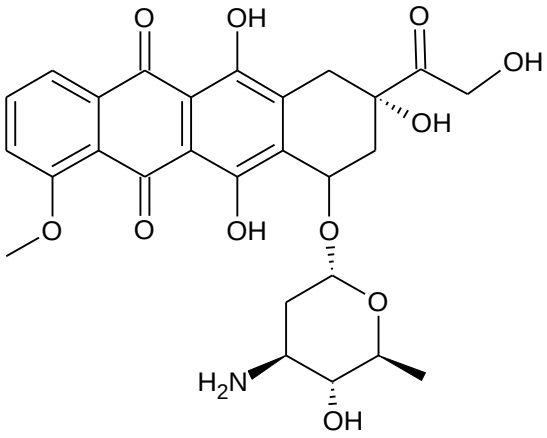
Extracted from *Streptomyces bacterium* in the early 1960s, anthracyclines play a major role in the treatment of many cancer types (142). Anthracyclines are listed among the World Health Organization (WHO) list of essential medicines (143). From the anthracycline family, doxorubicin (DOX) and daunorubicin (DAU), in particular, are the first choice drugs in the treatment of a wide variety of soft-tissue sarcomas and solid tumors, such as breast, stomach, uterine, ovarian, bladder cancer and lung cancers, in addition to haematological malignancies, such as leukemia and lymphomas (142, 144). Anthracyclines are used with a maximum recommended cumulative dose of 450 or 500 mg/m^2 (145). However, their clinical use is hindered by tumor resistance and toxicity, especially cardiotoxicity with cumulative dose of DOX at 550 mg/m^2 body surface area (145-149). Accordingly, numerous anthracycline analogues have been synthesized, such as epirubicin (EPI) and idarubicin (IDA), to overcome these limitations, but very few studies demonstrated improved clinical properties (150, 151). The structure differences in derivatives of anthracycline affected both their cytotoxicity and the time required to exert cytotoxic effect on tumor cells (152). Epirubicin is a stereoisomer of doxorubicin, having increased volume of distribution and longer half-life than doxorubicin, and idarubicin is a derivative of daunorubicin, which is more lipophilic with higher cellular uptake than daunorubicin (143) (Fig. 1.4).



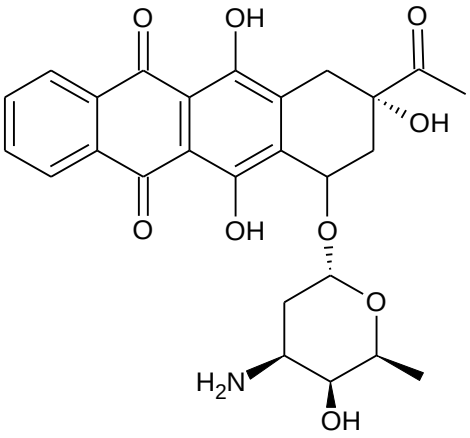
Doxorubicin



Daunorubicin



Epirubicin



Idarubicin

Fig. 1.4. Chemical structures Doxorubicin (DOX), Daunorubicin (DAU), Epirubicin (EPI) and Idarubicin (IDA) (153).

Structures are drawn using ACD/ChemSketch Freeware (https://www.acdlabs.com/resources/freeware/chemsketch/)

1.2.1.1 Anthracyclines Spectrum of Activity

The anti-tumor activity spectrum of anthracyclines include a wide variety of haematological and solid tumor malignancies (146). They are involved in the chemotherapy regimens of many cancers; for example, 32% of breast cancer patients (154), 57 to 70% of elderly lymphoma patients (155, 156), and 50 to 60% of childhood cancer survivors are treated with anthracyclines (157). Anthracycline regimens, in comparison with those of non-anthracyclines, improve response rates in adult and pediatric patients with acute lymphoblastic leukaemia, with DOX and DAU accounting for complete response rates of 56 to 88% (158, 159). Daunorubicin is active against acute myeloid leukemia, producing complete remission rates in 50% of patients. And, the combination of DOX with cyclophosphamide, vincristine and prednisone has become standard therapy for most patients with diffuse large cell non-Hodgkin's lymphoma (159). While for solid tumors, DOX and EPI are used in the treatment of advanced breast cancer and also used in adjuvant regimens with early-diseased patients (159-161). Moreover, DOX monotherapy produced responses in approximately 24% of small cell lung cancer (SCLC) patients in phase II studies, while reaching higher response rates (40 to 100%) in combination regimens with cyclophosphamide and vincristine (159). As well, DOX is effective in patients with sarcoma, gastric cancer and advanced ovarian cancer; however, anthracyclines, generally, have limited effect against prostate cancer (146, 162, 163).

1.2.1.2 Anthracyclines mechanism of action

The pharmacological activity of anthracyclines as anti-cancer drug is related to intercalation between adjacent DNA base pairs and inhibition of topoisomerase II (Top2). Anthracyclines, with their planar structure, intercalates into the DNA and bind to both DNA and Top2 through non-covalent interaction to form complexes that trigger cell death (146, 164, 165). This intercalation causes deformation of the DNA that leads to stabilization of the normally reversible topoisomerase II-DNA complex, resulting in the production of double-strand DNA breaks (146, 159, 166).

Another mechanism of anthracyclines is the production of free radicals through enzymatic reduction of the anthracycline ring that gives a semiquinone free radical, resulting in the production of a hydroxyl free radical that cause damage to both healthy and tumor tissues through membrane lipid peroxidation (146, 167, 168). Additionally, the conjugation between the hydroquinone portion of the anthracycline molecule and intracellular ferric iron may result in nonenzymatic

production of free radicals (168). This free radical damage is more closely associated with the associated cardiotoxic effects of anthracyclines (146, 168).

1.2.2 Anthracyclines Limitations

1.2.2.1 Anthracyclines Resistance

Anthracyclines and other Top2 inhibitors experience high levels of primary and acquired resistance in both solid tumors and haematological malignancies through P-Glycoprotein, the expression product of the MDR-l gene (169, 170). The protein functions as a transmembrane drug efflux pump, transporting anthracyclines out of the cancerous cell (169, 171). Some resistance modifying agents, such as verapamil, tamoxifen and cyclosporine has been used to reduce or reverse anthracycline resistance; however the clinical role of these agents is still unclear (169).

1.2.2.2 Anthracyclines Inflammation and Toxicities

DOX is a double-edged sword (145). DOX chemotherapy plays a prominent role in many cancer treatments and protects healthy cells; however, dose-dependent toxicity remains a critical side effect that limits its dosing and cancer treatment outcomes and damage non-target cells and tissues (143).

Conventional toxicities of DOX is similar to any other anti-neoplastic agent, including low therapeutic index, and other reversible adverse effects, such as nausea and vomiting, alopecia, and stomatitis, which are developed within 5 to 13 days of anthracycline treatment (137, 143, 146, 151, 172, 173). While, the classical dose-limiting adverse effects of this class of drugs are: acute myelosuppression and cumulative dose-related cardiotoxicity (146). Myelosuppression, especially neutropenia, occurs in 60 to 80% of patients on doxorubicin regimens (146). While, cardiac toxicity remains the most critical among all toxicities because of the increased cardiovascular morbidity and mortality (60, 143, 151).

1.2.3 DOX-induced cardiotoxicity

The use of DOX as antineoplastic agent is mainly limited by the dose-dependent, cumulative cardiotoxicity (174), which is often irreversible and manifested as dilated cardiomyopathy with or without symptoms of heart failure (HF) (175). Congestive heart failure (CHF) is developed in approximately 5% of DOX treated patients, with mortality of almost 50% (146, 175). Chronologically, cardiotoxic effects of anthracyclines range in severity, and are classified by their time of onset as acute, occurring during or immediately after infusion; early, occurring within 1 year of exposure; and late, occurring after 1 to 20 years of initial exposure (176). Acute DOX-induced cardiotoxicity is a reversible process manifesting itself by disturbances in electrical conduction of prolonged QT and nonspecific T-wave changes, and arrhythmias (176). In contrast, both early and late anthracycline-induced cardiotoxicity (AIC) is manifested as decreased left ventricular (LV) function, diminished exercise capacity, and progressive heart failure symptoms (176). The risk for early and late AIC is a function of cumulative anthracycline exposure (177). Although the pathogenesis behind DOX-induced cardiomyopathy is still not well recognized, several studies have shown that DOX exerts a complex of biochemical effects on the myocardium, ranging from binding to DNA and alteration of nucleic acids to protein synthesis and lipid peroxidation after free radical generation, as well as, generation of histamine and catecholamines, mitochondrial damage, excess calcium influx and changes on collagen matrix and different cellular membranes (178). Also, myocardial tissues lack free radical-scavenging enzymes, unlike normal tissues and, therefore, are susceptible to free radical damage (146, 168). Conclusively, a combination of these effects probably trigger DOX-mediated cardiomyopathy (178).

1.2.3.1 Mechanisms of DOX-induced Cardiotoxicity

The mechanism of DOX-induced cardiotoxicity is likely to be multifactorial (165). It can be direct cardiomyocyte injury (143), and/or indirect cardiac damage secondary to inflammation (179). It also involves free radical-induced oxidative stress through ROS generation, and mitochondrial dysfunction (180-182).

- *The Role of Oxidative Stress and Reactive Oxygen Species*

Production of hydroxyl free radicals is associated with DOX anti-tumor activity to cancerous cells and toxicity to healthy ones (146). Anthracyclines interfere with redox cycling and catalyze reactive oxygen species (ROS) generation, which results in DNA damage (183). Enzymatically, the quinone moiety of DOX is reduced to semiquinone free radical in myocardial cells through NADH dehydrogenase of the mitochondrial electron transport chain, then oxidizes to produce the parent anthracycline and superoxide anions in the mitochondria, causing cardiotoxicity (184, 185). This redox cycle is mainly due to cationic DOX affinity to a phospholipid located within the inner mitochondrial membrane at the site of the respiratory chain called *cardiolipin*, which is required for the proper functioning of electron transport chain proteins (151). Anthracycline-cardiolipin complex is reduced by NADH, drawing an electron away from the mitochondrial respiratory chain and subsequently reducing oxygen to form a superoxide anion radical (185). Subsequently, these formed free radicals cause damage to multiple cellular components, such as lipids of the cell membrane, proteins, and nucleic acids. Moreover, mitochondrial function impairment causes energetic metabolism change evidenced by a decrease of the adenosine triphosphate (ATP) production, which may contribute to abnormal contraction and relaxation in the failing heart (172, 186, 187). Thus, ROS generation is an important mechanism in anthracycline-induced cell death (188). Non-enzymatically, the direct interaction between anthracylines and intracellular ferric iron (Fe^{3+}) produces DOX-iron complexes that form toxic hydroxyl radicals, resulting in impaired mitochondrial function, cellular membrane damage, and cytotoxicity (189, 190). Increased ROS levels may be also due to the free cellular iron and resulting ferrous-ferric cycling of molecular iron from damaged cells (190). Other forms of DOX-induced ROS generation in the myocardium include NOSs oxidases pathway, which interact with DOX and induce nitrosative stress that worsen mitochondrial dysfunction (172, 191-193). NOS are a group of enzymes responsible for the NO production from L-arginine and oxygen (194). And, several studies illustrated the effect of DOX on the signaling of NOS evidenced by its increased transcription and protein activity (195, 196). There are two isoforms of the NOS enzymes involved in DOX-induced cardiotoxicity (DIC): eNOS, a membrane-bound enzyme, and iNOS, a soluble enzyme; thus the localization of these two enzymes in the cells is different (197). Alterations in NO production develop when DOX directly binds to eNOS reductase domain, leading to the reduction of DOX semiquinone radical which further reacts with oxygen and produces superoxide (151). In addition, recent studies

suggest that the other isoform iNOS is also involved with DOX-induced oxidative stress, evidenced by increased iNOS transcription and expression in mouse and rat hearts and isolated cardiomyocyte after DOX treatment (151, 198, 199). Altogether, these studies highlight the importance of NOS signaling in DIC (151).

- *The Role of Topoisomerase 2β (Top2β)*

Despite the fact that free radical production play a major role in the direct oxidative damage to cellular components, the aforementioned anthracycline intercalation into DNA remains a common postulated mechanism of DIC (165). DNA topoisomerases (Top) induce temporary single or double-stranded breaks to regulate the topological changes during DNA replication and transcription (200). Recent studies suggest that the isozyme Top2β, which is the only Top2 expressed in adult mammalian cardiomyocytes, plays a role in ROS production (201). Similar to Top2α, which is overexpressed in proliferating cancerous tissues, Top2β is another anthracycline target (143, 202). The cytotoxic effect of DOX takes place through binding to DNA and Top2 isoenzymes, giving a ternary Top2-doxorubicin-DNA complex that prevents further DNA and RNA synthesis, which is likely to contribute to the death of myocytes and mitochondrial mutations (203, 204). When bound to Top2α isoenzyme, this complex inhibits DNA replication and arrests the cell cycle in G1/G2 phase, inducing apoptosis as intended in proliferating malignant cells (204). When bound to Top2β isoenzyme, however, it suppresses peroxisome proliferator-activated receptor (PPAR), which regulates oxidative metabolism, resulting in mitochondrial dysfunction and apoptosis (143, 205). It is worth mentioning that Top2β is essential for DOX binding to DNA, which is confirmed by DIC protection in Top2β knockout mice (143, 204, 206, 207).

- *Apoptosis*

Oxidative stress and the resulting intracellular oxidants, such as hydrogen peroxide and superoxide from these events induced apoptosis of cardiomyocytes through the activation of P53 tumor suppressor pathway (143, 175).

1.2.3.2 Role of Immune Response in DOX-mediated Systemic Inflammation

Immunology plays an important role in DIC progression through activation of innate and adaptive immunity, by which DOX stimulates the release of cytokines and inflammatory markers, including TNF-α, IL-6, IL-1β, p38 MAPK and NF-κB (208). In addition, DOX up-regulates the expression of cell surface death receptors (DR), including DR1, DR4 and TNF1 receptors in cardiomyocytes, and most importantly, toll-like receptor (TLR) that activates NF-κB leading to cardiac damage (209). In contrast to the innate resident macrophages within the normal myocardium, polarized macrophages migrating from blood after being triggered by an inflammatory response have a different configuration (16). After a myocardial infarction incident, infiltrating pro-inflammatory M1 macrophages with activated TLR4 by necrotic myocardium invade the injured heart for clearance and functional restoration of cardiac homeostasis, then followed by anti-inflammatory macrophages M2 (16). Chronic activation of macrophage, however, can lead to adverse myocardial effects, which is confirmed by the direct involvement of macrophages in cardiomyocyte hypertrophy and the elevated cytokines in heart failure patients (16). Yet, the mechanism behind TLR pathway activation by DOX remains unclear (16). Therefore, the relationship between recruited macrophages, expressing TLR-4 and doxorubicin-induced cardiomyopathy is an open question that has been answered in part by Wang *et.al* study addressing the role of gut endotoxin leakage and TLR-4 upregulation in DIC (179). It has been shown that DOX disrupts the intestinal epithelium barrier, which results in endotoxin leakage from gut flora into circulation. Concurrently, DOX increased TLR4 expression in macrophages, which further enhanced the sensitivity of activated macrophages to endotoxin and exacerbated organ damage (179). Collectively, this led to DOX-induced systemic inflammation, which serves as a new mechanism for the multi-organ toxicity of DOX in the body (179). In support of this view, recent studies suggested that the circulating endotoxin originated from gut microflora, and its subsequent systemic inflammatory responses significantly contributed to the pathology of heart failure, which has been confirmed by the marked suppression of inflammatory response in multiple organs after the elimination of intestinal microflora in DOX-treated animals (179, 210). Besides DOX, LPS and ROS, and other inflammatory mediators, such as IL-6 and IFN-γ have been reported for their contribution to DOX-mediated systemic inflammation and to the upregulation of TLRs, such as TLR-9, TLR-2, and TLR-4 (179, 211, 212). Altogether, this illustrates the role of immunotoxicology in the pathogenesis of cardiotoxicity demonstrated in cancer patients

undertaking chemotherapy (213). Therefore, either antibiotics administration for elimination of gut-flora or inhibition of TLR-4 can be potential approaches to prevent or alleviate the adverse effects of DOX and other chemotherapeutic agents (179) (Fig. 1.5).

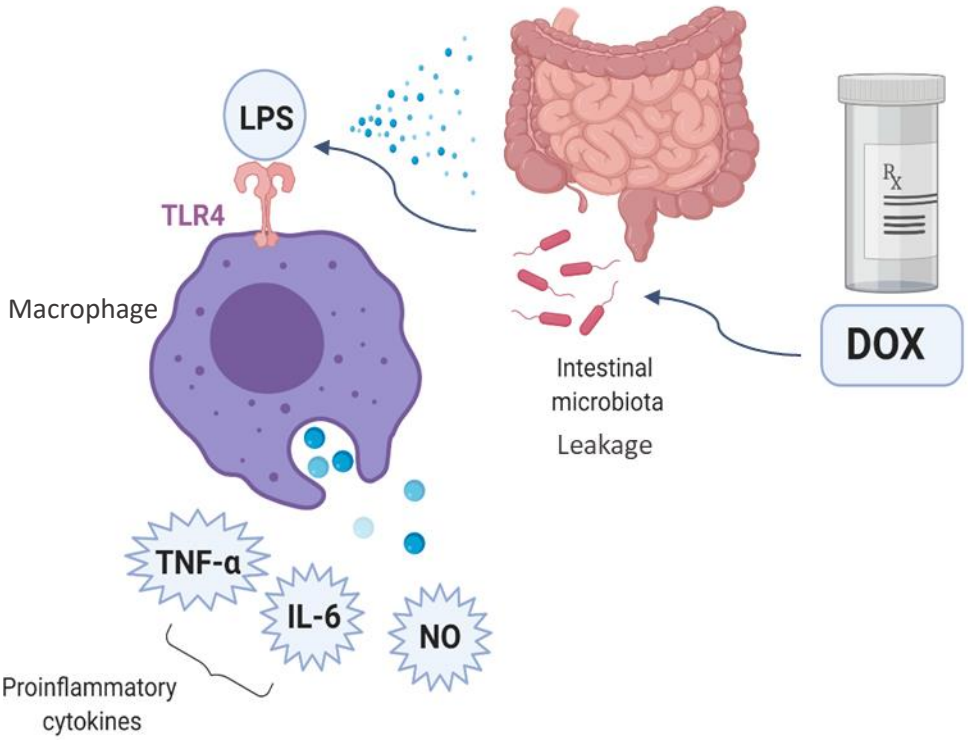


Fig.1.5. Schematic diagram illustrates the role of DOX in TLR4 signaling activation in macrophages.

(Created with Biorender.com)

1.2.3.3 DOX-Induced Cardiotoxicity Management

New trends have been presented in attempt to alleviate DOX toxicities and multidrug resistance in patients (214). AIC may be reduced or prevented by administration of DOX dosage that produces low peak plasma drug concentrations, or administration of dexrazoxane, an iron-chelating agent that provides cardioprotection (214). Also, liposomal encapsulation of DOX or DAU has been developed with promising results to improve the pharmacokinetic properties in preclinical studies; however, many nanodrugs fell short of expectations when tested in patients (146, 214). Interestingly, a new era of naturally plant-derived compounds in chemotherapy has been developed, exhibiting strong anti-inflammatory properties with less toxic side effects compared to the current conventional treatments (139). Today, medicinal plant-derived compounds became potential adjuvants in chemotherapy (30, 139).

1.3 Anti-inflammatory Compounds

Inflammation is a normal response to infection, but when it is chronic, it may result in autoimmune or auto-inflammatory disorders, neurodegenerative diseases, or cancer (17). Thus, different anti-inflammatory agents have been developed, including aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) (17, 215). In addition, biologics, such as anti-cytokine therapies and small molecules blocking the activity of kinases are recently involved (17). Other anti-inflammatories include statins, PPAR agonists, and plant-derived anti-inflammatory agents (15, 17).

3.11.1 Classes of Anti-inflammatory agents

- *Acetylsalicylate* or *Aspirin*

First, the world's most used therapeutic agent, Acetylsalicylate or Aspirin, was first synthesized by Fleix Hoffman in 1859 (215). Aspirin is commonly used as antipyretic, anti-inflammatory, and analgesic (215). It inhibits COX enzymes (COX-1 and COX-2) that synthesize the inflammatory mediators, PGs, especially PGE2 that is used to lower pain threshold, and thromboxanes, which have anti-thrombotic activity (17). In addition to COX system inhibition,

aspirin has also been found to inhibit NF-κB pathway (47). However due to their non-selectivity, aspirin and nonselective NSAIDs' exhibited critical side effects, including gastrointestinal upset, gastritis, ulceration, hemorrhage, and even death (47).

- *NSAIDs*

Generally, there are two COX isoenzymes, the constitutive COX-1, which makes vasodilator PGs that protect the kidney and intestinal mucosa from damage, and the inducible COX-2, which is associated with infections or inflammatory diseases and is induced by inflammatory cytokines, such as TNF-α and IL-6 to produce more PGs which contribute to the pain and swelling of inflammation (17, 215). The selective COX-2 inhibitors, NSAIDs have been developed with less side effects on the kidney and stomach, and have gained this name because they were distinct from glucocorticoids (the second major group of anti-inflammatory drugs) (17, 215, 216). The most common NSAIDs are antipyrine, phenacetin, phenylbutazone and, more recently, the fenamates, indomethacin, naproxen, etc. (215). Selective NSAIDs inhibit COX-2, which interfere with the synthesis of prostaglandins, particularly PGE2 and also inhibit NF-κB pathway (17, 47, 215). They effectively alleviate the swelling, redness, fever and pain of inflammation (215). COX-2 inhibitors have been used efficiently in reducing pain in patients with osteoarthritis or rheumatoid arthritis, and also used to decrease the risk of colon cancer (215, 217-219). Despite the fact that COX-2 inhibitors significantly reduced gastrointestinal side effects that are common with COX-1 inhibitors, however, their chronic use has been associated with kidney damage and cardiovascular/cerebrovascular diseases (17, 215).

- *Prostaglandin Agonists* and *Phosphodiesterase-4 Inhibitors*

From another prespective, prostaglandin agonists and phosphodiesterase-4 (PDE-4) inhibitors are used as anti-inflammatory agents (17). Despite the inflammatory effect of PGE2, it is considered one of the most immunosuppressive natural products of inflammation when agonist selectively bind to EP4, one of the four receptors for PGE2 (17). Therefore, selective EP4 agonists are anti-inflammatory agents, reducing levels of pro-inflammatory cytokines, chemokines, and adhesion molecules. PGE2 are effective inhibitors of IFN-γ , IL-2 and TNF-α production due to

upregulating intracellular levels of cyclic AMP (cAMP) (17). In addition, analogs of prostaglandin-I2, including iloprost, cicaprost, treprostinil, inhibit proinflammatory cytokines, including IL-12, TNF-α, IL-1α, and IL-6, as well as, chemokines, such as MIP-1α and MCP-1 through increasing cAMP levels (220).

Besides PGE2, PDE-4 inhibitors, such as rolipram, roflumilast, piclamilast, and pentoxifylline, are anti-inflammatory and immunosuppressive acting through inhibiting cAMP breakdown (17). PDE-4 inhibitors decrease NF-κB binding and suppress cytokine production and neutrophils degranulation, as well as, TNF-α-induced neutrophil adherence to endothelial cells, constitutively responsible for inflammation (17).

- *Glucocorticoids*

The synthetic forms of natural cortisol known as glucocorticoids are widely used to treat many inflammatory diseases, especially chronic inflammatory and autoimmune diseases, and also used in acute cases, such as gout (17). A major mechanism of glucocorticoids is reducing the expression of cytokine-induced genes; they act intracellularly through binding to the cytoplasmic steroid receptor, and then the glucocorticoid-steroid receptor complex is translocated into the nucleus and binds to specific DNA sequences that results in suppressing the transcription of the genes encoding for the pro-inflammatory cytokines *via* inactivating AP-1 and NF-κB transcription factors (17). Subsequently, they downregulate the expression of T cell growth factors encoding genes, such as IL-2, IL-4, IL-15, IL-17 and IFN-γ, as well as, the expression of genes encoding for COX-2, iNOS, and intracellular adhesion molecule-1 (ICAM-1), which are normally induced by IL-1β and TNF-α cytokines (17). They also increase the expression of genes encoding for anti-inflammatory molecules, such as IL-10 cytokine and IL-1 type 2 decoy receptor (17).

- *Biologicals*

Besides glucocorticoids, biologicals have been developed to treat inflammation, such as anti-cytokines, and agents that block lymphocyte trafficking into tissues, preventing the binding of monocyte-lymphocyte costimulatory molecules, or depleting B lymphocytes (17). As previously

mentioned, pro-inflammatory cytokines, including TNF-α, IL-1β, and vascular endothelial growth factor (VEGF) play central roles in inflammation (17). Therefore, *anti-cytokines* are used to neutralize the activity of specific cytokines or their receptors, including drugs that reduce the activity of TNF-α (e.g. adulimumab, golimumab, certolizumab and etanercept) and drugs that reduce IL-6 activities (e.g. MEDI5117 and toculizumab) (17). Other drugs are used to neutralize IL-12, IL-23, and IL-17 (e.g. ustekinumab and AIN457/LY24398) (17). Additionally, T or B lymphocytes acting agents (e.g. daclizumab and eplizumab , for T lymphocytes, and ituximab, crelizumab, ofatumumab, epratuzumab and belimumab, for B lymphocytes) (17). Collectively, anti-cytokines block the activity of the pro-inflammatory cytokines: IL-1, TNF-α, IL-6, IL-12, IL-17, IL-18, or IL-23 that further activate T cells, therefore, successfully reduce inflammation (17). Despite the benefits of neutralizing pro-inflammatory cytokines, anti-cytokine therapy may decrease host immune defense against infection and cancer, yet its less toxic effects, compared to glucocorticoids, outweigh the risks (17). Also, chemokines can be counteracted by a chemokine receptor antagonists used to treat Crohn's disease, which is an autoimmune inflammatory bowel disease (221).

- *Kinases Inhibitors*

Acting downstream of cytokine receptors, kinases have become new targets to mitigate inflammation (17, 222). Intracellular signaling kinases are involved in phosphorylating intracellular proteins, including auto-phosphorylation (17). One of the important kinases is p38 MAPK, which regulates several fundamental mediators of inflammation, including cytokines, chemokines, COX-2, and NOS (17, 222). Hence, inhibitors of the p38 MAPK, especially the α-isoform, managed to suppress IL-1β and TNF-α production in endotoxin-stimulated human monocytes (17). Since kinases are employed in normal cellular functions, the concentrations of kinases inhibitors require careful determination in order not to elicit organ toxicity, such as hepatic toxicity (17).

- *Complement system Inhibitors*

Another way to tame inflammation is through inhibition of activated complement (17). Complement activation gives the terminal component, complement 5a (C5a), which binds to its receptor C5aR and triggers the synthesis of cytokines, chemokines, and adhesion molecules with subsequent infiltration of myeloid cells into the area of injury (17). Being involved in almost all inflammatory processes, activated C5a is a major cause of acute inflammation, therefore, natural inhibitors of complement activation, such as C1 inhibitor and soluble complement receptor 1-related gene/protein y (Crry) successfully inhibit all complement activation pathways (223), improving multiple diseases, such as rheumatoid arthritis, immune complex diseases, psoriasis, lupus nephritis, acute lung injury, myocardial and cerebral infarction, and renal ischemia (17). In addition, the monoclonal antibodies (e.g. eculizumab and pexelizumab) against C5 have been used in many inflammatory conditions, including paroxysmal nocturnal hemoglobinuria and acute ST-elevation myocardial infarction (17). Besides monoclonal antibodies, serine protease inhibitors that target the formation of the C3/C5 convertases can be used in preventing the inflammatory sequelae of complement activation through the direct inhibition of serine proteases (17). Another approach is using orally active convertase inhibitors or C5aR antagonists for treating chronic inflammatory conditions that target inflammation by preventing the generation of C5a or blocking C5aR (17).

- *Statins*

Statins are synthetic, safe class of drugs that have anti-inflammatory properties. They are used normally to lower endogenous cholesterol synthesis, especially in patients at high risk for myocardial infarction with elevated levels of LDL (17). From anti-inflammatory perspective, Statins have been used to reduce inflammation and tame immune cell activation because they reduce cytokine production and expression of endothelial adhesion molecules, and they decrease MHC II expression, without significant effect on MHC I (17). Yet, not enough studies are available on the anti-inflammatory properties of statins (17).

- *Resolvins*

Also, resolvins, a group of molecules naturally derived from omega-3 fatty acids, are developed as anti-inflammatory agents (17). Physiologically, resolvins are employed in anti-inflammatory actions that coexist with inflammation, while synthetically, resolvins are currently in clinical trials for treating local inflammatory conditions (17, 224). The anti-inflammatory activity of omega-3 is known to be mainly through suppressing the infiltrating neutrophils and macrophages, and downregulating the expression of TNF-α, IL-1β, and VEGF (17, 224, 225).

- *Peroxisome Proliferator-activator Receptors (PPARs)*

In addition, PPARs are members of the nuclear receptor superfamily (17). They heterodimerize with retinoid X receptor (RXR), leading to downstream effects regulating glucose metabolism. (17). PPARs are expressed as: PPARα, PPARβ/δ, and PPARγ (17). PPAR agonists can be either endogenous, such as prostacyclin, or orally active synthetic ligands for PPARγ, such as rosiglitazone, pioglitazone, troglitazone, and fenofibrate, known as thiazolidinediones (glitazones) class that is used for type 2 diabetes (17). In addition to their anti-diabetic effect, PPAR agonists possess anti-inflammatory activity demonstrated through their intracellular mechanisms by targeting the gene expression of pro-inflammatory cytokines, cell adhesion molecules, COX-2 and iNOS (17). The anti-inflammatory effect of pioglitazone is evidenced by a reduction in the gene expression of TLR2, TLR4, IL-1β, TNF-α, IL-6 in human blood monocytes *in vitro*, as well as, the expression of LPS-induced TLR2 and TLR4 on peritoneal macrophages *in vivo* (226). Despite their significant anti-inflammatory activity, PPAR agonists, such as rosiglitazone clinically showed increased risk of heart failure, which is lower with pioglitazone (227).

- *Anti-inflammatory herbal medicines*

Lastly, the use of anti-inflammatory herbal medicines has been known since the ancient past and was documented in historical books (228). Nowadays, The use of plant products is becoming an effective and safer alternative for conventional steroidal and NSAIDs medications in chronic inflammatory diseases (47). In the past, tons of studies have addressed the potent anti-

inflammatory activities of different plant products (81). The next part will only highlight the recent data published on herbal medicines used in mitigating inflammation and will focus mainly on the use of curcumin, resveratrol, and sulforaphane and their underlying mechanisms generally in inflammatory response, and particularly in DOX-mediated systemic inflammation that leads to DIC.

1.3.2 Plant-derived anti-inflammatory compounds

Nowadays, half of the drugs in clinical use is of natural origin (229, 230). Natural products are becoming a promising source for the treatment of several inflammatory conditions (231, 232). In particular, the most promising anti-inflammatory herbal extracts were identified to influence key TLR signaling pathways and macrophage repolarization (233). Generally, diverse plant derived products are important secondary metabolites in plants that play key roles in defense, protection, nutrition and growth of the plant (229, 230, 232, 234). These secondary metabolites possess potent anti-inflammatory properties and are categorized as: I) phenolic compounds, including flavonoids, condensed tannins and gallotannins, II) glucosinolates, III) coumarins, IV) alkaloids, V) saponins, VI) sterols, and VII) terpenoids (235).

- *Phenolic Compounds*

Phenolic compounds act through inhibiting some molecular targets of pro-inflammatory mediators in inflammatory responses (236). For example, gallotannins and condensed tannins (proanthocyanidins) play a major pharmacological role as anti-oxidants because of their strong free radical-scavenging properties and inhibitory effect on particular enzymes such as COX enzymes (235, 236). They have anti-inflammatory and anticancer effects (235). Also, flavonoids are another class of polyphenolic compounds that are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids (235). Similarly, they act as antioxidants in inflammation and also block COX-2 activity, and they also act as blockers to cytokines, NF-κB and matrix metalloproteinases (235, 236). Additionally, flavonoids exhibit antimicrobial, antiviral, antiulcerogenic, cytotoxic, antineoplastic, mutagenic, antihepatotoxic, antihypertensive, hypolipidemic and antiplatelet activities (237).

- *Glucosinolates*

Glucosinolates are sulfur-containing secondary metabolite group in plants, mainly found in Brassicaceae family, including broccoli, cabbage, cauliflower, and mustard (238). Recent studies showed that glucosinolates have regulatory functions in inflammation and stress response, as well as, phase I metabolism, antioxidant activities, and direct antimicrobial properties (238). When unprocessed cruciferous plants are consumed, glucosinolates are hydrolyzed by myrosinase enzyme present in these plants into various metabolites, such as isothiocyanates, nitriles, oxazolidine-2-thiones, and indole-3-carbinols (239). From these metabolites, isothiocyanates are known for their potent anti-inflammatory properties, and apoptosis induction in cancer cells, as well as, their anti-fungal and anti-bactericidal activities (239).

- *Coumarins*

Coumarins represent a versatile family of compounds which has remarkable anti-inflammatory and antioxidant activities (235). Similar to phenolics, coumarin derivatives, such as olumbianetin (A) and libanoridin (B), possess some antioxidant capacity through scavenging superoxide anion radicals, and some of them block both lipoxygenase and cyclooxygenase pathways of arachidonic acid metabolism (235, 240).

- *Alkaloids*

Alkaloids, such as isoquinoline, indole and diterpene, are significant anti-inflammatory agents that act by inhibiting the synthesis or the action of some proinflammatory cytokines (235, 236). Also, Alkaloids have anti-inflammatory, immunomodulatory and antimalarial activities (235).

- *Saponins*

Saponins, including saikosaponins and ginsenosides, are steroid or triterpene glycosides that include many biological compounds (235). Their underlying anti-inflammatory mechanisms

involve indirect and direct corticomimetic activity, inhibiting glucocorticoid degradation, enzymatic formation, and inflammatory mediators release (235).

- *Phytosterols*

Phytosterols, being an important component of plant biomembranes, are biogenetic precursors of multiple metabolites, such as plant steroid hormones (235). They have anti-atherosclerotic properties, mainly due to lowering LDL cholesterol, and their effects on coagulation and antioxidant systems, as well as, hepatic and lipoprotein lipase activities (235). However, the only source to their consumption is through diet since they cannot be synthesized (235).

- *Terpenoids*

Terpenoids and essential oils, such as menthol and camphor, are volatile, natural, complex compounds called "terpenes", and are found in isoprene structured compounds (235). It has been reported in several studies that mono and sesquiterpenes have potent anti-inflammatory effect (241). Also, these compounds have antiseptic activity, bactericidal, and fungicidal activities, and they are used as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and as local anesthetic agents (242).

1.3.3 TLR4-acting Plant-derived Anti-inflammatory Compounds

In the context of TLR4 pathway in inflammation, TLR4 antagonists were developed synthetically (e.g. eritoran and TAK-242) and naturally (e.g. epigallocatechin-3-gallate and 6-shogaol) (233, 243-247). Recently, several studies started to focus on Chinese herbal medicines as a new oral treatment for inflammatory diseases, mainly due to their safety and easy oral administration (233). However, some of these herbal medicines have unidentified active compounds and potential targets, but certain phytochemicals, such as curcumin (CUR), resveratrol (RES), and sulforaphane (SFN) were reported to inhibit the activation of TLR4 signaling and reduce LPS-induced COX-2, NF-κB expression and pro-inflammatory cytokines production, (81).

With no structural similarity to LPS, CUR, RES, and SFN have been reported in many studies for their TLR4 antagonistic effects that are summarized in Table 1.1 (30, 233, 248).

1.3.3.1 Curcumin (CUR)

First identified in 1910, CUR (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also called diferuloylmethane, is a naturally occurring yellow pigment that is the main natural polyphenol obtained from turmeric *(Curcuma longa),* a flowering plant of the ginger family (249, 250). The lipophilic polyphenol, CUR has demonstrated activity on the cellular level, as well as, the molecular level through targeting multiple signaling molecules, which has supported the pleiotropic actions of curcumin (251, 252). The diverse bioactive effects of CUR include: anti-bacterial, anti-inflammatory, antioxidant, antitumor, and anti-atherosclerotic effects (253-257). Although CUR has infinite therapeutic benefits, its main advantage is mainly because of its antioxidant and anti-inflammatory effects (251, 258).

Traditionally, CUR is widely used in food as a curry coloring or flavoring spice and in Chinese and Indian medicine as an anti-inflammatory agent, for digestive disorders, and wound healing (47). CUR supplements are taken three times per day, with a dosage of 400–600 mg (259). Its side effects are few, but it may cause stomach upset with extended use, and gastric ulcers at very high doses (47). However, as indicated by many preclinical and clinical studies, CUR does not cause any adverse effects on liver or kidney functions, even at high doses (up to 12 g in humans) (260). CUR shows poor absorption and low bioavailability, especially if taken in its intact form, yet, this issue can be resolved when CUR is combined with enhancing agents, such as piperine (38, 261, 262). CUR is available in the forms of capsules, tablets, ointments and energy drinks, and has been approved by the US Food and Drug Administration (FDA) as “Generally Recognized As Safe” (GRAS), which is confirmed by clinical trials at doses between 4000 and 8000 mg/day (251, 263). From a clinical perspective, CUR was tested in humans in 77 studies, of them, 50 have been finished and 27 are in the recruiting phase; moreover, 100 clinical trials on the anti-inflammatory activity of CUR have been published in PubMed, showing CUR significance in clinical inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel diseases, nephropathies, and some cancers (251, 257).

- *CUR Mechanisms of action as anti-inflammatory agent*

CUR has a multifactorial mechanism of action against inflammation (252). Firstly, it has powerful antioxidant activity that inhibits lipid peroxide formation and lysosomal enzymes, such as acid phosphatase and cathepsin D (252, 264). Also, CUR increases the activities of serum antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GSH), as well as, scavenges different free radicals, including reactive oxygen and nitrogen species (ROS and RNS) and peroxyl radicals (250, 265-268). It can also inhibit different ROS generating enzymes, such as the iNOS, COX system (COX-1 and COX-2) , and 5-LOX, thus suppresses the activity of several PGs (47). Therefore, it may be considered a natural alternative to NSAIDs for the treatment of inflammation (47, 250, 269)

Secondly, CUR has potent anti-inflammatory effects that modulate TLR4 and MyD88 pathways in macrophages (270, 271), evidenced by blocking NF-κB activation and inhibiting MAPK and AP-1 signaling pathway (47, 159, 257, 271-274). It has been presented that CUR non-covalently binds to MD-2 (a lymphocyte antigen responsible for LPS binding to TLR-4), which results in a competition with LPS for TLR4/MD-2 complex that led to inhibition of both MyD88-dependent and TRIF-dependent pathways (81, 275, 276).

Interestingly, CUR inhibited M1 macrophage polarization in a dose dependent manner through downregulating the expression of TLR4 (277). These antagonistic effects to TLR4 signaling pathways and its downstream mediators are followed by an inhibition of the pro-inflammatory cytokines, including TNFα, IL-1β, and IL-6 (15, 257, 273, 277). Also, research studies suggest that CUR pretreatment protects against T cell-mediated hepatitis in mice and that the significant effect of CUR may be partly through inhibiting the expression levels of TLR2, TLR4 and TLR9 in the liver (278). Furthermore, CUR blocks the expression of cell adhesion molecules, such as ICAM-1, which are involved in the interaction between leukocytes and endothelial cells (257, 279).

In addition, on the miRNA level, studies done using RAW 264.7 macrophages revealed the inhibitory effect of curcumin on miRNA 155, which is a key transcriptional regulator of some

inflammation-related diseases and is strongly induced by different TLR ligands including, TLR4 ligand (e.g. LPS) (280, 281).

Last but not least, CUR has a dual activity when tested on human malignant cells, on one side it exhibited pyroptosis (caspase 1-dependent cell death) induction of cancerous cells, which is a strong inflammation phase through caspase 1 stimulation, yet on the other side it exhibited a protection against inflammation without pro-IL-1β induction due to NF-κB pathway inhibition (269, 282, 283). Also, because of its anti-oxidative and anti-inflammatory activities, CUR has been reported for its cardioprotective and hepatoprotective effects, and has been also involved in lowering cholesterol levels and having anti-diabetic activity (252, 260).

1.3.3.2 Resveratrol (RES)

Another polyphenol plant-derived molecule, resveratrol (5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol) is called Japanese Knot weed or *Polygonum cuspidatum*, and is found in various plant sources, such as peanuts, grapevines and mostly in the skins of red wine grapes (47, 257). RES, which is a stilbene derivative, is a phytoalexin that acts as a plant defense mechanism against infection (47, 257). Commercially, RES is available as dietary supplements, mainly from *P. cuspidatum* source in the Tans-active form (47). There is no standardized dosage for RES, but it is usually taken in the dose from 50 to 500 mg daily (47). Fortunately, no side effects were established, only antiplatelet effect that should be monitored if RES is taken with other prescribed antiplatelet or anticoagulant agents (47, 284-290). However, the RES limiting step is its pharmacokinetics, exhibited as poor bioavailability and rapid metabolism, which is a critical challenge for pharmaceutical industry (257, 291-297).

In addition, over 40 clinical trials were published in PubMed on the use of RES in inflammatory disorders, including diabetes, obesity, and coronary artery disease (257). In these studies, inflammation-related parameters, such as CRP, TNFα, IL-1β, and IL-6 in plasma, as well as, activated kinases, transcription factors in blood cells have been analyzed and reported (257, 298-303). Many of these trials announced the effect of RES on these parameters (257). Nonetheless, this information has not yet been translated into an approved clinical application

because no clinical improvements in the patients' status or reductions in disease-specific life-threatening events have been analyzed (257).

- *RES Mechanisms of action as anti-inflammatory agent*

Similar to CUR, RES has been widely recognized for its remarkable pharmacological activities as anti-mutation, anti-inflammatory, and antioxidant activities (47, 257). Linked to its anti-inflammatory effects, RES also has anti-tumor activity as it hinders cancer initiation and progression (269, 297, 304). Many studies demonstrated the protective role of RES in neuro and cardioprotection, as well as, arthritic joint pain (47). In support of this view, Elmali *et al*, in 2007, demonstrated the anti-inflammatory effects of RES in paw edema and knee osteoarthritis when injected intra-articular in animals, indicating an inhibitory effect on inflammatory PG synthesis (47, 284).

Numerous studies have been conducted to provide in-depth insights into the powerful anti-oxidant and anti-inflammatory underlying mechanisms of RES, it has been shown that RES, in a dose-effect relationship, exerts its effects at multiple levels: it inhibits TLR4 and MyD88 expression in activated RAW 264.7 macrophages (305, 306). As well, it inhibits NF-κB, MAPK, IRF-3, AP-1, iNOS, COX-2, and 5-LOX pathways, which modulate the secretion of pro-inflammatory cytokines (15, 257, 306-309). Subsequently, it reduces NF-κB induced pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, as well as, the inflammatory mediators, NO and ROS (15, 47, 257, 269, 305, 310, 311). It also lowers LTs and PGs levels due to suppressing COX-2 signaling (15, 257, 311). These results are confirmed in LPS-stimulated RAW 264.7 cells, and macrophages from C57BL/6 and BALB/c mice (312).

In addition to studies performed on macrophages, others were conducted on heart tissues of rats to investigate the anti-oxidant effect of RES, highlighting the role of TLR4/NF-κB signaling in early inflammation associated with DIC (313). These studies showed the effective cardioprotection effect of RES, which has been demonstrated in lowering left ventricular peroxidation and enhancing antioxidants, such as GSH and SOD, and decreasing TNF-α levels (314). Consistent with the results of previous studies, RES has been shown to inhibit TLR4/NF-κB signaling in ischemic injured rat heart model, confirmed by TLR4 and NF-κB downregulation,

and reduced myocardial TNF-α production (313). Lastly, RES was reported to downregulate the levels of several proinflammatory miRNAs, such as miR-155, miR-21 and miR-146, which are upregulated in LPS-induced macrophages (304, 315, 316).

Conclusively, polyphenols , such as CUR and RES are involved in numerous signaling pathways, and most importantly, in regulating the redox system and modulating immune response through inhibiting inflammatory cytokines synthesis (248). Unfortunately, polyphenols, in general, have poor bioavailability, thus encouraging research to be directed to aliphatic isothiocyantes, such as SFN, which exhibit significantly higher bioavailability and potent anti-inflammatory response (248).

1.3.3.3 Sulforaphane (SFN)

SFN: 1-isothiocyanato-4-(methylsulfinyl) butane is one of the highly studied plant-derived isothiocyanate organosulfur compounds (317). SFN is characterized by the presence of a sulfocyanate group (N=C=S), and is found in cruciferous vegetables from the Brassicaceae family, including broccoli, cabbage, cauliflower, and kale (317, 318). A precursor of SFN, glucoraphanin is a glucosinolate compound which is composed of a sulfonated oxime group (β-D-thioglucose group) and an amino acid-derived side chain (317). Through enzyme dependent hydrolysis, glucosinolates are activated to give their respective isothiocyanates; in case of SFN, glucoraphanin is hydrolyzed by myrosinases (317, 319).

Usually, SFN is found as L-isomer, however, it is used as a synthetic racemic mixture of D, L-SFN in research studies (318, 320, 321). In contrast to polyphenols, SFN was reported to have relatively high bioavailability (around 80%) (248, 322-324). Studies showed that SFN peaks in blood after only one hour of ingestion, thus can be considered for development as a nutraceutical compound (248, 325, 326). It has also been stated that SFN oral dosage in clinical trials ranges from 20 to 40 mg in preliminary clinical studies (248). To date, SFN has low toxicity profile without any serious side effects (317, 322, 327). Due to SFN effects on different cellular targets, its pharmacological effects are pleiotropic, including: anti-microbial, antioxidant, anti-inflammatory, anti-cancer, anti-aging, neuroprotective, antidiabetic, and cardioprotective (317, 328-334). Several studies have revealed its potential anti-inflammatory and antioxidant properties

(322). SFN is a potent immunomodulatory in chronic inflammatory diseases through targeting monocytes/macrophages lineage and triggering nuclear factor erythroid-derived 2-like 2 (Nrf2) antioxidant defense pathway (335, 336). Also, SFN is involved in ameliorating microglial-mediated neuro-inflammation and aging-associated oxidative stress (337).

In addition, SFN anti-cancer effect has been shown in hepatocellular carcinoma through hindering cell proliferation, and in lung cancer through inhibiting histone deacetylase (HDAC) activity (338, 339). While, in hypoxic injuries, SFN demonstrated cardioprotective activity, and protected the vascular endothelial cells through Nrf2 mediated antioxidative defense (340, 341). In the same manner, SFN mitigates vascular impairment and progression of high cholesterol diet-induced atherosclerosis through its antioxidant capacity and suppression of NF-κB-mediated inflammation (342). SFN is reported to prevent cardiac hypertrophy by downregulating MAPK signaling pathways (343). Furthermore, SFN manages to suppress inflammation in a rat model of focal cerebral ischemia by inhibiting NF-κB signaling pathway (344). In another study, SFN reduced systemic inflammation in cardiovascular bypass-porcine model through downregulating NF-κB expression (322, 345). Also, SFN exhibited noticeable anti-inflammatory and antioxidant effects in LPS-induced liver injury (346). These effects has been also confirmed by Sing et al. study in autism disorder, in which SFN successfully reversed neuro-inflammation, oxidative stress, low antioxidant capacity, reduced glutathione synthesis, increased lipid peroxidation, impaired mitochondrial function and oxidative phosphorylation (327). And clinically, over 1900 trials on SFN are published in PubMed (248).

- *SFN Mechanisms of Action as anti-inflammatory agent*

SFN has a dual action in modulating redox system and immune imbalance through interacting with Nrf2 and NF-κB signaling pathways (248). These two key transcription factors (Nrf2 and NF-κB) act both independently and dependently *via* their "cross talk", which is not yet fully understood (248, 347). SFN is considered an indirect antioxidant because it is not involved in quenching free radicals and ROS; however, it upregulates some phase II enzymes by enhancing Nrf2 activity (318, 348-351). Nrf2 is involved in cytoprotection through recruiting direct antioxidants, increasing GSH metabolism synthesis, and inhibiting inflammation. The activity of Nrf2, however, is

negatively regulated by the Kelch-like ECH-associated protein 1 (Keap1) which promotes its degradation by the ubiquitin–proteasome pathway (318, 349, 352).

Furthermore, SFN activates Nrf2-Keap-1 complex with its sulfur chemistry (353). The chemistry of sulfur plays an integral role in Nrf2 activation, because Keap-1 has sulfur-rich cysteine residues that regulate its oxidation/reduction (248, 353). In this context, SFN abrogates the binding of Nrf2 to Keap1, allowing the translocation of Nrf2 to the nucleus, and as a transcriptional activator, Nrf2 modulates the gene expression of the genes containing the antioxidant response elements (ARE) in their DNA sequence (318, 354-356). Thus, by destabilizing the bond between Nrf2-keap1 protein, SFN enhanced the expression of these ARE sequences and activated the transcription of antioxidant enzymes genes including: nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), γ-glutamylcysteine ligase (γGCL), thioredoxin, GSH and SOD (248, 318, 322, 357-361). These Phase II enzymes are important in ROS neutralization (322, 362). HO-1, in particular, is involved in cytoprotective effects through antioxidant and anti-inflammatory properties in cardiovascular and other tissues (248). This mechanism has been confirmed in mouse peritoneal macrophages, where SFN exhibited its anti-inflammatory activity mainly *via* activation of Nrf2 (322).

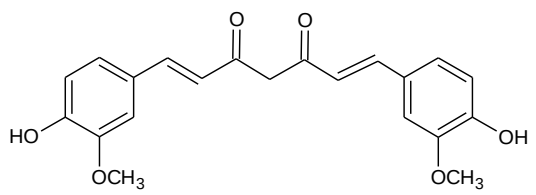
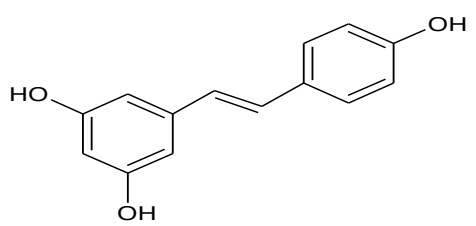
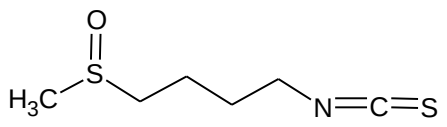
In parallel, the anti-inflammatory effects of SFN has been demonstrated in the form of reduced levels of LPS-induced pro-inflammatory mediators, such as TNF-α, iNOS, and COX-2 (81, 318, 363, 364). While SFN directly activates cytosolic Nrf2, its action on NF-κB is to inhibit NF-κB binding to the DNA (248, 363). These findings has been reported *in vitro* on LPS-stimulated RAW 264.7 macrophages and *in vivo* on C57BL/6 mice (365). A recent study showed for the first time the ability of SFN to suppress the direct binding between NF-κB and its consensus sequence in DNA *via* its thiol groups, therefore suppressing LPS-induced pro-inflammatory mediators in macrophages (366). SFN inhibits DNA binding of NF-κB without interfering with nuclear translocation of NF-κB or degradation of IKB-α in LPS-stimulated macrophages (363, 367). And, it has been suggested that SFN might directly inactivate NF-κB subunits by binding to essential cysteine residues (363). In addition, SFN has been identified as a potent bioactive molecule that can suppress inflammation through inhibition of LPS-TLR4 binding (248, 368, 369). One of the novel underlying anti-inflammatory mechanisms of SFN molecule is its ability to suppress TLR4

oligomerization in a thiol dependent manner in macrophages, where SFN formed adducts with cysteine residues in the extracellular domain of TLR4 (81, 248, 363, 364, 367). SFN suppressed both ligand-induced and ligand-independent oligomerization of TLR4 (367). Oligomerization is an important step for TLR4 activation and recruitment of adaptor molecules; therefore, the reactivity of SFN to sulfhydryl moiety contributes to its inhibitory activities and subsequent downregulation of NF-κB activation (367). Similar to CUR, SFN antagonizes LPS binding to TLR4/MD-2 complex by selectively competing on MD-2, a large hydrophobic pocket where LPS binds and mediates TLR4 dimerization (81, 363, 364, 367, 370). This in-depth investigations successfully connected the gut-immune relationship to systemic disease (248, 371). In leukocytes, increased phosphorylation of p38 and p65 (RelA) subunit of NF-κB was resolved upon SFN treatment, associated with downregulation of TNF-α, IL-6, and IL-8 expression levels (318, 345). In contrast, SFN showed no effect on p38 and p65 phosphorylation in organs, including heart, lung, and kidney; therefore, SFN effect was suggested to be at the systemic, not local, level (318, 345). Furthermore, previous studies have shown that SFN successfully prevented carcinogenesis, which is relevant in part to its potent anti-inflammatory properties (322).

In accordance with SFN anti-inflammatory activities, several studies pinpointed the crosstalk between Nrf2 and NF-κB pathway (318). SFN is commonly known to induce its anti-inflammatory activity through the activation of Nrf2 transcription factor (322). Through activating Nrf2/ARE pathway which equilibrates ROS imbalance, SFN inhibits the expression of pro-inflammatory mediators as ROS overproduction is known to activate NF-κB and AP-1 inflammatory-regulatory factors (322, 372). This has been confirmed by Li et.al study showing that SFN activates Nrf2 pathway through inhibiting Nrf2 ubiquitination, and concomitantly reducing NF-kB expression and AP-1, thus restoring endogenous antioxidant levels and reducing inflammatory damage in an experimental autoimmune encephalomyelitis mice model (322, 368, 373-376). Furthermore, Sun et al. study correlated Nrf2 activation by SFN with the downregulation of inflammatory mediators (IKK-α, p-IKK-β, and NF-κB) and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) with increased expression of IκB-α in skeletal muscle (318, 377). Similarly in LPS-stimulated RAW 264.7 cells, SFN inhibited IκB-α degradation and hence NF-κB activation (41). Importantly, studies on the miRNA level indicated that SFN significantly attenuated miRNA-155 and 146a levels in LPS-stimulated RAW264.7 macrophages in a dose-dependent manner (365, 378).

In addition, SFN has illustrated cardioprotective effect against DIC; it reduced DOX-induced oxidative stress and enhanced mitochondrial respiration in the cardiomyocytes of DOX-treated rats (180). Lastly, SFN showed chemopreventive action through epigenetic inhibitory effect on HDACs, providing DNA protection (248, 379-381). This has been demonstrated as a selective downregulation of class I and II HDAC enzymes, and HDAC 6 (322).

Table 1.1 Summary of key information on curcumin, resveratrol, and sulforaphane reporting their main antioxidant and anti-inflammatory mechanisms

Name	Structure	Source	Anti-oxidant Mechanisms	Anti-inflammatory Mechanisms	Reference
Curcumin		A polyphenol found in Turmeric (*Curcuma longa)*	Inhibits lipid peroxide formation and lysosomal enzymes, such as acid phosphatase and cathepsin D Increases the serum antioxidants, such as SOD and GSH Scavenges ROS and RNS, Inhibits ROS generating enzymes (iNOS and COX-2)	Modulates TLR4 and MyD88 in macrophages through blocking NF-κB, AP-1, and MAPK Inhibits M1 macrophage depolarization through TLR4 downregulation Inhibits proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β Inhibits TLR4-induced miR-155, a transcription regulator of inflammation	(47, 159, 250, 252, 257, 264-268, 270-274, 277, 280, 281)
Resveratrol		A polyphenol mainly found in skin of red grapes	Inhibits iNOS and COX-2, and lowers PGs Inhibits ROS and NO release	Inhibits TLR4 and MyD88 expression, and NF-κB, MAPK, AP-1, and IRF-3 Inhibits proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β Inhibits miR-155, miR-21, and miR-146a	(15, 47, 257, 269, 305-311)
Sulforaphane		An isothiocyanate found in cruciferous plants, such as broccoli	Activates Nrf2 pathway and upregulate phase II enzymes, and reducing ROS Reduces iNOS and COX-2	Inhibits NF-κB binding to DNA Inhibits LPS-TLR4 Binding, and inhibit TLR4 oligomerization Inhibits proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β Inhibit miR-155 and miR-146a	(81, 248, 317, 318, 345, 348-351, 363-365, 367-369, 378)

Structures are drawn using ACD/ChemSketch Freeware (https://www.acdlabs.com/resources/freeware/chemsketch/)

1.4 Rationale

In this current study, we have selected these three natural products namely CUR, RES, and SFN because of their effectiveness and prominent anti-inflammatory properties through antagonizing TLR4 signaling pathway, responsible for the exaggerated innate immune response and systemic inflammation induced by DOX (30).

1.5 Hypotheses

We hypothesize that the three naturally occurring compounds CUR, RES, and SFN will differentially attenuate the DOX-mediated inflammation in the murine macrophages cell line RAW 264.7. Furthermore, we hypothesize that these differential effects are occurring in part due to an effect at the epigenetic level through affecting miRNAs levels.

1.6 Objectives and Aims

The objective of the present study is to compare the effect of three naturally derived compounds with anti-inflammatory effects, which are CUR, RES, and SFN, against DOX-mediated inflammation through affecting TLR4 signaling pathway in RAW 264.7 murine macrophages.

In addition, the specific aims of the current study are to:

1. Examine the effects of CUR, RES, and SFN on LPS/IFN-γ-mediated effects on TLR4, TNF-α, IL-6, and iNOS mRNA expression levels in the absence and the presence of DOX using real-time PCR.
2. Compare the effects of CUR, RES, and SFN on LPS/IFN-γ-mediated induction of nitric oxide (NO), in addition to TNF-α and IL-6 protein levels in the absence and presence of DOX using Griess reagent and ELISA, respectively.
3. Identify the underlying mechanism(s) involved in this modulation of these inflammatory markers utilizing several molecular biology techniques.

- *Novelty of this research*

The novelty of this project is deciphered in its ability to answer an important question with regards to the capabilities of three naturally derived compounds of well-known anti-inflammatory effects to differentially protect against DOX induced inflammation. This project identified the use of SFN as a novel therapeutic adjuvant to protect against DOX induced inflammation and subsequent toxic effects. Furthermore, the results of this thesis showed for the first time a comparison between three naturally derived compounds of known anti-inflammatory effects namely CUR, RES, and SFN on DOX induced inflammation in RAW 264.7 macrophages.

2. MATERIALS AND METHODS

2.1 Materials

The murine monocyte/macrophage RAW 264.7 cell line ((ATCC® TIB71TM) was obtained from the National Research Centre (NRC) Cairo, Egypt. LPS (*Escherichia coli* 0111: B4; L2630) and Curcumin (458-37-7) were purchased from Sigma Chemical Co. (MO, USA). Murine Interferon-γ (315-05) was obtained from PeproTech (NJ, USA). Dulbecco's Modified Eagle Medium Gibco™ DMEM, High Glucose (41965-039), Fetal Bovine Serum Gibco™ FBS (10270-106), Dimethyl sulfoxide DMSO (67-68-5), Chloroform (HPLC grade; C607SK-1), Isopropanol (HPLC grade; BP26324), Ethanol (HPLC grade; 64-17-5), RevertAid cDNA kit (K1621), Maxima SYBR Green qPCR (K0251), mRNA primers (10629186; designed by NCBI primer blast tool), were all purchased from ThermoFisher Scientific (MA, USA). Griess Reagent Kit (G7921) was purchased from Invitrogen (CA, USA). DMEM media (with 4.5 g/L Glucose, without L-Glutamine, without phenol red) (12-917F), Penicillin-Streptomycin Mixture Pen/Strep (09-757F), and Phosphate Buffered Saline (10X) PBS (17-516Q) were obtained from Lonza-Bioscience (Basel, Switzerland). Doxorubicin (25316-40-9) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; 298-93-1) were purchased from TOCRIS (Bristol, UK). Sulforaphane (10496), Resveratrol (70675), murine TNF-α (500850) and IL-6 (583371) Enzyme-linked immunosorbent assays **(**ELISA) were purchased from Cayman Europe OÜ (Tallinn, Estonia). QiAzol lysis buffer (79306), RNAse/DNAse free water (129114), miScript II RT kit (218161), miScript SYBR Green PCR kit (218073), miScript Primer Assays: Mm_miR-155_1 (MS00001701), Mm_miR-21_2 (MS00011487), Mm-miR-146a*_1 (MS00024220), Hs_RNU6-2_11 (MS00033740) were purchased from Qiagen (Hilden, Germany).

2.2. Cell culture

RAW 264.7 cells (cell information: see Table 2.1 and Fig. 2.1) were grown in 75 cm^2 flasks until 80% confluence at 37 °C in a 5% CO_2 humidified incubator. As suggested by ATCC, cultured cells were maintained in DMEM high glucose media supplemented with 10% heat inactivated fetal bovine serum FBS and 1% Pen-Strep (100 units/mL penicillin, and 100 μg/mL streptomycin) at 37 ^oC in humidified air with 5% CO_2 (382, 383). When cells reached a density of 2-3×10^6 cells/mL they were seeded in 96 well plate for 2 h and then stimulated by new medium containing *E. coli*

LPS (10 ng/mL) and murine Interferon-γ (10 U/mL) for 24 h with/without treatments (383). Then, DOX (0.1 µM), CUR, RES, and SFN (5, 10, and 20 µM) dissolved in dimethyl sulfoxide (DMSO, as final concentration of 0.1%) were added separately with/without LPS/IFN- γ. Cells were treated with 0.1% DMSO as vehicle control.

Table 2.1: RAW 264.7 cell line information(382)

Cell line RAW 264.7
Species Mus musculus
Strain BALB/c
Tissue origin ascites (Abelson murine leukemia virus induced tumor)
Age adult
Gender male
Morphology monocyte/macrophage
Growth property adherent
Medium DMEM high glucose, supplemented with 10 % FCS
Sub culturing 1:3 to 1:6 (up to 1:10 in this study; when reaching 80 % confluence)
Medium renewal every 2 to 3 days
Culture conditions 37 °C, 5% CO_2 in air atmosphere (7% CO_2 in this study)
Biosafety level 2

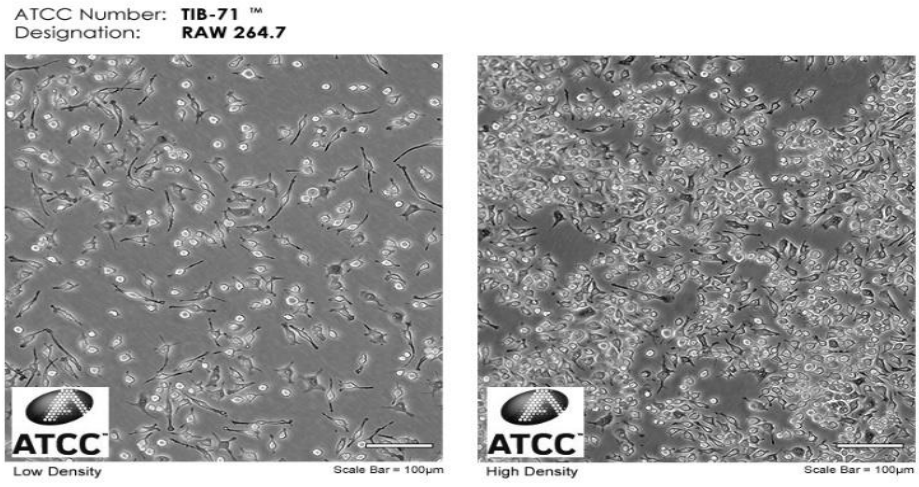


Fig. 2.1: RAW 264.7 ATCC® TIB-71™ (382).

1.3. Cell viability: MTT assay

RAW 264.7 cells were seeded in the density of (2×10^5 cells/well) in a 96-well plate for 2 h. For optimization, cells were treated with LPS (10 ng and 100 ng) and IFN- γ (5 and 10 U/ mL) separately and in mix and match combinations to determine the non-cytotoxic concentration for stimulation of Raw 264.7 (384). In another experiment, cells were treated with increasing concentrations of DOX (0.005, 0.01, 0.05, 0.1, or 0.5 µM). Also, different concentration of treatments CUR, RES, and SFN (5, 10, and 20 µM) have been tested.

Based on the optimization results, RAW 264.7 cells in further experiments were stimulated with 10 ng/ml LPS plus 10 U/mL IFN-γ in the presence or absence of 0.1 µM DOX, and exposed to herbal treatments CUR, RES, or SFN at concentrations (5, 10, or 20 µM) for 24 h. Cellular viability assay was analyzed using the MTT colorimetric assay as previously described (385). First, cells were seeded in a 96 well-plate with density of ($2x10^5$ cells/well) and then incubated for 2 h. Then, DMEM media was discarded and replaced with fresh new DMEM media without phenol-red and treatments were added in the concentrations mentioned above. After 24 h of incubation with different treatments, culture medium from each well were drawn and replaced with 100 µL of MTT solution (1 mg/mL in serum-free media) were added to each well. After 2 h of incubation at 37 °C, MTT media was removed from wells and replaced by 200 µL of isopropyl alcohol to dissolve formazan crystals resulting from the reductases activity of viable cells. The absorbance was read at 540 nm using Nano SPECTROstar microplate reader (BMG LABTECH, Ortenberg, Germany), and the percentage of macrophages viability was calculated as relative from control (386).

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\ \% \quad (387)$$

2.4. Nitrite Assay: Griess method

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction. Following the same conditions as previously mentioned with MTT assay, Griess method was done on cell supernatant as follows: 150 µL of each supernatant medium was drawn from the wells and diluted with 130 µL of deionized water and 20 µL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) was added. Then the plate was kept in dark for 30 min. Absorbance of the mixture at 550 nm was measured with Nano SPECTROstar microplate reader (383, 388). The intensity of the color is directly proportional to the nitrite concentration. Finally, standard curve was plotted with absorbance (y-axis) versus nitrite standard (S1-S4) concentrations (x-axis). Using line equation, concentration of nitrite was calculated for each sample (Fig. Supl.2.1C).

2.5 Treatment and isolation of total RNA

Raw 264.7 cells were seeded in the density of (2×10^6 cells/well) in a 6-well plate for 2 h. Then cells were treated with CUR, RES, and SFN at concentrations (5 and 20 µM) alone and with LPS/IFN-γ induction in the absence or the presence of DOX, then incubated for additional 6 h. After 6 h of incubation with the test compounds, total RNA was extracted using QIAzol Lysis Reagent according to the manufacturer's guidelines (358). In each well, 600 µL of QIAzol were added, and cell lysates were collected in different microcentrifuge tubes, then 120 µL chloroform was added, and incubated for 2-3 min, then undergone 15 s vigorous shaking, followed by centrifugation at 12,000 x *g* at 4 °C for 15 min. The mixtures separates into 3 layers; lower red phenol red-chloroform, interphase, and a colorless upper aqueous phase. Aqueous phase containing RNA was transferred to a new tube. Next, 300 µL of Isopropanol was added, incubated for 10 minutes and then centrifuged for another 10 min at 12000 xg at 4 °C . Then, total RNA precipitated at the bottom of the tube and the supernatant was discarded. The precipitated pellet was washed with 75 % Ethanol, vortexed briefly and then centrifuge at 7500 x *g* at 4 °C for 5 min. Then, supernatant was discarded and the tube was left to air dry the RNA pellet for 5-10 min (air dry time should not extend beyond 10 min to avoid RNA crystallization). Finally the pellet was re-suspended in 30 µL Nuclease Free water and incubated in heat block at 60 °C for 15 min. Then,

RNA samples was quantified through measuring their absorbance at 260 nm (ng/µl) and the A260/280 ratio (to check phenol contamination) by NanoDrop Spectrophotometer (Table Supl.2.2). Finally, RNA was stored at -80 °C or proceeded in downstream applications (389).

2.6 cDNA synthesis

2.6.1 cDNA synthesis for qualitative analysis of mRNA

For the mRNA, the first-strand cDNA was synthesized using the Revertaid cDNA synthesis kit according to the manufacturer's guidelines (390). Briefly, 1 µg of the total RNA of each sample was diluted with nuclease-free water up to 10 µL (Table 2.2). Then 10 µL from cDNA reaction (Table 2.3) was added to each sample for a final volume reaction of 20 µL. The cDNA reaction Mastermix is composed of of 4.0 µL 5× reverse transcription (RT) buffer, 2 µL 10mM dNTP mix (100 mM), 1.0 µL RT random Hexamer primers and 1.0 µL Oligo $(dt)_{18}$ primer, 1.0 µL RevertAid M-MuLV RT (200 U/µL) reverse transcriptase, 1.0 µL RiboLock RNase Inhibitor (20 U/µL). The final reaction mixture will be undergo the following conditions: 25 °C for 5 min, followed by 42 °C for 60 min, and then 70 °C for 5 min to terminate the reaction, and finally cooled to 4 °C in 96 well Thermal cycler (Applied Biosystems, CA, USA) (390). The completed reaction was stored at -20°C to prevent cDNA degradation.

Prior to quantification by qPCR, the newly synthesized cDNA was diluted by 1:3 ratio with nuclease free water by adding 40 µL nuclease-free water to the 20 µL cDNA reaction, from which 3 µL will be taken for real-time PCR reaction.

Table 2.2: Revertaid cDNA reaction

Kit component	Per reaction (µL)
5X Reaction Buffer	4
Oligo $(dT)_{18}$ primer	1
Random Hexamer primer	1
RiboLock RNase Inhibitor (20 U/µL)	1
10 mM dNTP Mix	2
RevertAid M-MuLV RT (200 U/µL)	1
Total volume	10

2.6.2 cDNA synthesis for qualitative analysis of miRNA

Reverse transcription is performed for miRNAs. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of mature miRNA in the real-time PCR step. cDNA synthesis was performed using miScript II RT kit according to the manufacturer's instructions (391). 2 µg of RNA from each sample was diluted with nuclease-free water up to 12 µL according to the RNA volume taken (Table 2.4). Then, 8 µL of miScript RT reaction (Table 2.5) was added to each sample for a final reaction volume of 20 µL. The reaction contains 4 µL of 5x miScript HiSpec buffer, 2 µL of 10x miScript Nucleics mix and 2 µL of miScript Reverse Transcriptase enzyme. This method synthesized cDNA from mature microRNAs. The RT reaction was incubated at 37 °C for 1 h followed by inactivation of the RT enzyme at 95 °C for 5 min. The completed reaction was stored at -20 °C to prevent cDNA degradation.

Prior to quantification by q-PCR, the newly synthesized cDNA was diluted 1:100 with nuclease-free water, by taking 2 µL from the 20 µL synthesized cDNA and adding 198 µL of nuclease-free water. Then, store the diluted cDNA into -20°C. For real-time PCR reaction, 2 µL were taken from the diluted cDNA.

Table 2.3: miScript II RT Reaction

Kit component	Per reaction (µL)
5X miScript HiSpec Buffer	4
10X miScript Nucleics Mix	2
miScript Reverse Transcript	2
Total volume	8

2.7. Quantification of mRNA and miRNA using real-time PCR (q-PCR)

Raw 264.7 cells were seeded in the density of (2×10^6 cells/well) in a 6-well plate for 2 h. On the following day cells were treated with the tested compounds and incubated for 6 h. Then, cells were harvested and their total RNA was extracted, reverse transcribed, quantified, and analyzed as previously described in sections 2.5 and 2.6 (392, 393). Using q-PCR, quantitative analysis was performed for the expression of specific mRNAs (TLR4, TNF-α, IL-6, and iNOS) normalized against GAPDH and miRNAs (miR-21, miR-146a, and miR-155) normalized against RNU6-6p. The resulting cDNA was further amplified using the ABI Prism 7500 system (Applied Biosystems, CA, USA).

2.7.1 Quantification of mRNA

For mRNA, Maxima SYBR Green qPCR Mater Mix (2X) was used. The SYBR green mRNA reaction (Table 2.6) was of total volume 12.5 µL which contain: 0.375 µL of 10 µM forward primer and 0.375 µL of 10 µM reverse primer (equivalent to a final primer concentration of 0.3 µM), 6.25 µL of SYBR Green Universal Mastermix, 2.5 µL of nuclease-free water, and 3 µL of cDNA sample (1:3 dilution; equivalent to 50 ng cDNA). Then, the plate was covered with an optical adhesive cover, and the thermocycling conditions were conducted as follows: initiation at 95 °C for 10 min, followed by 40 PCR repeated cycles of denaturation at 95 °C for 15 s and anneal/extension at 60 °C for 1 min. A melting curve was released by the end of each cycle to verify the primers specificity and the final PCR product purity (394). Specific pairs of

primer that were utilized for quantification of mRNAs was described in the (Table 2.7). Primers were generated using the online NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and purchased from ThermoFischer.

For mRNA primer reconstitution: Forward and reverse primers from ThermoFischer (in nano-moles) were briefly centrifuged and then suspended in 10 times the number of their molecular weight (X nmoles * 10) µL of nuclease free water to give 100 µM stock. From this stock, a working stock of 10 µM (1:10 dilution) is prepared by taking 5 µL from 100 µM primer stock and diluted with 45 µL nuclease free water. From this working solution, 0.375 µL will be taken for real-time PCR reaction.

Table 2.4: SYBR green mRNA PCR reaction

Component	Per Reaction (µL)
Maxima SYBR Green qPCR Master Mix (2X)	6.25
Forward Primer (0.3 µM)	0.375
Reverse Primer (0.3 µM)	0.375
cDNA	3
Nuclease-free water	2.5
Total Volume	12.5

Note: Add 2 µl of ROX Solution to 18 µl of Water, nuclease-free, mix and use 0.05 µl for 25 µl qPCR reaction.

Table 2.5: List of mRNA primers used for RT-PCR:

	Sequence 5'…..3'	**Primer**	**Tm**
1	CTTTGTCAAGCTCATTTCCTGG	GAPDH-F	57
2	TCTTGCTCAGTGTCCTTGC	GAPDH-R	58
3	GATGCTACCAAACTGGATATAATCAG	IL6-F	55
4	CTCTGAAGGACTCTGGCTTTG	IL6-R	58
5	GGAACCTACCAGCTCACTCTGG	iNOS -F	63
6	TGCTGAAACATTTCCTGTGCTGT	iNOS -R	60
7	TTCAGAACTTCAGTGGCTGG	TLR4-F	58
8	TGTTAGTCCAGAGAAACTTCCTG	TLR4-R	56
9	GAACTCCAGGCGGTGCCTAT	TNF-α-F	63
10	TGAGAGGGAGGCCATTTGGG	TNF-α-R	63

2.7.2 Quantification of miRNA

For miRNA, miScript SYBR® Green PCR Kit and miScript Primer Assays were used. A total reaction volume of 10 µL containing 5 µL of 2x QuantiTect SYBR Green PCR Master mix, 1 µL of 10 x miScript universal primer, 1 µL of 10x miScript specific primer assay and 2 µL of cDNA template (1:100 dilution) and 1 µL of RNase/DNase-free water was used in 96 well plates (Table 2.8) (391). Specific forward miRNA primers were selected based on previously published reports and purchased custom-made from Qiagen (Table 2.9). Then, the plate was covered with an optical adhesive cover, a standard protocol designed by Qiagen was followed with initial denaturation step at 95 °C for 15 min, followed by 40 PCR repeated cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 70 °C for 34 s. Fluorescence data collection was performed at the end of each elongation step. All samples were tested in duplicates and nuclease

free water was used as a non-template control (391). A universal reverse primer for microRNA quantification was used for all reactions and was supplied in miScript SYBR Green PCR Kit.

For miRNA primer reconstitution, miScript Primer Assay vial was briefly centrifuged and suspended in 550 µL of nuclease free water, then vortexed 5-6 times as described by Qiagen protocol (391).

Table 2.6: SYBR green mRNA PCR reaction

Component	Per Reaction (µL)
2x QuantiTect® SYBR Green PCR Master Mix	5
10x miScript Universal Primer	1
10x miScript Primer Assay	1
Template cDNA	2
Nuclease-free water	1
Total Volume	10

Table 2.7: List of mature miRNA primers used for qRT-PCR

	Sequence	MiRNA name	Catalog no.
1	UAGCUUAUCAGACUGAUGUUGA	mmu-miR-21a-5p	MS00011487
2	CCUGUGAAAUUCAGUUCUUCAG	mmu-miR-146a-3p	MS00024220
3	UUAAUGCUAAUUGUGAUAGGGGU	mmu-miR-155-5p	MS00001701
4	Housekeeping gene	RNU6-6P	MS00033740

2.8. Real-time PCR data analysis

The real-time PCR amplification data was analyzed by relative gene expression (ΔΔCT method). The second-derivative maximum algorithm $2^{-\Delta\Delta CT}$ method (395), by which fold change was calculated as the ratio of the relative change between a treated sample and the average of the relative change values of untreated samples. Briefly, the used primers were tested against nonspecific amplification, primer dimers, or self-priming formation. To confirm quality measurements for each gene, each plate included the following: no template control, negative control, and a positive control. For each tested sample, a threshold cycle (CT) was calculated depending on the number of PCR cycles after which the reporter fluorescence emission increased beyond a threshold level (determined by the background fluorescence of the system). Each sample was measured in triplicate, and an averaged CT values was taken for each group after outliers' removal. The samples were diluted in such a manner so that the CT values will be detected between 15 and 30 cycles. As described in User Bulletin 2 of Applied Biosystems, results were expressed using the comparative CT method. For each gene of interest, ΔCT values were calculated in every sample (CT gene of interest − CT reporter gene), using GAPDH as the reporter gene for mRNA and RNU6-6P as the reporter gene for miRNA. Then, relative changes in the expression level of each specific gene (ΔΔCT) were calculated by subtracting the ΔCT of the untreated control group from the ΔCT of the respective treatment groups. The values and ranges given in different figures were determined as $2^{-\Delta\Delta CT}$ with (ΔΔCT + SE) and (ΔΔCT – SE), where SE is equivalent to the standard error of the mean of the ΔΔCT value (User Bulletin 2; Applied Biosystems) (396).

2.9. Quantification of protein using ELISA

ELISA kits were utilized for the measurement of TNF-α and IL-6 proteins in the supernatants (397). RAW 264.7 cells were cultured at a density of 2×10^6 cells/well in 6-well plates for 2 h. Then, treated with 20 µM of CUR, RES, and SFN alone or in combination LPS/IFN-γ in the presence or absence of DOX. After 24 h of incubation, the supernatant was collected, centrifuged and transferred to new microcentrifuge tubes to measure the levels of released TNF-α and IL-6.

2.9.1. Quantification of TNF-α

The levels TNF-α for cell culture supernatants were quantified by ELISA kits according to the manufacturer's instructions (398).

Buffers preparation:

Assay Buffer: Immunoassay buffer B concentrate (10X) (Item No. 400054) was diluted with 90 mL deionized water.

Wash Buffer: 5 ml vial (Item No. 400062) was diluted to a total volume of 2 L with deionized water and then 1ml Polysorbate 20 was added.

The anti-TNF-α (mouse) ELISA strip 96 well plate (Item No. 400852) is coated with a rat monoclonal antibody specific for mouse TNF-α. First step, 100 µL of (1:20 diluted with deionized water) supernatant samples were added to corresponding wells, and 100 µL of TNF-α ELISA standard (Item No. 400854, reconstituted with 0.5 ml assay buffer) were added in duplicate to wells (to be used for further standardization), then the plate was incubated for 2 h at room temperature on an orbital shaker (analytic jena, Jena, Germany), then the plate was washed 4 times with wash buffer. Next, plate was incubated for the second time with 100 µL anti-TNF-α biotin conjugate rat monoclonal antibody (Item No. 400850, reconstitutes with 12 ml assay buffer) for 1 h. at RT on orbital shaker. Again the plate was washed 4 times with wash buffer. Third incubation was done with 100 µL of diluted TNF-α streptavidin-HRP (Item No. 400853) (10X TNF-α streptavidin-HRP was diluted with 10.8 mL assay buffer) for 30 min at RT on an orbital shaker. Then wells are emptied and washed 4 times. Then, 100 µL of the chromogenic TMB (3,3',5,5'-tetramethylbenzidine) substrate solution (Item No. 400072) was added to each well for 30 min at RT followed by 100 µL HRP Stop Solution (Item No. 10011355) to stop the reaction, and then the plate developed a yellow color. Finally, TNF-α concentration was determined spectrophotometrically at 450 nm. The intensity of the color is directly proportional to the TNF-α concentration. Finally, standard curve was plotted as absorbance (y-axis) versus TNF-α standard (S1-S7) concentrations (x-axis). Using line equation, concentration of TNF-α was calculated for each sample (Fig. Supl.2.1A).

Note: TNF-α ELISA kit sensitivity: minimum detectable concentration is 15.6 pg/ml

2.9.2 Quantification of IL-6

The levels IL-6 for cell culture supernatants were quantified by ELISA kits according to the manufacturer's instructions (399).

Buffer Preparation:

Assay Buffer: Immunoassay buffer B concentrate (10X) (Item No. 400054) was diluted with 90 mL deionized water.

Wash Buffer: 5 mL vial (Item No. 400062) was diluted to a total volume of 2 L with deionized water and then 1 mL Polysorbate 20 was added.

The anti-IL-6 (mouse) ELISA strip 96 well plate (Item No. 483372) is coated with a rat monoclonal antibody specific for mouse IL-6. First, 100 µL of supernatant samples (1:10 diluted with deionized water) were added to corresponding wells and 100 µL of IL-6 ELISA standard (Item No. 483375, reconstituted with 1ml assay buffer) were added in duplicate to wells and incubated for 1 h at room temperature on an orbital shaker, then the plate was washed 4 times with wash buffer. Next, plate was incubated for the second time with 100 µL of anti-IL-6 Biotin Conjugate rat monoclonal antibody (Item No. 483370, reconstitutes with 12 mL assay buffer) for 1 h. at RT on orbital shaker. Again the plate was washed 4 times with wash buffer. Third incubation with 100 µL of diluted IL-6 streptavidin-HRP (Item No. 483373) (1.2ml (10X) IL-6 streptavidin-HRP was diluted with 10.8 ml assay buffer) was done for 30 min at RT on an orbital shaker. Then wells were emptied and washed 4 times. Then, 100 µL of the chromogenic TMB (3,3',5,5'-tetramethylbenzidine) substrate solution (Item No. 400072) was added to each well for 30 min at RT followed by 100 µL HRP Stop Solution (Item No. 10011355) to stop the reaction, and then the plate developed a yellow color. Finally, IL-6 concentration was determined spectrophotometrically at 450 nm. The intensity of the color is directly proportional to the IL-6 concentration. Finally, standard curve was plotted as absorbance (y-axis) versus IL-6 standard (S1-S8) concentrations (x-axis). Using line equation, concentration of IL-6 was calculated for each sample (Fig. Supl.2.1B).

Note: IL-6 ELISA kit sensitivity: minimum detectable concentration is 23 pg/ml

2.10. Statistical Analysis:

Data are presented as means ± SE for the indicated number of independently performed experiments. One way ANOVA with Student–Newman–Keuls (SNK) post-hoc test was used to identify the statistical significance between multiple groups. A *P*-value < 0.05 was considered statistically significant. The comparative analysis of results between different experimental groups respective to their corresponding controls was conducted using SigmaPlot (Version 14.0; Systat Software, Chicago, IL, USA). Furthermore, linear regression was performed for all values obtained of Griess and ELISA assays.

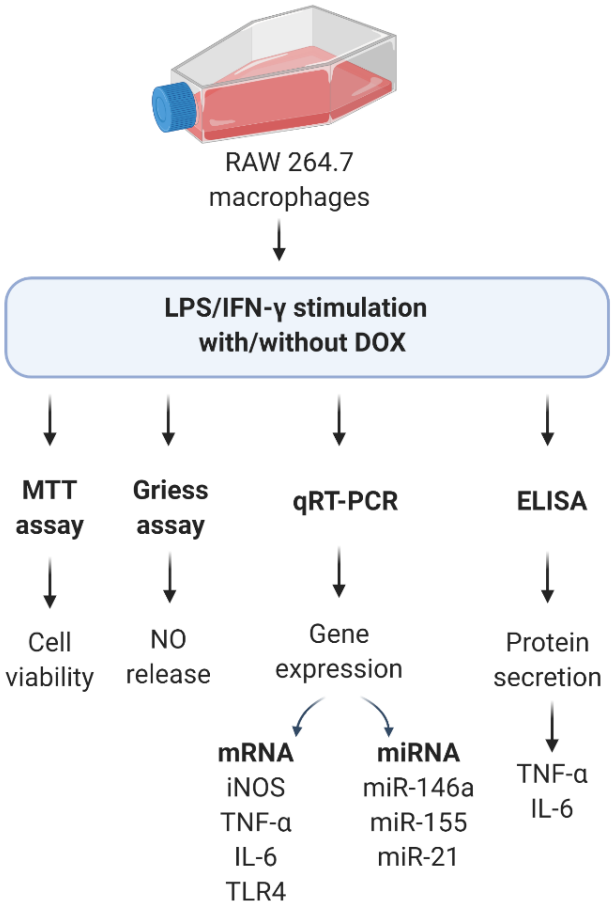


Fig. 2.2. Schematic diagram showing the methods used according to the aims of the study.

(Created with Biorender.com)

3. RESULTS

3.1 Effect of different time points and concentrations of LPS and IFN-γ on cell viability in RAW 264.7 macrophages.

To decide the nontoxic concentrations of LPS and IFN- γ to be used in the present study, RAW 264.7 cells were treated for 24 and 48 h with two concentrations of LPS (10 and 100 ng/mL) in the absence and presence of IFN-γ at 5 and 10 U/mL; thereafter, cytotoxicity was measured using MTT assay. Figure 3.1A shows that LPS and IFN-γ did not negatively affect cell viability after 24 h, alone or in combination. Exposure to LPS (10 ng/ mL) and IFN-γ (10 U/ mL), individually, had lower cytotoxicity by 19 and 31%, respectively, compared to control. Co-exposure of LPS (10 ng/ mL) with IFN-γ (5 and 10 U/ mL) showed lower cytotoxicity by 31 and 21%, respectively, in respect to control. And, co-exposure of LPS (100 ng/ mL) with IFN-γ (5 and 10 U/ mL) had lower cytotoxicity by 23 and 32%, respectively, compared to control. On the other hand, Fig. 3.1B showed decreased cell viability after 48 h with some treatments. LPS (10 ng/mL) in the presence of 5 or 10 U/mL IFN-γ decreased cell viability by 42 and 30 %, respectively. Similarly, LPS (100 ng/mL) in the presence of 5 or 10 U/mL IFN-γ decreased cell viability by 23 and 19%, respectively. Also, individual IFN-γ at concentration (10 U/mL) decreased cell viability by 20%, whereas LPS (10 and 100 ng/ mL) and IFN-γ (5 U/ mL) did not show a significant effect on cell viability. Based on these information, all subsequent experiments were conducted at 24 h.

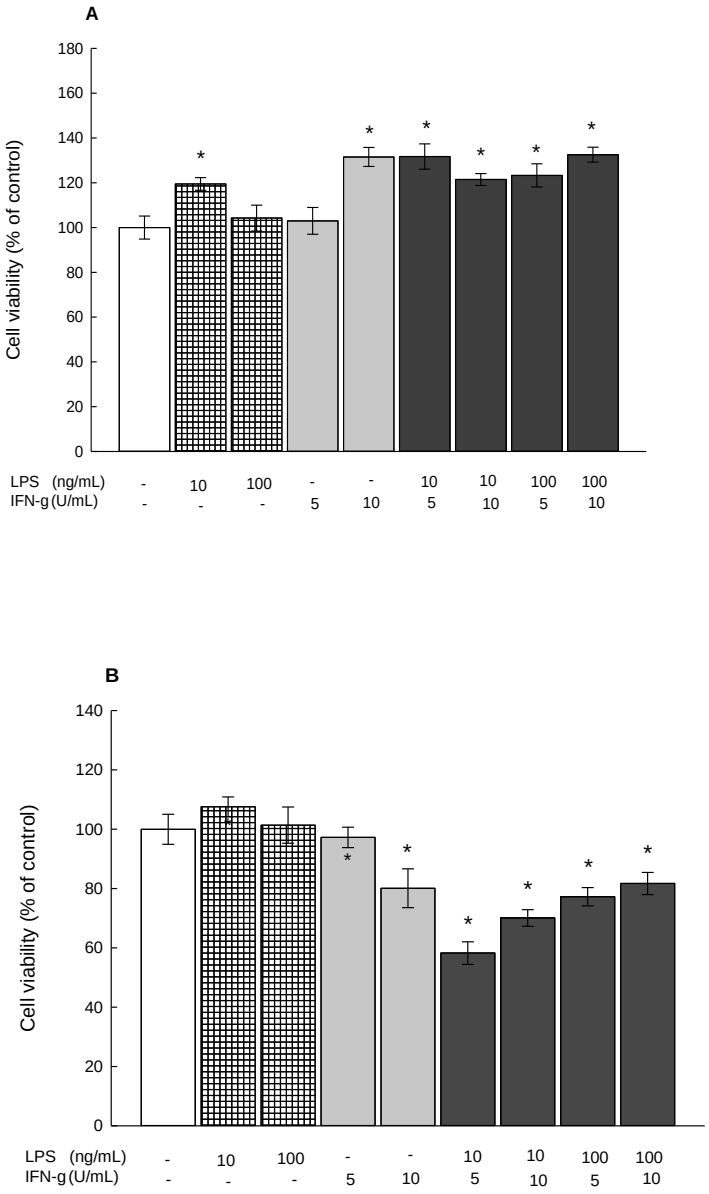
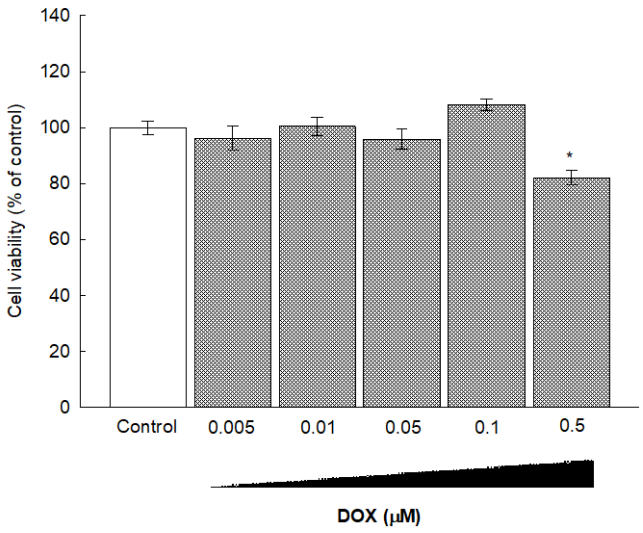


Fig. 3.1. Effect of LPS and IFN-γ on cell viability. RAW 264.7 macrophages were exposed to LPS (10 or 100 ng/mL) in the presence or absence of IFN- γ (5 or 10 U/mL) for 24 h (A) and 48 h (B). Cell cytotoxicity was measured using MTT assay. Data are expressed as a percentage of control (at 100%) ± S.E. (n=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control.

3.2 Effect of DOX on cell viability in RAW 264.7 macrophages

To decide the nontoxic concentrations of DOX to be used in the present study, RAW 264.7 cells were exposed to increasing concentrations of DOX (0.005 – 0.5 µM); thereafter, cytotoxicity was measured using the MTT assay. It has been shown that DOX alone at concentrations (0.005 - 0.1 µM) did not significantly affect cellular viability, whereas 0.5 µM DOX decreased cell viability by 18% (Fig. 3.2). Therefore, DOX was used in all subsequent experiments at concentration of 0.1 µM.



3.2. Effect of DOX on cell viability. RAW 264.7 macrophages are exposed to increasing concentrations of DOX (0.005, 0.01, 0.05, 0.1, or 0.5 µM) for 24 h. Cell cytotoxicity was measured using MTT assay. Data are expressed as a percentage of control (at 100%) ± S.E. (n=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control.

3.3 Effect of CUR, RES and SFN on cell viability in RAW 264.7 macrophages.

To decide the nontoxic concentrations of CUR, RES, and SFN to be used in the present study, RAW 264.7 cells were treated for 24 h with increasing concentrations of treatments (5 – 20 µM) alone and in combination with LPS/IFN-γ (10 ng/ 10 U/ mL) in the presence or absence of DOX (0.1 µM); thereafter, cytotoxicity was measured using the MTT assay. Figure 3.3A shows that exposure to CUR (5 and 10 µM), RES (5 – 20 µM), or SFN (20 µM) alone did not significantly affect cell viability, whereas CUR (20 µM) and SFN (5 and 10 µM) increased cell viability by 17, 17, and 18%, respectively. On the other hand, when cells are stimulated with LPS/IFN-γ, SFN (10 and 20 µM) conserved its proliferative effect and increased cell viability by 16 and 14%, respectively in the presence of DOX, and similarly by 15 and 18%, respectively in the absence of DOX (Fig. 3.3B, Supl.3.1). While, SFN (5 µM), CUR and RES (5 – 20 µM) did not significantly affect cell viability.

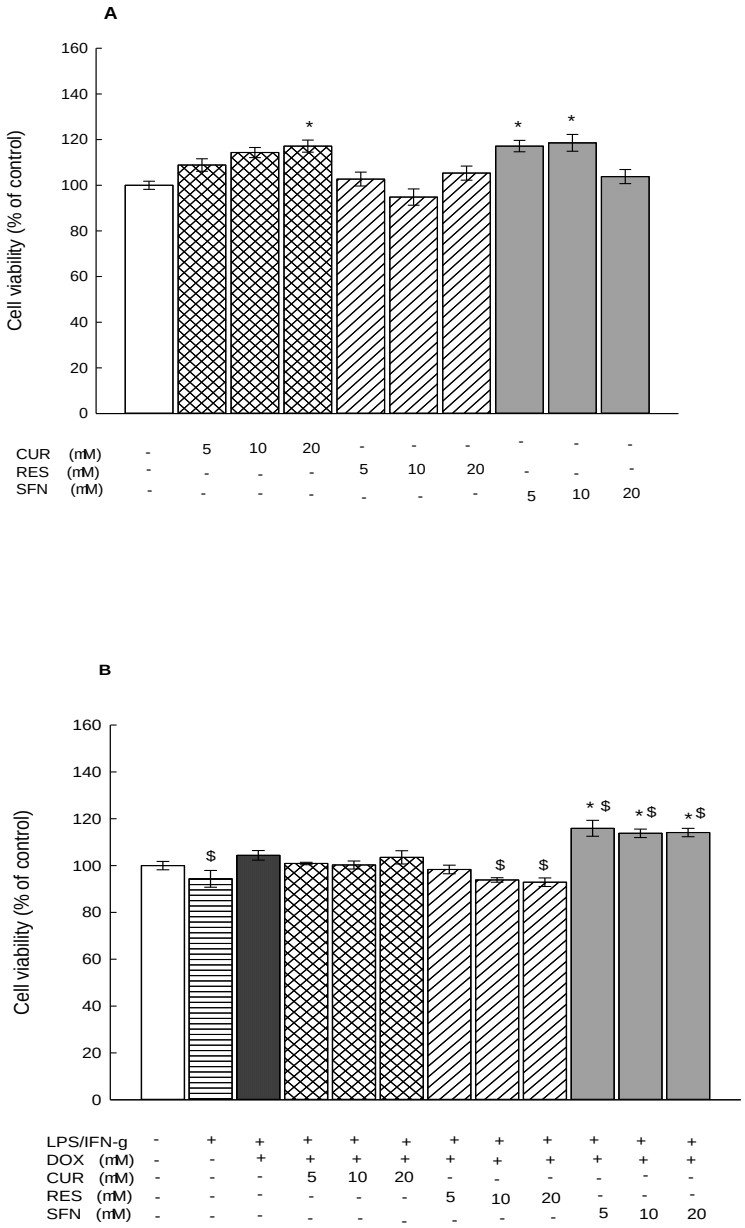


Fig. 3.3. Effects of CUR, RES and SFN on cell viability. RAW 264.7 macrophages were exposed for 24 h to CUR, RES or SFN at increasing concentrations of (5, 10 or 20 μM) alone (A) or with LPS (10 ng/mL) plus IFN-γ (10 U/mL) in the presence of DOX (0.1μM) (B). Cell cytotoxicity was measured using MTT assay. Data are expressed as a percentage of control (at 100%) ± S.E. (n=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN-γ plus DOX group.

3.4 Effect of different concentrations of LPS and IFN- γ on nitrite production in RAW 264.7 macrophages.

To determine the concentrations of LPS and IFN-γ to be used for RAW 264.7 cells activation, the individual and combined effects of LPS and IFN-γ were examined on RAW 264.7 macrophages. Cells were treated for 24 h with increasing concentrations of LPS (10 and 100 ng/mL) in the absence or presence of IFN-γ at 5 and 10 U/mL; thereafter nitrite production was assessed as an indicator for macrophage activation using the Griess method. Figure 3.4A shows that individual exposure to LPS at concentrations of 10 and 100 ng/mL induced nitrite production by nearly 500 and 400%, reaching a level of (7.1 ± 0.8) and (5.9 ± 0.4) µM, respectively, while IFN-γ at 5 and 10 U/mL showed a concentration-related NO production by 1000 and 1150%, evident at (14.4 ± 2.0) and (16.2 ± 1.1) µM, respectively.

Also, different combinations of LPS/IFN- γ showed a seemingly corresponding additive effect in respect to each individual component. Co-exposure to LPS (10 ng/mL) and IFN-γ (5 U/mL) showed higher stimulation at (19.4 ± 1.3) µM than each of them alone. Other combinations with increasing concentrations showed higher nitrite induction by 1600% for both LPS/IFN-γ (10 ng/10 U/mL) and (100 ng/5 U/mL), evident at (23.1 ± 1.1) and (23.4 ± 1.7) µM, respectively. The highest tested combination, LPS/IFN- γ (100 ng/10 U/mL) elevated nitrite by 1700%, evident at (25.3 ± 1.1) µM, which is not significant from LPS/IFN-γ (10 ng/10 U/mL). Based on these information, all subsequent studies were conducted using the concentrations of LPS at 10 ng/ mL and IFN-γ at 10 U/ mL.

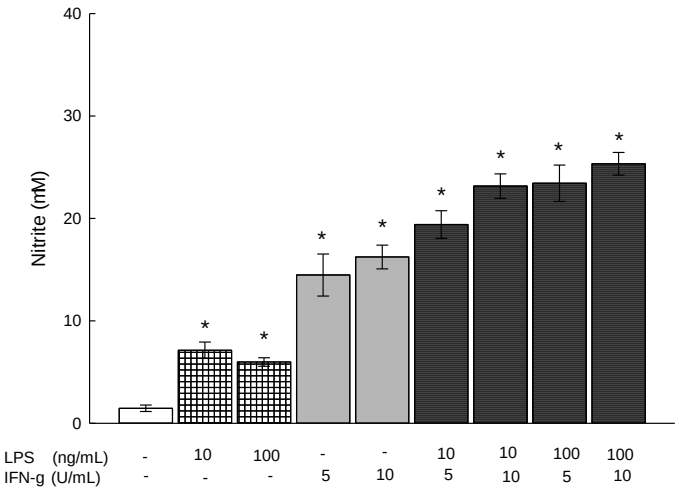


Fig. 3.4. Effect of LPS and IFN-γ on nitrite production. RAW 264.7 macrophages were exposed to LPS (10 or 100 ng/mL) in the presence or absence of IFN-γ (5 or 10 U/mL) for 24 h. Data are expressed as mean ± S.E. (n=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control.

3.5 Effect of CUR, RES and SFN on nitrite production in RAW 264.7 macrophages.

To examine the effect of herbal treatments on nitrite production, RAW 264.7 cells were exposed for 24 h to increasing concentrations of CUR, RES, or SFN (5 - 20 µM) in the presence or absence of LPS/IFN-γ (10 ng/ 10 U/ mL) with or without DOX (0.1 µM). Thereafter, nitrite levels were assessed using Griess method. Figure 3.5 shows that LPS/IFN-γ with/without DOX induced nitrite levels by 300%. When cells are co-exposed to LPS/IFN-γ plus DOX in the presence of the tested phytochemicals, SFN (5 – 20 µM) inhibited induced nitrite levels by 58, 75, and 87%, respectively in a concentration-dependent manner, and CUR only at 20 µM decreased nitrite induction by 23% decrease, whereas RES did not show any significant effect on nitrite production (Fig. 3.5).

To assess the difference in effect of tested phytochemicals in the absence of DOX, RAW 264.7 cells were cotreated with LPS/IFN-γ alone in the presence of CUR, RES, or SFN at the same conditions. Our results did not show much difference in the percent changes of nitrite levels between DOX $^{+}$ and DOX $^{-}$ treated groups (Supl.3.2A). Also, it has to be mentioned that DOX alone (without LPS/IFN-γ) did not show a direct stimulatory effect on nitrite levels (Supl.3.3).

To examine the effects of CUR, RES and SFN on basal nitrite levels, RAW 264.7 cells were treated with tested herbal treatments without LPS/IFN-γ stimulation at the same conditions. SFN (5 – 20 µM) conserved its inhibitory effect on nitrite production, accounting for 63, 75, and 63% inhibition of basal nitrite levels, whereas CUR and RES only at the highest tested concentration (20 µM) exhibited an inhibition by 44 and 24%, respectively (Supl.3.2B).

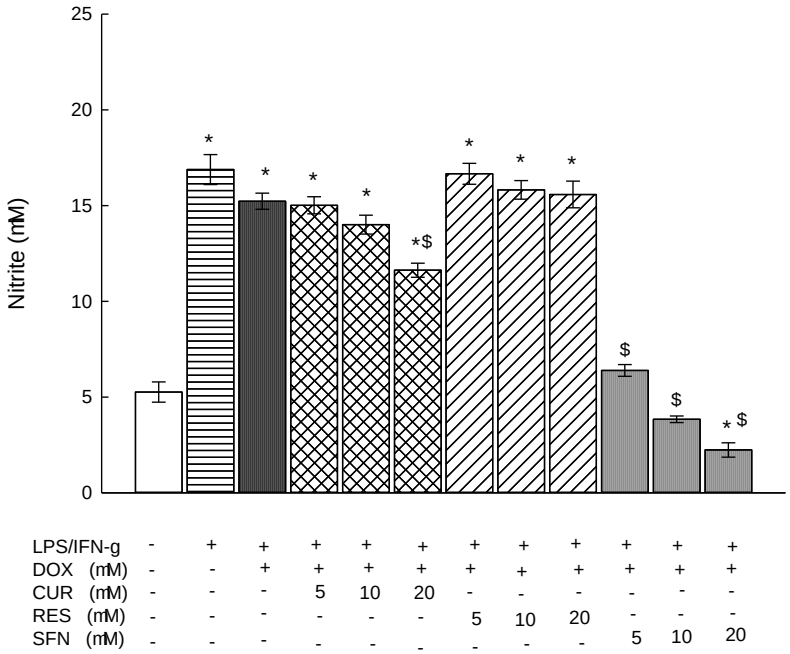


Fig. 3.5. Effects of CUR, RES and SFN on nitrite production. RAW 264.7 macrophages were exposed for 24 h to CUR, RES or SFN at increasing concentrations of (5, 10 or 20 μM) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with DOX (0.1μM). Nitrite production was determined using Griess method. Data are expressed as mean ± S.E. (n=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN-γ plus DOX group.

3.6 Effect of CUR, RES and SFN on iNOS mRNA expression in LPS/IFN- γ-stimulated RAW 264.7 macrophages.

To examine whether the inhibitory effect of herbal treatments on nitrite production is due to interfering with iNOS mRNA expression, RAW 264.7 cells were exposed for 6 h to increasing concentrations of CUR, RES, or SFN (5 and 20 μM) in the presence of LPS/IFN-γ (10 ng/ 10 U/ mL) with or without DOX (0.1 μM). Thereafter, iNOS mRNA levels were measured using real-time PCR. Figure 3.6 illustrates LPS/IFN-γ in the presence and absence of DOX induced iNOS mRNA expression similarly by 650%. When cells are cotreated with LPS/IFN-γ plus DOX in the presence of the tested phytochemicals, SFN (5 and 20 μM) downregulated induced iNOS mRNA levels by 74 and 94%, respectively, whereas CUR 20 did not show any significant effect at any concentration (Fig. 3.6). On the other hand, CUR (5 μM) and RES (5 and 20 μM) showed even higher expression than that of LPS/IFN-γ treatment by approximately 100 %.

Similarly, the same treatments were studied on LPS/IFN-γ induced macrophages in the absence of DOX, showing the same percent changes of iNOS levels with all the tested concentrations of SFN and 20 μM CUR as those observed in DOX^+ treated group, but CUR (20 μM) and RES (5 and 20 μM) showed a decrease in iNOS induced levels by 9, 65 and 70%, respectively (Supl.3.4A). It has to be mentioned that DOX alone did not affect iNOS expression in the absence of LPS/IFN-γ (Supl.3.5A).

The effect of herbal treatments on basal iNOS expression was examined on RAW 264.7 cells without LPS/IFN- γ stimulation. Interestingly, SFN (5 and 20 μM) downregulated iNOS mRNA expression levels by 30 and 60%, respectively, while CUR (5 and 20 μM) and RES (5 and 20 μM) enhanced its expression by 750, 690, 270, and 300%, respectively (Supl.3.5A).

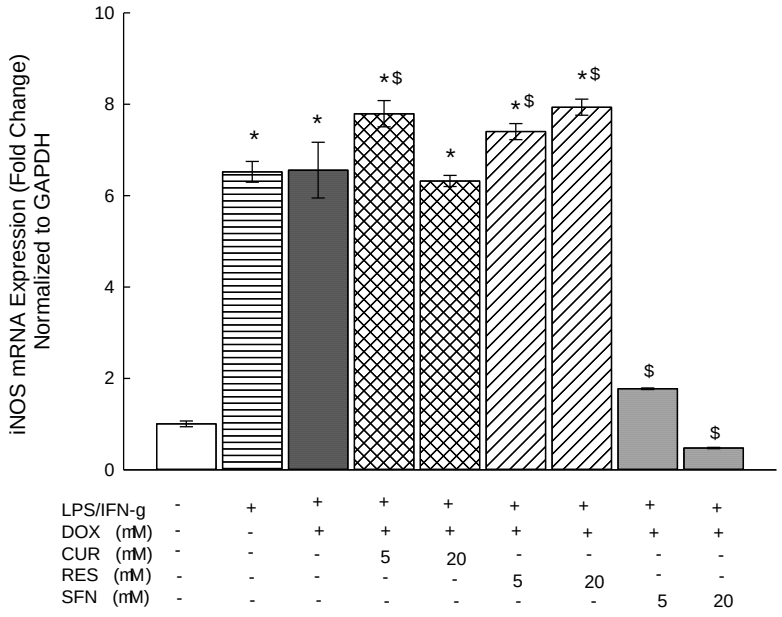


Fig.3.6. Effect of CUR, RES or SFN on iNOS mRNA in LPS/IFN-γ-mediated RAW 264.7 macrophages. RAW 264.7 cells were treated for 6 h with CUR, RES or SFN (5 and 20 M) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with DOX (0.1 μM). iNOS mRNA levels were measured using qRT-PCR and were normalized to GAPDH. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN- γ plus DOX treatment.

3.7 Effect of CUR, RES and SFN on induced TLR4 mRNA expression in LPS/IFN-γ-stimulated RAW 264.7 macrophages.

To examine the effect of herbal treatments on TLR4 mRNA levels, RAW 264.7 cells were exposed for 6 h to two concentrations of CUR, RES, or SFN (5 and 20 µM) in the presence or absence of LPS/IFN- γ (10 ng/ 10 U/ mL) with or without DOX (0.1 µM). Thereafter, TLR4 mRNA levels were measured using real-time PCR. Figure 3.7 demonstrates that LPS/IFN-γ in the presence or absence of DOX non-significantly downregulated TLR4 mRNA levels by 50 and 30%, respectively. When cells are co-exposed to LPS/IFN-γ plus DOX in the presence of CUR, RES or SFN, tested phytochemicals upregulated TLR4 mRNA levels by approximately 630% for CUR (5 and 20 µM), 570 and 640% for RES (5 µM and 20 µM), respectively, and 560 and 700% for SFN (5 and 20 µM), respectively (Fig. 3.7).

To assess the difference in effect of the experimented herbal treatments without DOX, RAW 264.7 cells were induced by LPS/IFN-γ alone in the presence of CUR, RES, or SFN at the same conditions. Results did not show much difference in the percent changes of TLR4 levels between DOX^{+} and DOX^{-} treated groups (Supl.3.4D). Also, it has to be mentioned that DOX alone (without LPS/IFN-γ) did not affect TLR4 expression levels (Supl.3.5D).

To examine the individual effects of herbal treatments on basal TLR4 expression, RAW 264.7 cells were treated with CUR, RES, and SFN alone without LPS/IFN-γ stimulation. Phytochemicals conserved their stimulatory effect on TLR4 expression. CUR (5 and 20 µM) induced TLR4 by 650 and 700%, respectively, RES (5 and 20 µM) by 670 and 760%, respectively, and SFN (5 and 20 µM) by 600 and 780%, respectively (Supl.3.5D).

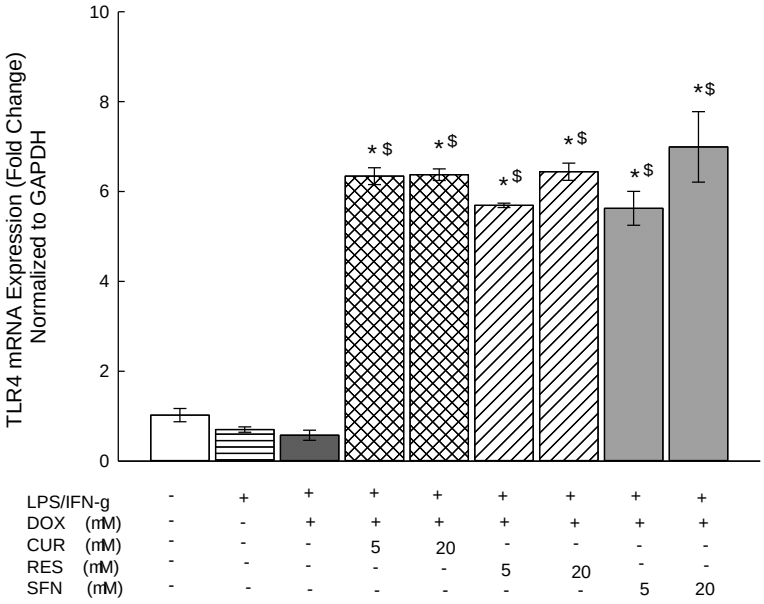


Fig. 3.7. Effect of CUR, RES or SFN on TLR4 mRNA in LPS/IFN-γ-mediated RAW 264.7 macrophages. RAW 264.7 cells were treated for 6 h with CUR, RES or SFN (5 and 20 M) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with DOX (0.1 μM). TLR4 mRNA levels were measured using qRT-PCR and were normalized to GAPDH. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN- γ plus DOX treatment.

3.8 Effect of CUR, RES and SFN on TNF-α and IL-6 proinflammatory cytokines mRNA expression in LPS/IFN- γ-stimulated RAW 264.7 macrophages.

To examine the effect of herbal treatments on TNF-α and IL-6 mRNA levels, RAW 264.7 cells were exposed for 6 h to two concentrations of CUR, RES, or SFN (5 and 20 μM) in the presence of LPS/IFN-γ (10 ng/ 10 U/ mL) with or without DOX (0.1 μM). Thereafter, TNF-α and IL-6 mRNA levels were measured by real-time PCR. Figure 3.8 shows that LPS/IFN-γ in the presence and absence of DOX significantly induced IL-6 and TNF-α expression by 6300 and 500%, respectively.

When cells were co-treated with experimented herbal treatments in the presence of LPS/IFN-γ plus DOX, SFN (5 and 20 μM) significantly attenuated the induced mRNA levels of TNF-α by 26 and 78%, respectively, and of IL-6 by 84 and 100%, respectively. On the other hand, CUR and RES did not show any significant inhibition on TNF-α induced mRNA levels at any concentration tested, but both downregulated induced IL-6 mRNA expression levels by approximately 50% at 5 and 20 μM (Fig. 3.8). Similarly, the same treatments were studied on LPS/IFN-γ induced macrophages without DOX, showing approximately the same percent changes of TNF-α and IL-6 levels with all treatments (Supl.3.4B & C). It has to be mentioned that DOX alone did not affect TNF-α or IL-6 mRNA expression in the absence of LPS/IFN-γ (Supl.3.5B & C).

We further examined the effect of tested herbal treatments on the basal expression levels of TNF-α and IL-6 in RAW 264.7 cells without LPS/IFN-γ stimulation. Unexpectedly, CUR and RES (5 and 20 μM) upregulated TNF-α and IL-6 levels by approximately 150%. On the other hand SFN (5 μM) increased TNF-α level by 150%, but did not affect IL-6 level. While SFN (20 μM) had no effect on TNF-α and decreased IL-6 expression by 28% (Supl.3.5B & C).

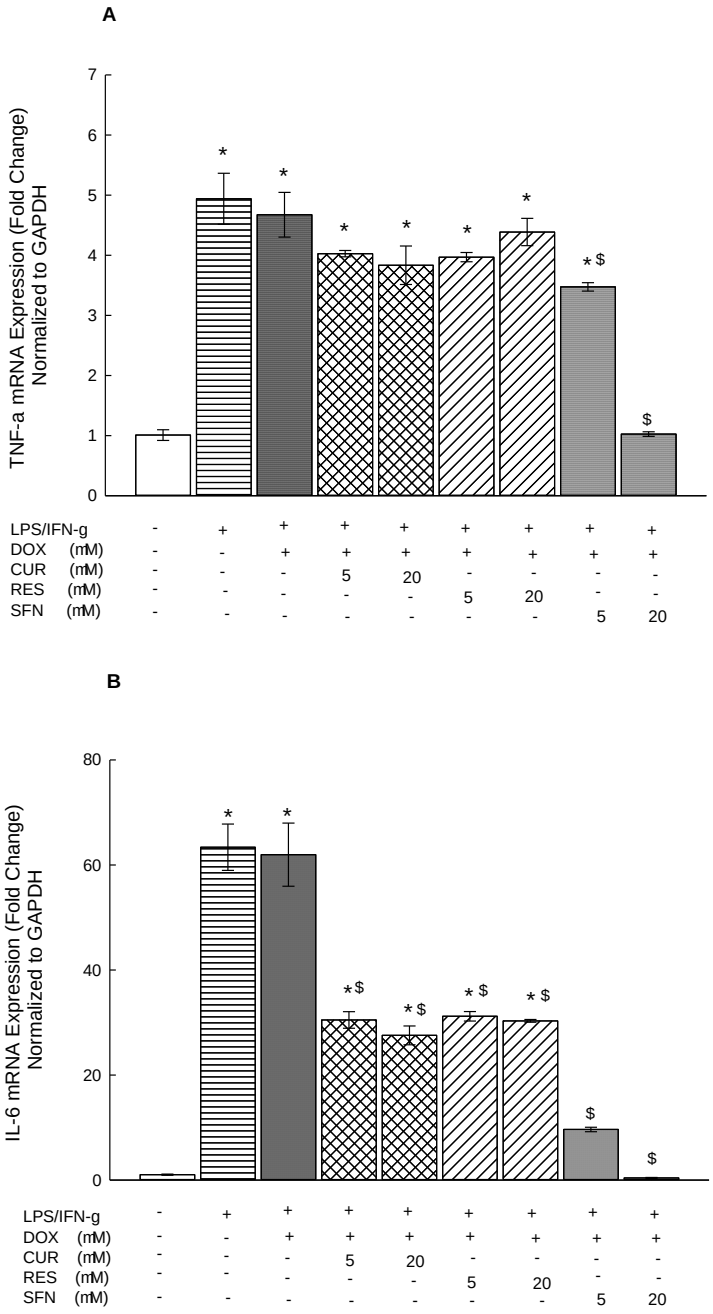


Fig. 3.8. Effect of CUR, RES or SFN on TNF-α and IL-6 mRNA in LPS/IFN-γ-mediated RAW 264.7 macrophages. RAW 264.7 cells were treated for 6 h with CUR, RES or SFN (5 and 20 M) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with DOX (0.1 μM). TNF-α (A) and IL-6 (B) mRNA levels were measured using qRT-PCR and were normalized to GAPDH. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN- γ plus DOX treatment.

3.9 Effect of CUR, RES and SFN on TNF-α and IL-6 protein levels in LPS/IFN- γ-stimulated RAW 264.7 macrophages.

To determine whether the observed inhibition of LPS/IFN-γ-induced TNF-α and IL-6 mRNA levels by SFN, in comparison to CUR and RES, is further translated to the protein level, RAW 264.7 cells were exposed for 24 h to 20 µM of CUR, RES, and SFN in the presence of LPS/IFN-γ (10 ng/ 10 U/ mL) with or without DOX (0.1 µM). Thereafter, the protein levels of TNF-α and IL-6 was assessed using ELISA. Figure 3.9A shows that LPS/IFN-γ in the presence or absence of DOX increased TNF-α levels to 1822.41 and 1694.82 pg/mL, respectively (TNF-α levels were undetected in untreated control cells). On the other hand, IL-6 protein levels were induced by 1943 %, in the presence of DOX, and by 2022 %, in the absence of DOX (Fig. 3.9B).

When cells were cotreated with tested phytochemicals in the presence of LPS/IFN-γ with DOX, SFN (20 µM) downregulated both TNF-α and IL-6 induced expression by almost 98 %, whereas CUR and RES (20 µM) did not show any inhibitory effect on neither IL-6 nor TNF-α induced expression levels (Fig. 3.9). Similar results were observed in LPS/IFN-γ-induced cells without DOX treatment (Supl.3.6A & B). It has to be mentioned that DOX alone did not affect TNF-α (data not shown) or IL-6 protein levels in the absence of LPS/IFN-γ (Supl.3.6 C).

The effect of the tested phytochemicals was examined in the absence of LPS/ IFN-γ stimulation. TNF-α protein levels were undetected with all treatments (data not shown) and IL-6 was released in a negligible amount, which was totally undetectable with SFN at 20 µM (Supl.3.6 C).

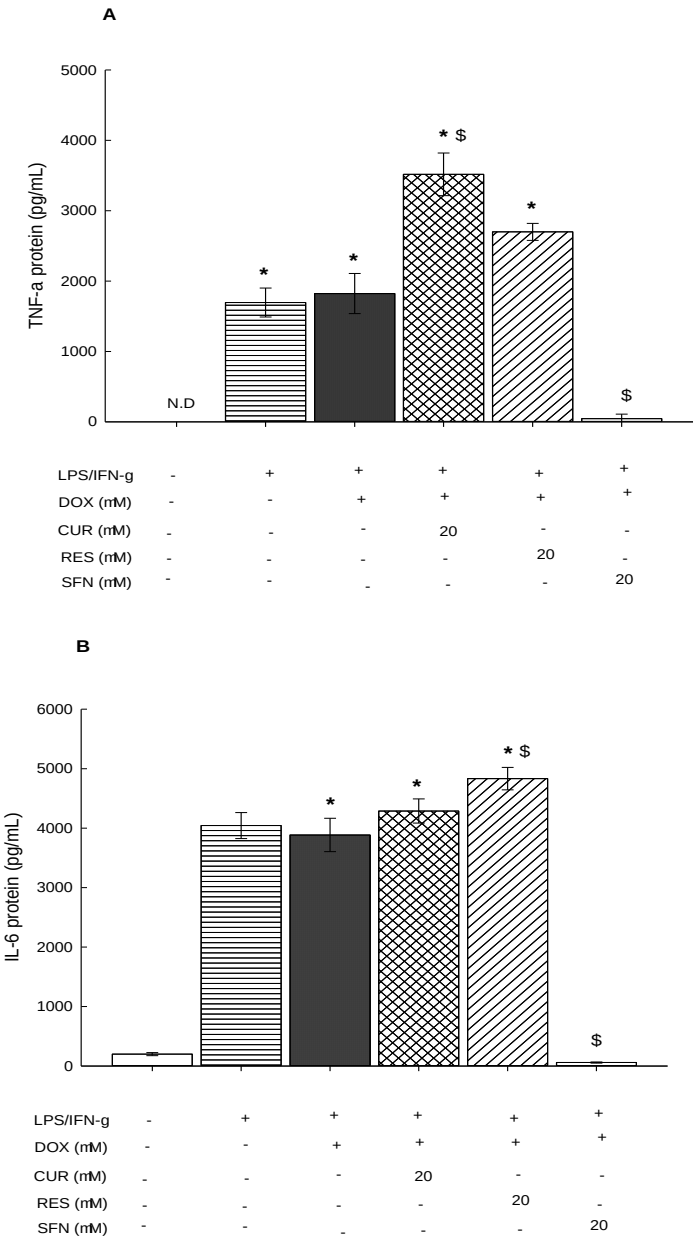


Fig. 3.9. Effect of CUR, RES or SFN on TNF-α and IL-6 protein in LPS/IFN-γ-mediated RAW 264.7 macrophages. RAW 264.7 cells were treated for 24 h with CUR, RES or SFN (5 and 20 M) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with DOX (0.1 µM). TNF-α (A) and IL-6 (B) protein levels were quantified using ELISA. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN- γ plus DOX treatment.

3.10 Basal and LPS/IFN-γ-induced expression of miR-146a, miR-155 and miR-21 miRNAs in RAW 264.7 macrophages.

A novel comparison was conducted between the relative expression levels of the tested miRNAs in RAW 264.7 macrophages using real-time PCR and data were presented as $\log_{10}$ fold change. On the basal level without LPS/IFN-γ stimulation, Fig. 3.10 shows that miR-21 is the most expressed miRNA in RAW 264.7 macrophages relative to miR-155 and miR-146a, with 1- and 5-log higher in fold change, respectively. On the other hand, miR-155 expression was 3.5-log higher in fold change relative to miR-146a, which is the least expressed miRNA in RAW 264.7 cells. Upon LPS/IFN-γ (10 ng/10 U/mL) stimulation, miR-146a was induced by only 0.5-log fold change, whereas both miR-155 and miR-21 are increased by 1-log fold change, relative to their respective controls, which corresponds to their relative basal expression (Fig. 3.10).

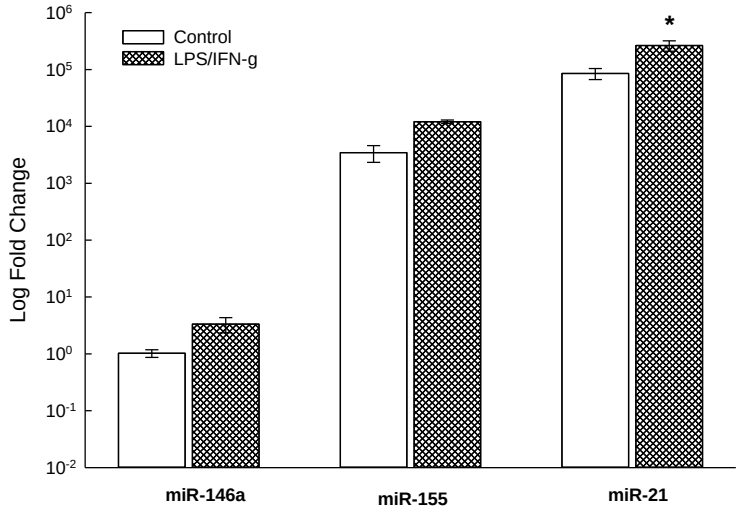


Fig. 3.10. Relative expression of miR-146a, miR-155, and miR-21 in RAW 264.7 macrophages. RAW 264.7 cells were treated for 6 h with LPS (10 ng/mL) plus IFN-γ (10 U/mL). The basal and induced expression levels of miR-146a, miR-155, and miR-21 were measured using qRT-PCR and were normalized to RNU6. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with corresponding control.

3.11 Effect of SFN on miR-146a, miR-155 and miR-21 miRNAs induced expression in LPS/IFN- γ-stimulated RAW 264.7 macrophages.

In an effort to determine whether SFN-mediated effect on the LPS/IFN-γ-induced stimulation of different inflammatory markers is occurring through an epigenetic mechanism, we examined the potential effect of SFN on miR-146a, miR-155, and miR-21 expression using real-time PCR. For this purpose, RAW 264.7 cells were exposed for 24 h to SFN (5 and 20 μM) in the presence of LPS/IFN-γ (10 ng/ 10 U/ mL) plus DOX (0.1 μM). Our results showed that miR-146a and miR-155 were upregulated by almost 500 and 300%, respectively, in response to LPS/IFN-γ plus DOX (Fig. 3.11A & B). On the other hand, when cells were coexposed to SFN (5 and 20 μM) in the presence of LPS/IFN-γ plus DOX, SFN attenuated the induced levels of miR-146a and miR-155 by 85 and 40%, respectively (Fig. 3.11A & B), whereas no significant effect was observed on miR-21 expression levels (Fig. 3.11C).

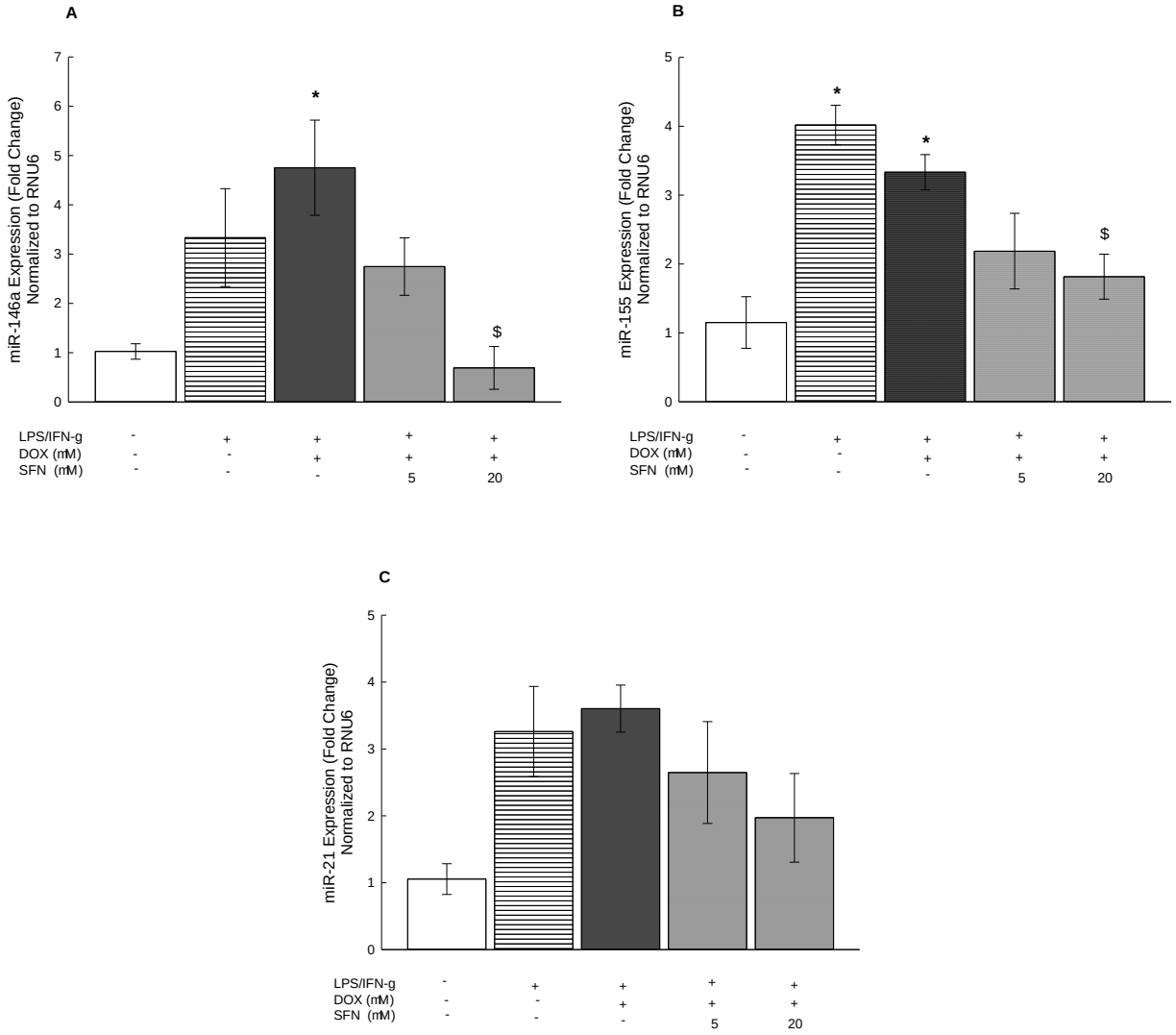


Fig. 3.11. Effect of SFN on miR-146a, miR-155, and miR-21 expression in LPS/IFN-γ-mediated RAW 264.7 macrophages. RAW 264.7 cells were treated for 6 h with SFN (5 and 20 M) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) with DOX (0.1 μM. The expression levels of miR-146a (a), miR-155 (b), and miR-21 (c) were measured using qRT-PCR and were normalized to RNU6. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN-γ plus DOX treatment.

4. DISCUSSION

In the present study, we studied the direct and indirect effect of DOX on the activation of RAW 264.7 macrophages through TLR4 signaling pathway. Qualitative studies on NO production and gene expression analysis of inflammatory mediators, such as iNOS, TNF-α, and IL-6 has been conducted, showing no direct effect of DOX on TLR4 signaling in RAW 264.7 macrophages, and confirmed its role in macrophages' activation to be primarily dependent on endotoxin leakage (179). Our second main finding is that SFN effectively attenuated RAW 264.7 macrophage stimulation, which is reflected in suppressing the gene expression levels of proinflammatory enzymes and cytokines, including iNOS, TNF-α, and IL-6 on the transcriptional level, as well as, NO levels, and TNF-α and IL-6 proteins at the post-transcriptional level. Interestingly, we also found that SFN downregulated LPS/IFN- γ-induced expression of miR-155 and miR-146a, which are key regulatory miRNAs in inflammatory response

DOX is a member of the anthracycline family used in the treatment of both hematologic and solid tumors (143). Cardiotoxicity, however, is its major limiting side effect (143). DOX-induced cardiotoxicity has been previously proposed to be mediated through several mechanisms, such as oxidative stress through free radical generation, mitochondrial dysfunction through membrane lipid peroxidation, or LPS-induced systemic inflammation *via* intestinal disruption (143, 179). Recent studies have linked cardiometabolic diseases with intestinal dysfunction that result from increased levels of intestinal lipopolysaccharides released from gut microbiota and bind to TLR4 receptor in macrophages, thus activating NF-κB pathway followed by inflammatory cytokines generation (248, 400). In a correlative study, DOX administration to mice has been reported to directly cause intestinal epithelium damage, which resulted in endotoxin leakage and, therefore, severe systemic inflammation through TLR4 signaling in macrophages (179). This inflammation can be in part a major factor of DOX severe adverse effects, ranging from cardiotoxicity and cardiomyopathy to life-threating heart failure (179). Few data, however, are provided on the exact role of macrophages in chemotherapy-induced myocarditis (401).

Our data revealed no significant direct or additive effect of DOX on the activation of RAW 264.7 cells, when used alone or in combination with LPS/IFN- γ, which has been indicated by assessing NO production levels and the gene expression levels of inflammatory mediators, including iNOS, TNF-α and IL6. This observation could be related, in part, to the absence of

intestinal epithelium in *in vitro* studies, which is the primary target for DOX to induce inflammation in mice as demonstrated by Wang et al (179). Additionally, Wang et al. showed that isolated peritoneal macrophages treated with LPS plus DOX induced higher levels of TNF-α and IL-6 than those stimulated with LPS alone, which is inconsistent with our results (Fig. 3.8), showing no inducing effect of DOX on the upregulation of TNF-α or IL-6 (179). It worth mentioning that the concentration of DOX used in Wang et al study was higher than that used in the current study, which was determined based on MTT results (Fig. 3.2). Also our experimented RAW 264.7 cell line has acquired genetic and phenotypic differences in comparison to peritoneal macrophages (122), which might explain the variation in results. Nevertheless, in the current study, we induced RAW 264.7 macrophages with LPS/IFN-γ in the presence of DOX in order to mimic the physiological environment maintained in the body and to investigate if there is a direct effect for DOX on macrophages. However, the direct activity of DOX might be more prominent on other types of inflammatory cytokines rather than TNF-α and IL-6. A study done by Sauter et al. highlighted the enhanced expression of pro-IL-1β in LPS-primed BMDM upon DOX treatment, and also reported DOX induced IL-6 and TNF-α levels only in mice (402).

In the present study, RAW 264.7 cells were exposed to LPS and IFN-γ alone and in combination. According to macrophage activation and polarization guidelines, IFN-γ cytokine together with LPS are involved in M1 polarization of RAW 264.7 macrophages (403, 404). As shown in Fig. 3.4, combinations of LPS and IFN- γ enhanced the release of NO, which is consistent with Reis et.al study disclosing a synergistic effect for LPS and IFN-γ on NO release by RAW 264.7 macrophages; however, they used a higher concentration for IFN-γ and did not indicate cellular viability data (405). Hence, we propose a model whereby LPS and IFN-γ combination is optimized to fine tune RAW 264.7 macrophage activation, independent of any cytotoxic effect (Fig.3.1 & Fig. 3.4).

In macrophages, NO production is induced primarily by LPS and IFN-γ through the activation and transcription of the gene encoding for iNOS enzyme (383). After LPS/IFN-γ exposure, macrophages showed significant increase in NO production, observed at about 24 h (Fig. 3.4) and the rate of its production was elevated time-dependently for at least 48 h (data not shown). Cell viability was determined for the 24 and 48 h time points (Fig. 3.1). Decreased cell viability was observed after 48 h incubation with LPS/IFN-γ combinations at all tested concentrations, which is

consistent with Shi et al. results showing a time-dependent significant negative effect between LPS different incubation time and cell relative growth rate (406). Another study by Liu et al. explained that LPS/IFN-γ negatively regulate cell proliferation, which is evident by low percentage of macrophages in S-phage and mitosis and subsequent downregulation of cell cycle regulators upon LPS/IFN-γ in macrophages (407). Accordingly, we continued with the 24 h time period in the subsequent experiments. An additional study by Fan et al. demonstrated the effect of LPS on cell growth regulation to be multifunctional. They showed that LPS inhibitory and stimulatory effects on macrophage proliferative regulation are based on the coexisting cytokines, which might explain the observed increase in cell viability with LPS/IFN-γ tested concentrations (Fig. 3.1) (408)

Furthermore, iNOS mRNA induction by LPS/IFN-γ (Fig. 3.6) was consistent with increased NO production by LPS/IFN-γ shown in (Fig. 3.4). Altogether, these findings are compatible with Chiou et.al study showing an induced NO accumulation and iNOS upregulation by LPS/IFN-γ after 24 h (383).

Macrophage phenotype is influenced by the release of pro-inflammatory cytokines, such as IL-6 and TNF-α through activating TLR4 signaling pathway and inducing the so-called "classically activated" macrophages that are characterized by pronounced inflammatory responses (409). In highlight of this, LPS/IFN-γ treated RAW 264.7 macrophages showed a striking overexpression of IL-6 and TNF-α, on both the mRNA and protein levels (Fig. 3.8 and 3.9), affirming the cause-effect relationship between pro-inflammatory cytokines and enhanced inflammatory reaction in response to LPS stimulation (18).

Macrophage sensitivity to LPS is partly regulated at the level of TLR4 expression (410, 411). Although TLR4 upregulation is reported in several experimental models of inflammation including intestinal inflammation (412), our findings confirmed the suppressive effect of LPS on TLR4 mRNA expression (Fig. 3.7). This finding is in agreement with that of Tsatsanis et.al, demonstrating that TLR4 showed decreased expression after 2 h following LPS stimulation in RAW 264.7 macrophages, which represents a negative regulatory mechanism through TLR4 downregulation (410). In this context, Matsuguchi et al. study showed increased TLR2, but constant TLR4 mRNA expression levels upon treatment with LPS and IFN- γ in RAW 264.7 cell line, *in vitro* and in mouse splenic macrophages, *in vivo* (413). Several studies have implicated the effect of LPS on TLR4 expression to be a possible compensatory mechanism that leads to the

development of macrophage tolerance towards further LPS stimulation and to help in the containment of inflammatory response (414, 415). The effect of LPS on TLR4 expression is a form of a dynamic balance between the process of activation and suppression of immune response, which is critical in host-immunity homeostasis (416). Upon LPS exposure, different regulatory feedback mechanisms take place to limit the strength of innate immune response signaling transduction, and one of these regulatory mechanisms is the downregulation of TLR expression (410, 414-416).

On top of this, our data present a first time comparison between the protective effects of CUR, RES, and SFN on DOX-induced inflammation in RAW 264.7 macrophages. The polyphenols (CUR and RES) and the isothiocyanate (SFN) are previously proved to have a potent anti-inflammatory activity, as well as, antioxidant and chemopreventive effects (30, 248). These phytochemicals have been chosen, in specific, for their efficacy, safety and easy oral administration (30, 233). However, the comparative variations in their efficacy and the differences in their anti-inflammatory mechanisms is still unknown, particularly with DOX-induced inflammation (30, 248).

In the present study, it has been observed that SFN significantly increased cell viability at all tested concentrations (Fig. 3.3), which could be explained by Shih et al. study demonstrating a promoting effect on T and B cells upon SFN treatment, which play a role in modulating the immune responses (417).

In addition, we show that in a process independent of cytotoxicity, SFN exhibited a dose-depended inhibition on LPS/IFN-γ-induced NO production in RAW 264.7 cells, while only the highest tested concentration of CUR (20 μM) managed to suppress NO, yet still not as much as SFN; however, RES did not show any inhibitory effects at any concentration (Fig. 3.5).

NO, which is an important signaling molecule produced by activated macrophages, plays a main role in the pathogenesis of inflammation and inhibition of pathogen replication (36). Although important as a defense molecule against infection, its excessive production is cytotoxic (36, 418). Therefore, NO inhibitors present a principal therapeutic advance in the management of inflammatory diseases (418). Several studies presented a persuasive evidence for the involvement of NO in the progression of inflammatory response and suggested its attenuation as an effective approach to alleviate mediated inflammatory reactions (419). Several methods exist for measuring

NO in biological systems, of which is Griess diazotization reaction used to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions (420-424). Knowing that NO production is catalyzed by iNOS enzyme (425), we also investigated the effect of CUR, RES, and SFN treatments on the enzyme expression in LPS/IFN-γ activated macrophages treated with DOX. Our observations indicated the marked effectiveness of SFN in inhibiting iNOS mRNA expression in a dose-dependent manner, whereas no significant effect was shown with neither CUR nor RES (Fig. 3.6). This finding demonstrated that iNOS mRNA downregulation may be the factor contributing to the inhibitory effect of SFN on iNOS enzyme activity as evident by its effect on NO production. Additionally, SFN efficacy was eminent on basal NO production and iNOS expression in inactivated macrophages (Fig. Supl.3.3B & 3.5A).

Furthermore, the expression of immuno-stimulatory cytokines have been stated to activate iNOS and generate high concentrations of NO through NF-κB (425). LPS-induced pro-inflammatory cytokines, such as TNF-α and IL-6, are known signaling molecules that mediate and regulate inflammation (18). Specifically, TNF-α and IL-6 are known to be rapidly evoked upon LPS stimulation to initiate inflammatory immune response (426). However, their excessive production eventually leads to a systemic inflammation (426). Based on this information, the pharmacological inhibition of these inflammatory mediators is an important target in the treatment of DOX-mediated endotoxemia.

In the current study, we examined the effect of CUR, RES, and SFN on TNF-α and IL-6 mRNA and protein levels. Our data revealed that SFN inhibited the production of TNF-α and IL-6 production in a dose-dependent manner in LPS/IFN-γ stimulated RAW 264.7 cells in the presence or absence of DOX treatment. Given all together, SFN demonstrated a corresponding inhibitory effect on TNF-α and IL-6, at the transcriptional and the translational levels. It is worth mentioning that SFN efficacy at 20 μM concentration managed to effectively drop TNF-α and IL-6 expression levels to nearly their basal levels in unstimulated macrophages. On the other hand, RES and CUR showed a noticeable inhibitory effect on IL-6 mRNA expression levels, but this effect did not extend to the protein levels. While they showed no recognizable effect on TNF-α in the presence of DOX, yet a slight decrease was observed in the absence of DOX. Besides IL-6 induced mRNA expression, SFN at its highest tested concentration (20 μM) inhibited IL-6 basal mRNA expression in RAW 264.7 macrophages, which confirmed the inhibitory effect of SFN on IL-6. All in all, SFN

remarkable inhibitory effect was in agreement with Ruhee et al, exhibiting the suppressive effect of SFN on the release of NO, TNF-α, and IL-6 in LPS-primed RAW 264.7 macrophages (427).

It has been shown previously that macrophage sensitivity to LPS is partly regulated at the level of TLR4 expression (410, 411). TLR4 is a transmembrane receptor that is a member of the toll-like receptor family, which belongs to the pattern recognition receptor (PRR) family. When TLR4 is activated, an intracellular signaling pathway NF-κB is turned on and inflammatory cytokines are produced, activating the innate immune system (428). CUR, RES, and SFN have been previously reported to display an antagonistic effect on TLR4 receptor in macrophages (81, 429). In highlight of this information, we determined the effect of equivalent concentrations of CUR, RES, and SFN on TLR4 mRNA expression in RAW 264.7 cells. Interestingly, our results revealed that all the experimented herbal treatments upregulated TLR4 mRNA expression in activated and inactivated macrophages. This effect is likely to indicate the direct antagonistic effect of CUR and SFN on TLR4 receptor, which was previously mentioned in several studies, (81). A direct competitive inhibition of CUR and SFN on TLR4-LPS binding site, known as MD2, was previously reported (81). And although direct targets of RES in TLR4 pathway have not been identified, inhibition of TRIF-induced NF-κB activation has been introduced as a molecular target for RES in RAW 264.7 cells (429). Altogether, this might explain the mRNA levels induction of TLR4 after exposure to CUR, RES, and SFN to be a possible refractory upregulation of the receptor in response to their antagonistic activity.

Collectively, our findings raised the possibility that the observed weak activity of CUR and RES, in comparison to SFN, might be due to having a modest efficacy, and a dose-effect relationship that limited their effects at low concentrations. As illustrated by Chen et al., a weak inhibition of TNF-α and IL-1β was reported in RAW 264.7 cells with low concentrations of CUR at 5 and 10 μM (430). Also, a study by Matsuguchi et al. highlighted the necessity of the use of high concentrations of CUR (50 μM or higher) for a significant inhibitory effect on TLR signaling (413). Another paper by Nelson and his colleagues criticized the instability, low water solubility, lack of efficacy and selective target activity of CUR, and questioned the results published in literature that demonstrate CUR activity (81, 431). Similarly, RES at high concentration of 60 μM was used to induce TLR4 inhibition in LPS-induced RAW 264.7 cells as proposed by Yang et al.

(305). It has to be noted that low concentrations of CUR and RES at 5 and 20 µM only were determined based on our cellular viability data (Supl.3.1A).

Given all the evidence obtained so far, our results present a novel comparative observation that nominates SFN, over CUR and RES, to be a promising TLR4 antagonist that can counteract DOX-mediated systemic inflammation due to its marked pharmacological efficacy.

Going one step further, we assessed the mechanism of action underlying such a function by conducting an in depth mechanistic investigation on the expression of the selected trio: miR-146a, miR-155, and miR-21. These miRNAs are selected, in specific, because of their obvious upregulation in response to TLR activation, and their key role as immune modulators in TLR signaling pathway (120). The expression of miR-146a, miR-155, and miR-21 is induced by TLR agonists, such as LPS, therefore, they are directly controlled by the NF-κB-dependent pathway, and they usually employ a feedback control over the NF-κB-dependent response by fine tuning the expression of key mediators of this pathway (432). Post-transcriptionally, miRNAs are used to mediate translational repression or degradation of mRNA through their base pairing to 3' UTR of the target mRNA region (433).

Differential expression of miRNAs influence macrophage activation, polarization and differentiation through regulating gene sets that are responsible for different biological pathways (432). Our results presented a novel comparison between the relative expression levels of miR-146a, miR-155, and miR-21 in RAW 264.7 macrophages, showing a relatively higher expression of miR-155 and miR-21 over miR-146a on the basal and induced states of macrophages (Fig.3.10).

The induced expression of the tested miRNAs was examined in LPS/IFN-γ stimulated macrophages (Fig. 3.11). In line with a recent study showing increased levels of miR-146a in LPS-treated macrophages, our results showed an upregulation of miR-146a, which plays a protective role against LPS-induced damage (Fig.3.11A) (434). Mechanistically, NF-κB activation induced by LPS has been reported to upregulate miR-146a, which, in turn, downregulated IRAK1 and TRAF6 proteins and suppressed the pro-inflammatory cytokines, including iNOS and TNF-α, thus inhibiting TLR pathway activation in macrophages (88, 435).

Additionally, miR-155 was also upregulated (Fig.3.11B), which agrees well with Bala and his colleagues showing enhanced miR-155 levels upon LPS stimulation in RAW 264.7 cells (436). In

macrophages, TLR agonists (e.g. LPS) and pro-inflammatory cytokines induce miR-155 expression, which exerts a positive feedback effect on NF-κB signaling (117, 120). Also, miR-155 indirectly enhances the production of TLR-induced proinflammatory cytokines, including IL-6 and TNFα through increasing their mRNA half-life and translation (432, 437). This information correlates well with the observed upregulation of TNF-α and IL-6 in LPS/IFN-γ-induced RAW 264.7 macrophages (Fig. 3.8 and 3.9).

Also, miR-21 was overexpressed with LPS/IFN-γ stimulation in RAW 264.7 macrophages (Fig.3.11C), which conform to Lu et al. and Feng et al. studies, presenting an upregulation of miR-21 level in LPS-treated RAW 264.7 macrophages (438, 439). Similar to miR-146a and miR-155, miR-21 expression is triggered by TLR agonists, such as LPS (440). It has been mentioned that miR-21 impairs MyD88-dependent NF-κB activation and IL-6 expression, but enhances the expression of the anti-inflammatory cytokine, IL-10, through inhibition of PDCD4, which is an IL-10 inhibitor (114).

In order to determine the effect of DOX on the expression of these miRNAs, we investigated their expression for the first time in LPS/IFN-γ activated macrophages treated with DOX. An enhanced upregulation of miR-146a was observed after DOX treatment (Fig. 3.11A). This finding was consistent with Horie et al. study on cardiomyocytes treated with DOX, suggesting a direct relationship between miR-146 upregulation and DOX-induced cardiotoxicity (441). While miR-155 and miR-21 did not show a significant difference with DOX.

Interestingly, SFN effect has been examined on miR-146a, miR-155, and miR-21 in LPS/IFN-γ primed macrophages treated with DOX. In line with Deramaudt et al. study (378), SFN downregulated the LPS/IFN-γ -mediated induction of miR-146a (Fig. 3.11A), which is a critical brake on pro-inflammatory NF-κB signaling in macrophages. This might be explained in terms of SFN inhibitory effect on TLR4 signaling in RAW 264.7 macrophages, which in turn exhibited a dependent-downregulation of miR-146a as a part of a negative feedback loop (442).

Also, SFN exhibited a dose dependent inhibition on miR-155 expression (Fig. 3.11B), which is consistent with Wagner et al. and Eren et al. studies illustrating a dose-related downregulation of miR-155 by SFN in LPS-treated RAW 264.7 cells and N9 microglial cells, respectively (365)

(368). Also, Wagner et al. study deduced an existing crosstalk between miR-155, NF-κB and Nrf2 signaling pathways that might need further investigation in the presence of SFN, which is known to exert its anti-inflammatory activity through inhibiting NF-κB pathway and activating Nrf2 signal transduction cascade (365, 443).

In addition, SFN showed no significant effect miR-21 expression levels in activated RAW 264.7 macrophages, which is a novel data presented for the first time excluding the role of miR-21 to be a possible mechanism for SFN antagonistic effect on TLR4 pathway (Fig. 3.11C). A recent study by Cho and his colleagues showed that SFN ameliorated LPS-mediated PDCD4 mRNA reduction in RAW 264.7 cells, but they could not reveal the underlying mechanism behind this finding and speculated to be related to miR-21 overexpression (444). In contrast to their speculation, our finding provides a clue for miR-21 insignificant role in PDCD4 suppression in RAW 264.7 macrophages, which still needs further verification, may be with higher concentrations of SFN.

Collectively, by analyzing and comparing the expression of the experimented miRNAs in RAW 264.7 macrophages, we suggest a model in which miR-146a and miR-155 play an efficient fine-tuning mechanism in the regulation of LPS/IFN- γ stimulated TLR4 signaling in murine macrophages. In this context, our observations suggest a novel post-transcription mechanism underlying the effectiveness of SFN as an anti-inflammatory agent against TLR4 mediated inflammation due to its multiple sites of action.

5. CONCLUSION AND FUTURE PERSPECTIVES

In summary, we have shown that SFN, in comparison to CUR and RES, successfully modulates TLR4 signaling activation in RAW 264.7 macrophages and effectively inhibits NO, iNOS, TNF-α, and IL-6 gene expression induced by LPS/IFN- γ. Also, our findings introduced the inhibitory role of SFN on the expression of miR-155 and miR-146a, proposing a novel post-transcriptional mechanism for SFN that might implicate a regulatory relationship with TLR4/ NF-κB signaling pathway in LPS/IFN-γ-activated macrophages. This link between miRNAs and TLR4 pathway will need further investigations through overexpression of miR-155 and miR-146a to ascertain the direct activity of SFN on miRNA regulation. Also, an *in vivo* study in mice using higher concentrations of CUR, RES, and SFN would be recommended to rule out the concentration-related effects as a limiting factor in the present study and confirm the anti-inflammatory effectiveness of SFN over CUR and SFN. Moreover, our results indicated no significant direct activity of DOX in enhancing inflammatory mediators, suggesting its effect on TLR4 stimulation in RAW 264.7 macrophages to be indirect through endotoxin leakage. Again, *in vivo* experiments are recommended to confirm the effect of DOX on macrophages stimulation in the presence of CUR, RES, and SFN. All in all, our study indicates the therapeutic potential of SFN as an immunomodulatory agent in DOX-mediated inflammation.

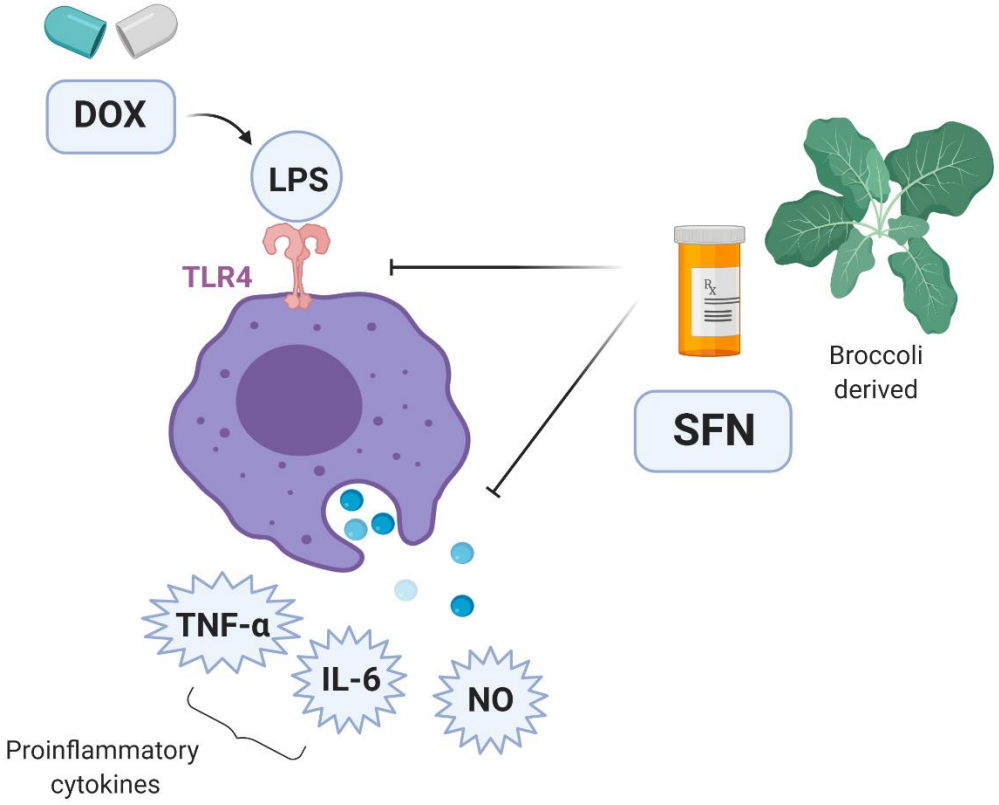


Fig.5.1. Schematic diagram illustrates the role of SFN in mediating DOX-induced TLR4 signaling cascade

(Created with Biorender.com)

Supplementary Figures

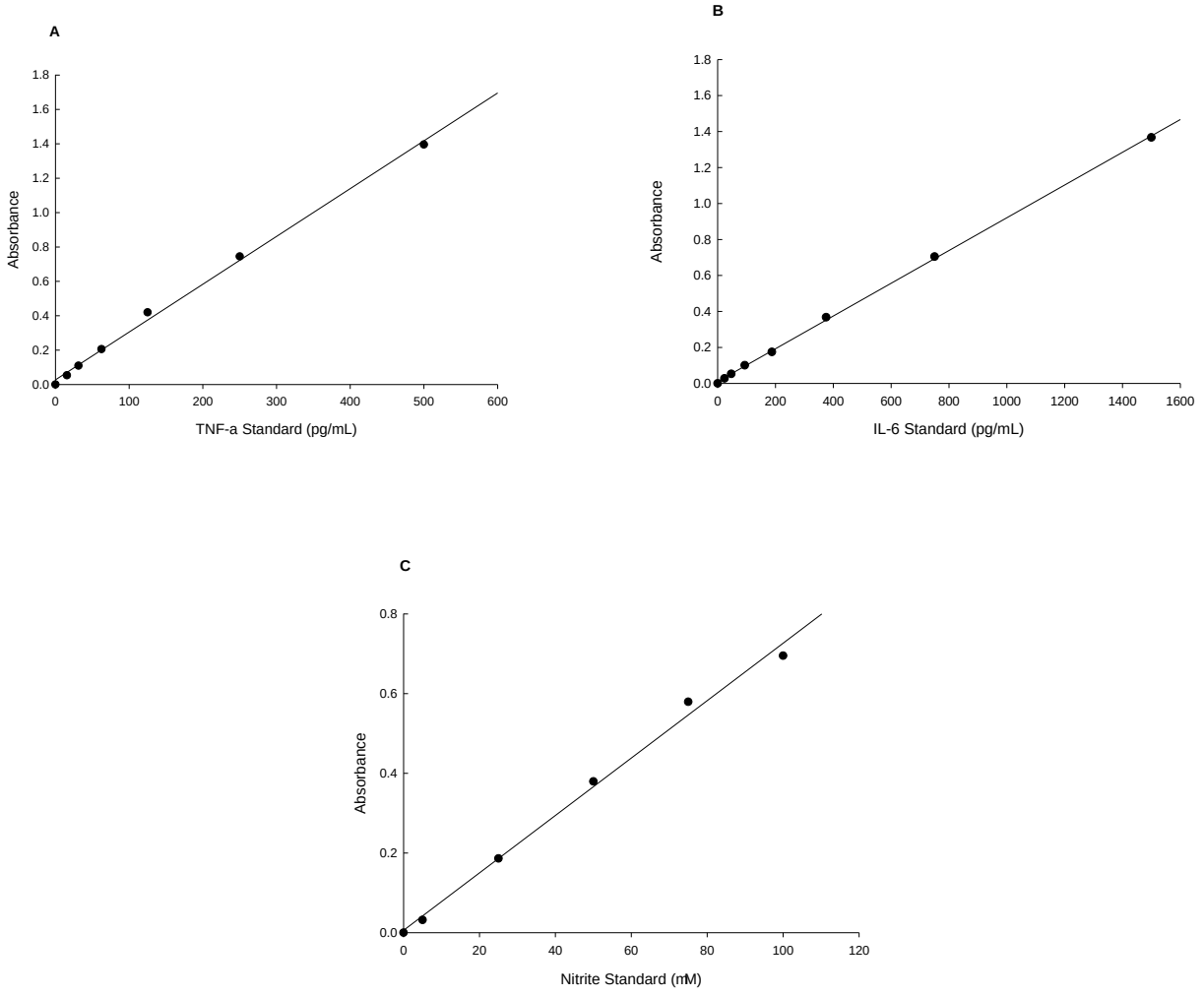


Fig. Supl.2.1. Standard curves of ELISA in (pg/mL) and Nitrite in (µM). (a) TNF-α protein standard curve. (b) IL-6 protein standard curve. (c) Nitrite standard curve

(a) Equation: Y=0.0029 X and R^2= 0.9953

(b) Equation: Y=0.0009 X and R^2= 0.9992

(c) Equation: Y=0.0073 X and R^2= 0.994

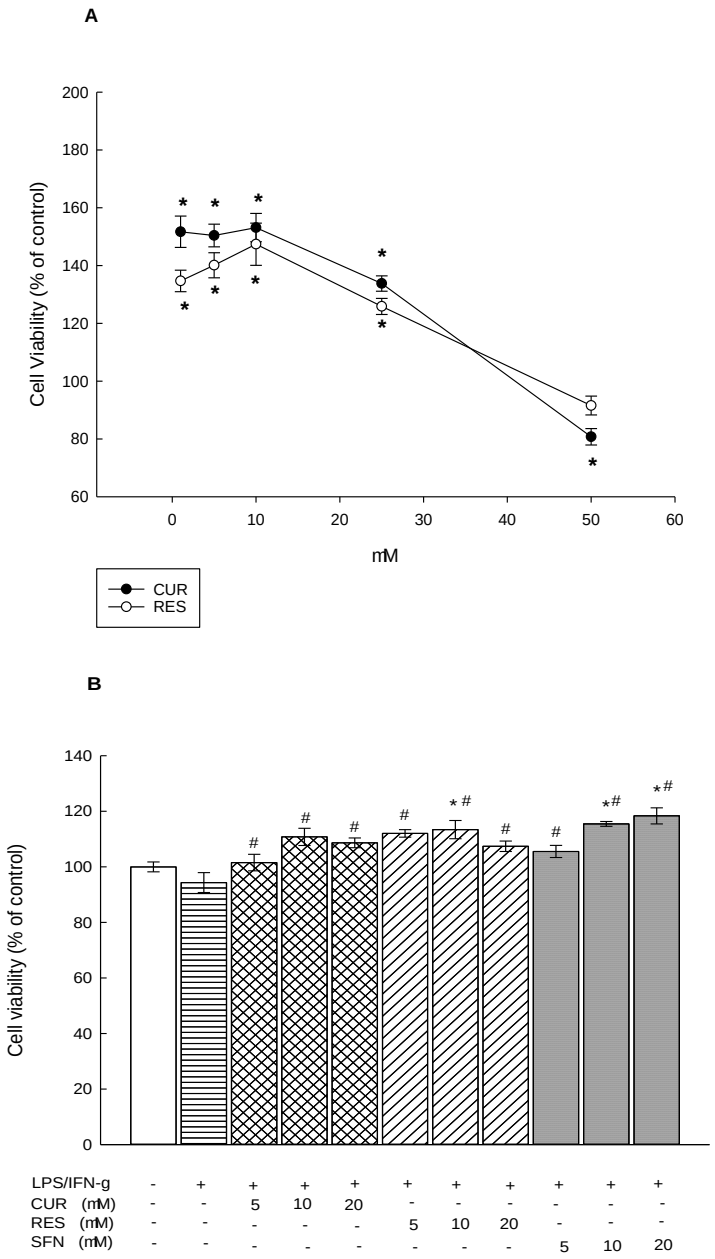


Fig. Supl.3.1. Effects of CUR, RES and SFN on cell viability. RAW 264.7 macrophages were exposed for 24 h to (A) CUR and RES alone at increasing concentrations (1, 5, 10, 25 or 50 µM), and (B) CUR, RES, or SFN at increasing concentrations of (5, 10 or 20 µM) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL). Cell cytotoxicity was measured using MTT assay. Data are expressed as a percentage of control (at 100%) ± S.E. (n=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; $, P <0.05, compared with LPS/IFN- γ group.

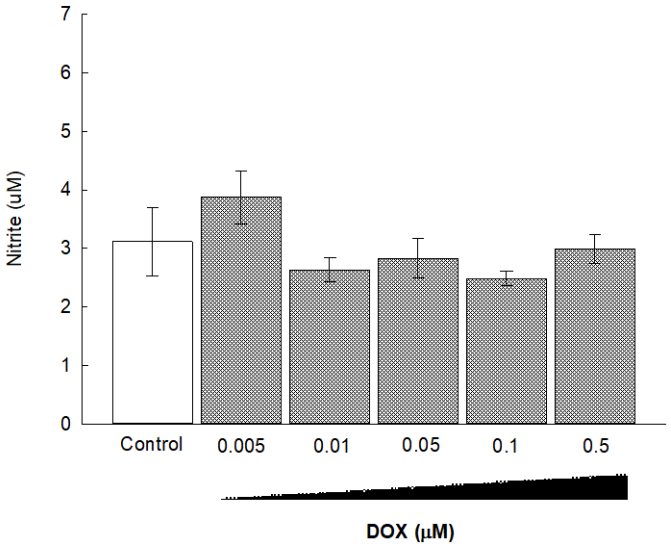


Fig. Supl.3.2. Effect of DOX on nitrite production. RAW 264.7 macrophages were exposed to increasing concentrations of DOX (0.005, 0.01, 0.05, 0.1, or 0.5 µM) for 24 h. Nitrite production was quantified using Griess method. Data are expressed as mean ± S.E. (n=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control.

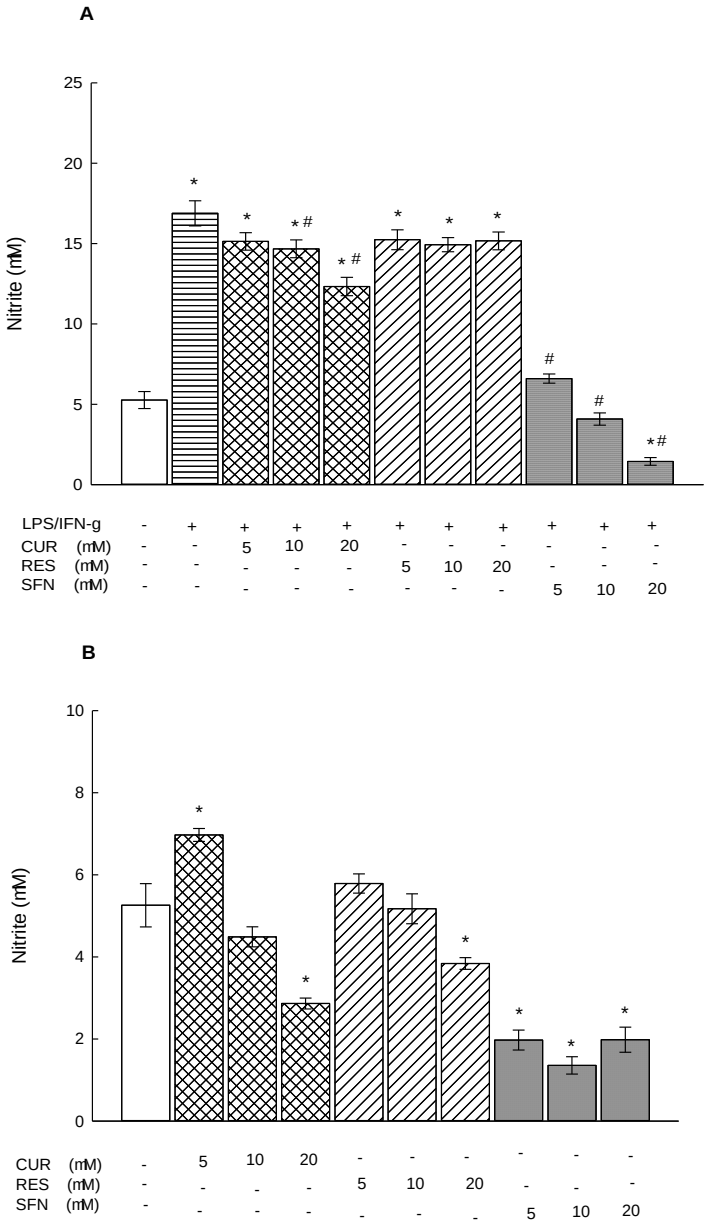


Fig. Supl.3.3. Effects of CUR, RES and SFN on nitrite production. RAW 264.7 macrophages were treated for 24 h to increasing concentrations of CUR, RESV or SFN (5, 10 or 20 µM) alone (A) or in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) (B). Nitrite production was quantified using Griess method. Data are expressed as mean ± S.E. (*n*=8). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, *P* <0.05, compared with control; #, *P* <0.05, compared with LPS/IFN- γ group.

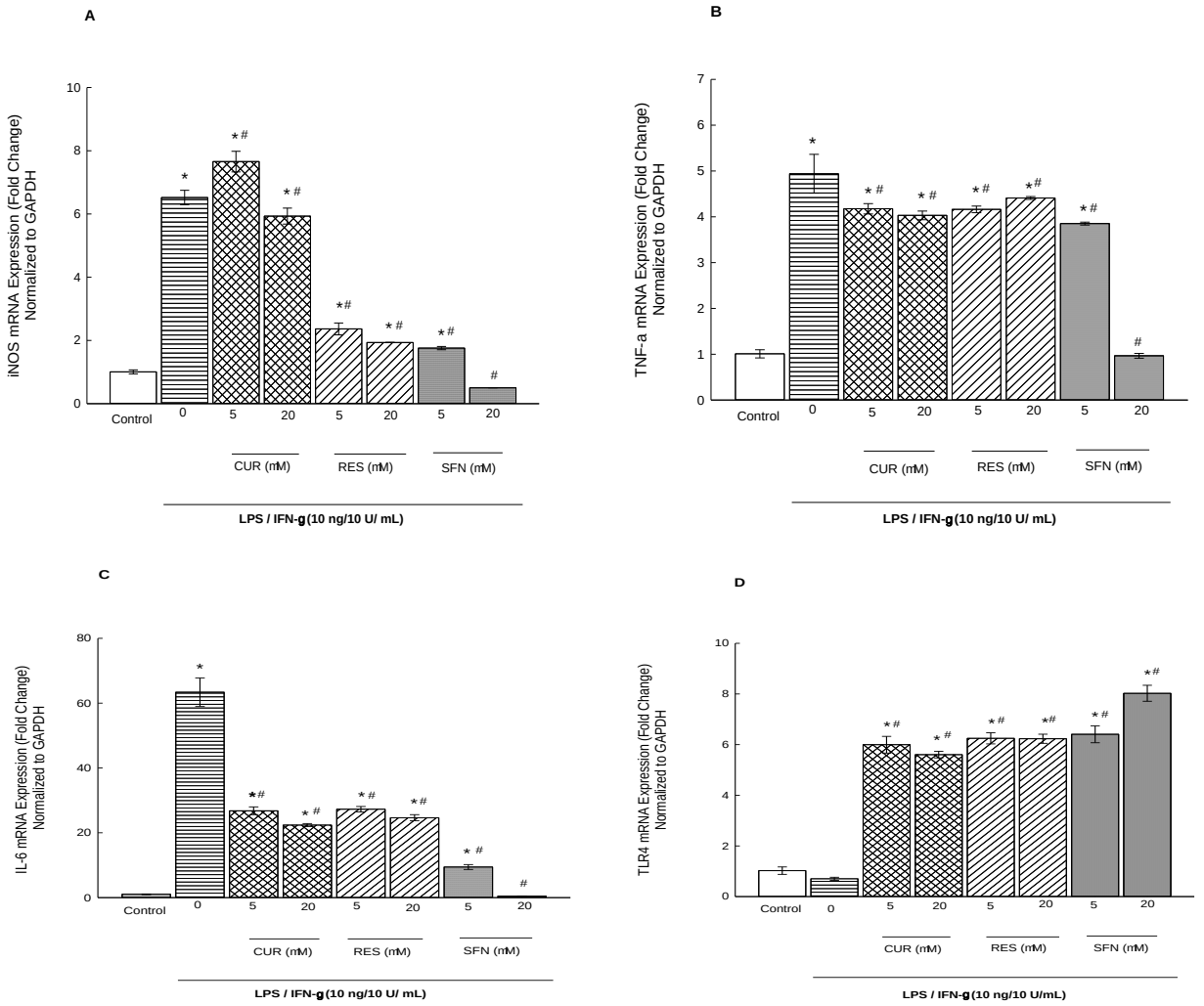


Fig. Supl.3.4. Effect of CUR, RES and SFN on the mRNA expression of iNOS, TNF-α, IL-6, and TLR4 in LPS/IFN-γ-mediated RAW 264.7 macrophages. RAW 264.7 cells were treated for 6 h with CUR, RES or SFN (5 and 20 M) in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL). iNOS (a), TNF-α (b), IL-6 (c), and TLR4 (d) mRNA expression levels were measured using qRT-PCR and normalized against GAPDH. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; #, P <0.05, compared with LPS/IFN- γ group.

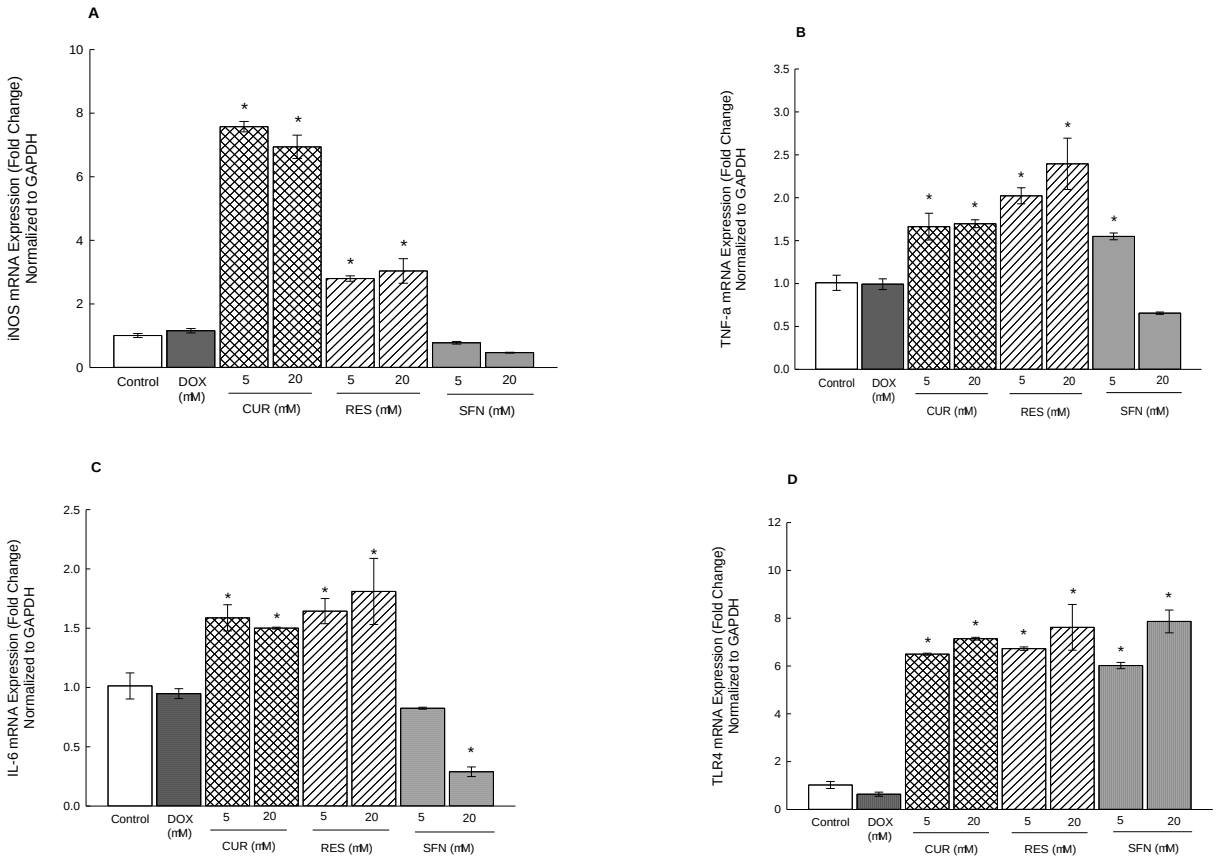


Fig. Supl.3.5. Effect of DOX, CUR, RES, and SFN on the mRNA expression of iNOS, TNF-α, IL-6, and TLR4 in RAW 264.7 macrophages. RAW 264.7 cells were treated for 6 h with DOX (0.1 μM), or CUR, RES or SFN (5 and 20 μM). iNOS (a), TNF-α (b), IL-6 (c), and TLR4 (d) mRNA expression levels were quantified using qRT-PCR and normalized against GAPDH. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control.

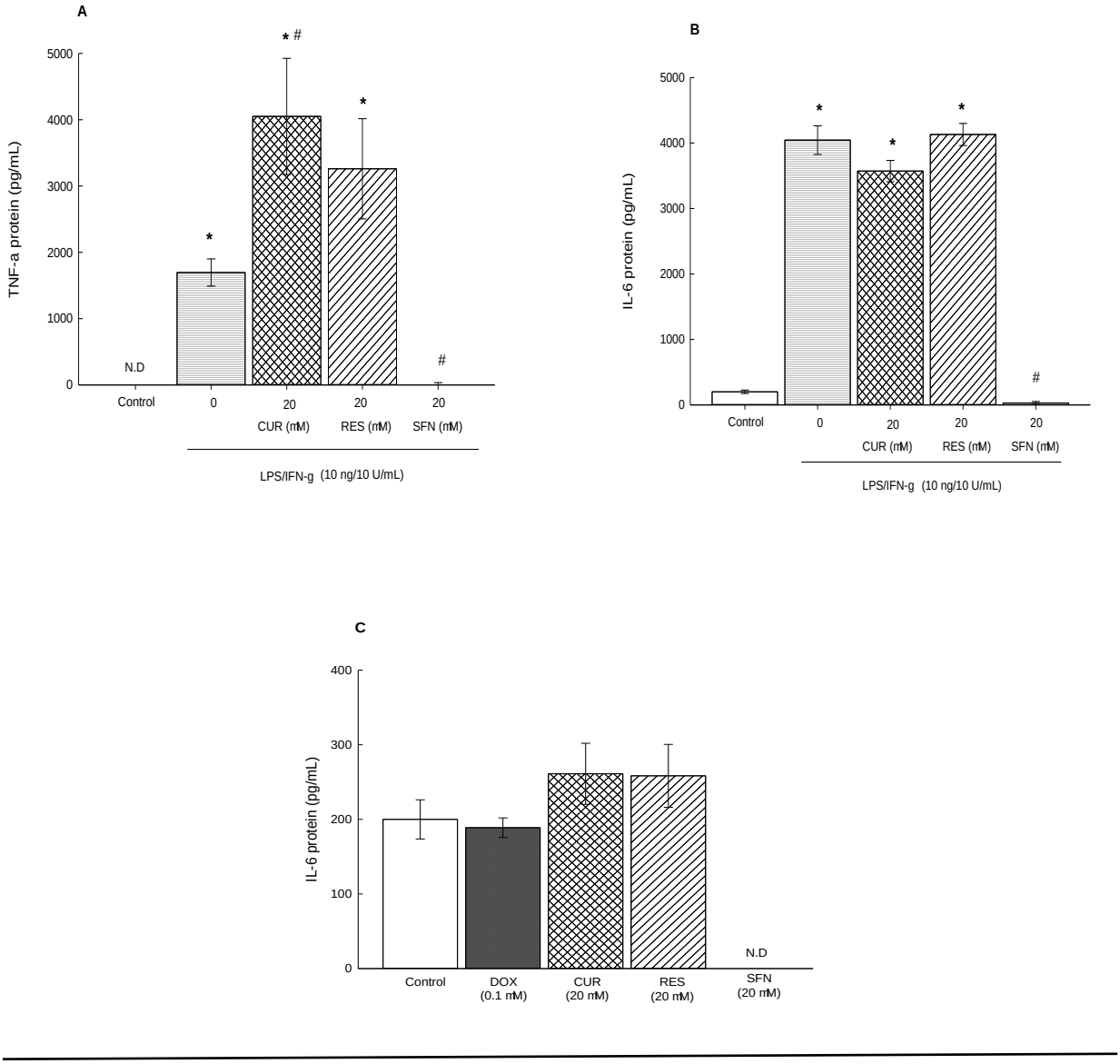


Fig. Supl.3.6. Effect of CUR, RES and SFN on TNF-α and IL-6 protein levels in LPS/IFN-γ-mediated macrophages. RAW 264.7 cells were treated for 24 h with CUR, RES or SFN (20 M) alone (C) or in the presence of LPS (10 ng/mL) plus IFN-γ (10 U/mL) (A & B), TNF-α (A) and IL-6 (B) protein levels were quantified using ELISA. Data are expressed as mean ± S.E. (n=3). Comparisons are made with ANOVA followed by Student–Newman–Keuls (SNK) post-hoc test; *, P <0.05, compared with control; #, P <0.05, compared with LPS/IFN- γ group; N.D (not detected).

References

1. Chaplin DD. Overview of the immune response. Journal of Allergy and Clinical Immunology. 2010;125(2):S3-S23.

2. Nicholson LB. The immune system. Essays in biochemistry. 2016;60(3):275-301.

3. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. Allergy, Asthma & Clinical Immunology. 2018;14(2):49.

4. Turvey SE, Broide DH. Innate immunity. Journal of Allergy and Clinical Immunology. 2010;125(2):S24-S32.

5. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.

6. Bonilla FA, Oettgen HC. Adaptive immunity. Journal of Allergy and Clinical Immunology. 2010;125(2):S33-S40.

7. Chesnut R, Grey H. Antigen presenting cells and mechanisms of antigen presentation. Critical reviews in immunology. 1985;5(3):263-316.

8. Cano RLE, Lopera HDE. Introduction to T and B lymphocytes. Autoimmunity: From Bench to Bedside [Internet]: El Rosario University Press; 2013.

9. Oliveira C, Silveira I, Veiga F, Ribeiro AJ. Recent advances in characterization of nonviral vectors for delivery of nucleic acids: impact on their biological performance. Expert opinion on drug delivery. 2015;12(1):27-39.

10. Newton K, Dixit VM. Signaling in innate immunity and inflammation. Cold Spring Harbor perspectives in biology. 2012;4(3):a006049.

11. Cronkite DA, Strutt TM. The regulation of inflammation by innate and adaptive lymphocytes. Journal of immunology research. 2018;2018.

12. Kizil C, Kyritsis N, Brand M. Effects of inflammation on stem cells: together they strive? EMBO reports. 2015;16(4):416-26.

13. Xiao TS. Innate immunity and inflammation. Nature Publishing Group; 2017.

14. Cignarella A, Bolego C. Innate Immunity in Inflammation. In: Riccardi C, Levi-Schaffer F, Tiligada E, editors. Immunopharmacology and Inflammation. Cham: Springer International Publishing; 2018. p. 179-90.

15. Azab A, Nassar A, Azab AN. Anti-inflammatory activity of natural products. Molecules. 2016;21(10):1321.

16. Frantz S, Falcao-Pires I, Balligand JL, Bauersachs J, Brutsaert D, Ciccarelli M, et al. The innate immune system in chronic cardiomyopathy: a European Society of Cardiology (ESC) scientific statement from the Working Group on Myocardial Function of the ESC. European journal of heart failure. 2018;20(3):445-59.

17. Dinarello CA. Anti-inflammatory agents: present and future. Cell. 2010;140(6):935-50.

18. Zhang J-M, An J. Cytokines, inflammation and pain. International anesthesiology clinics. 2007;45(2):27.

19. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2018;9(6):7204.

20. Montgomery SL, Bowers WJ. Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system. Journal of neuroimmune pharmacology. 2012;7(1):42-59.

21. Zelová H, Hošek J. TNF-α signalling and inflammation: interactions between old acquaintances. Inflammation Research. 2013;62(7):641-51.

22. Fenton MJ. transcriptional and post-transcriptional regulation of interleukin 1 gene expression. International journal of immunopharmacology. 1992;14(3):401-11.

23. Rider P, Carmi Y, Voronov E, Apte RN. Interleukin-1α. Seminars in immunology. 2013;25(6):430-8.

24. Vignali DA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. Nature immunology. 2012;13(8):722.

25. Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, Cua DJ. IL-12 and IL-23: master regulators of innate and adaptive immunity. Immunological reviews. 2004;202(1):96-105.

26. Duvallet E, Semerano L, Assier E, Falgarone G, Boissier M-C. Interleukin-23: a key cytokine in inflammatory diseases. Annals of medicine. 2011;43(7):503-11.

27. Sabat R. IL-10 family of cytokines. Cytokine & growth factor reviews. 2010;21(5):315-24.

28. Ng T, Britton GJ, Hill EV, Verhagen J, Burton BR, Wraith DC. Regulation of adaptive immunity; the role of interleukin-10. Frontiers in immunology. 2013;4:129.

29. Kwilasz A, Grace P, Serbedzija P, Maier S, Watkins L. The therapeutic potential of interleukin-10 in neuroimmune diseases. Neuropharmacology. 2015;96:55-69.

30. Chen CY, Kao CL, Liu CM. The Cancer Prevention, Anti-Inflammatory and Anti-Oxidation of Bioactive Phytochemicals Targeting the TLR4 Signaling Pathway. International Journal of Molecular Sciences. 2018;19(9):2729.

31. Goetzl EJ, An S, Smith WL. Specificity of expression and effects of eicosanoid mediators in normal physiology and human diseases. The FASEB Journal. 1995;9(11):1051-8.

32. Leff JA, Busse WW, Pearlman D, Bronsky EA, Kemp J, Hendeles L, et al. Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction. New England Journal of Medicine. 1998;339(3):147-52.

33. Peters-Golden M, Henderson Jr WR. Leukotrienes. New England Journal of Medicine. 2007;357(18):1841-54.

34. Zhao J, Quyyumi AA, Patel R, Zafari AM, Veledar E, Onufrak S, et al. Sex-specific association of depression and a haplotype in leukotriene A4 hydrolase gene. Psychosomatic medicine. 2009;71(7):691.

35. Biswas SK. Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? Oxidative medicine and cellular longevity. 2016;2016:5698931.

36. Tripathi P. Nitric oxide and immune response. Indian Journal of Biochemistry and Biophysics. 2007;44(5):310-9.

37. Moncada S, Bolaños JP. Nitric oxide, cell bioenergetics and neurodegeneration. Journal of neurochemistry. 2006;97(6):1676-89.

38. Hewlings SJ, Kalman DS. Curcumin: a review of its' effects on human health. Foods. 2017;6(10):92.

39. Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer. Oncogene. 1999;18(49):6938-47.

40. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-κB signaling pathways. Nature immunology. 2011;12(8):695.

41. Hwang J-H, Lim S-B. Antioxidant and anti-inflammatory activities of broccoli florets in LPS-stimulated RAW 264.7 cells. Preventive nutrition and food science. 2014;19(2):89.

42. Ling J, Kumar R. Crosstalk between NFkB and glucocorticoid signaling: a potential target of breast cancer therapy. Cancer letters. 2012;322(2):119-26.

43. Gupta I, Parihar A, Malhotra P, Gupta S, Lüdtke R, Safayhi H, et al. Effects of gum resin of Boswellia serrata in patients with chronic colitis. Planta medica. 2001;67(05):391-5.

44. Bonis P, Chung M, Tatsioni A, Sun Y, Kupelnick B, Lichtenstein A, et al. Effects of omega-3 fatty acids on organ transplantation. Evid Rep Technol Assess (Summ). 2005;115:1-11.

45. Shin JS, Noh YS, Lee YS, Cho YW, Baek NI, Choi MS, et al. Arvelexin from Brassica rapa suppresses NF-κB-regulated pro-inflammatory gene expression by inhibiting activation of IκB kinase. British journal of pharmacology. 2011;164(1):145-58.

46. Tsoyi K, Park HB, Kim YM, Chung JI, Shin SC, Lee WS, et al. Anthocyanins from black soybean seed coats inhibit UVB-induced inflammatory cylooxygenase-2 gene expression and PGE2 production through regulation of the nuclear factor-κB and phosphatidylinositol 3-kinase/Akt pathway. Journal of agricultural and food chemistry. 2008;56(19):8969-74.

47. Maroon JC, Bost JW, Maroon A. Natural anti-inflammatory agents for pain relief. Surgical neurology international. 2010;1(80).

48. Majumdar S, Aggarwal BB. Methotrexate suppresses NF-κB activation through inhibition of IκBα phosphorylation and degradation. The Journal of Immunology. 2001;167(5):2911-20.

49. Moro C, Palacios I, Lozano M, D'Arrigo M, Guillamón E, Villares A, et al. Anti-inflammatory activity of methanolic extracts from edible mushrooms in LPS activated RAW 264.7 macrophages. Food Chemistry. 2012;130(2):350-5.

50. Raghav SK, Gupta B, Shrivastava A, Das HR. Inhibition of lipopolysaccharide-inducible nitric oxide synthase and IL-1β through suppression of NF-κB activation by 3-(1′-1′-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin isolated from Ruta graveolens L. European journal of pharmacology. 2007;560(1):69-80.

51. Karlmark K, Tacke F, Dunay I. Monocytes in health and disease–Minireview. European journal of microbiology & immunology. 2012;2(2):97.

52. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer research. 2006;66(2):605-12.

53. Dimitrievska S, Petit A, Ajji A, Bureau MN, Yahia LH. Biocompatibility of novel polymer-apatite nanocomposite fibers. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2008;84(1):44-53.

54. Liu G, Xia XP, Gong SL, Zhao Y. The macrophage heterogeneity: difference between mouse peritoneal exudate and splenic F4/80+ macrophages. Journal of cellular physiology. 2006;209(2):341-52.

55. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nature Reviews Immunology. 2005;5(12):953.

56. Chamberlain LM, Godek ML, Gonzalez-Juarrero M, Grainger DW. Phenotypic non-equivalence of murine (monocyte-) macrophage cells in biomaterial and inflammatory models. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2009;88(4):858-71.

57. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nature immunology. 2010;11(10):889.

58. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nature reviews immunology. 2008;8(12):958.

59. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496(7446):445.

60. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. The Journal of clinical investigation. 2012;122(3):787-95.

61. Taciak B, Białasek M, Braniewska A, Sas Z, Sawicka P, Kiraga Ł, et al. Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages. PloS one. 2018;13(6):e0198943.

62. Siegemund S, Sauer K. Balancing pro- and anti-inflammatory TLR4 signaling. Nat Immunol. 2012;13(11):1031-3.

63. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. Journal of leukocyte biology. 2004;76(3):509-13.

64. Stout RD, Suttles J. T Cell Signaling of Macrophage Function in Inflammatory. Frontiers in Bioscience. 1997;2:d197-206.

65. Gordon S. Alternative activation of macrophages. Nature reviews immunology. 2003;3(1):23.

66. Fernández N, Renedo M, García-Rodríguez C, Crespo MS. Activation of monocytic cells through Fcγ receptors induces the expression of macrophage-inflammatory protein (MIP)-1α, MIP-1β, and RANTES. The Journal of Immunology. 2002;169(6):3321-8.

67. Baig MS, Zaichick SV, Mao M, de Abreu AL, Bakhshi FR, Hart PC, et al. NOS1-derived nitric oxide promotes NF-kappaB transcriptional activity through inhibition of suppressor of cytokine signaling-1. J Exp Med. 2015;212(10):1725-38.

68. Banerjee S, Cui H, Xie N, Tan Z, Yang S, Icyuz M, et al. miR-125a-5p regulates differential activation of macrophages and inflammation. J Biol Chem. 2013;288(49):35428-36.

69. Espinoza-Jimenez A, Peon AN, Terrazas LI. Alternatively activated macrophages in types 1 and 2 diabetes. Mediators Inflamm. 2012;2012:815953.

70. Gong D, Shi W, Yi SJ, Chen H, Groffen J, Heisterkamp N. TGFbeta signaling plays a critical role in promoting alternative macrophage activation. BMC Immunol. 2012;13:31.

71. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity. 2005;23(4):344-6.

72. Takeda K, Akira S. Toll-like receptors in innate immunity. International immunology. 2005;17(1):1-14.

73. Blander JM, Medzhitov R. Regulation of phagosome maturation by signals from toll-like receptors. Science. 2004;304(5673):1014-8.

74. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, et al. RIP1 is an essential mediator of Toll-like receptor 3–induced NF-κB activation. Nature immunology. 2004;5(5):503.

75. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKKε and TBK1 are essential components of the IRF3 signaling pathway. Nature immunology. 2003;4(5):491.

76. Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-κB and IFN-regulatory factor-3, in the Toll-like receptor signaling. The Journal of Immunology. 2003;171(8):4304-10.

77. Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu L-C, Wang GG, et al. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature. 2006;439(7073):204.

78. Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, et al. Critical role of TRAF3 in the Toll-like receptor-dependent and-independent antiviral response. Nature. 2006;439(7073):208.

79. Li H, Sun B. Toll-like receptor 4 in atherosclerosis. J Cell Mol Med. 2007;11(1):88-95.

80. Toshchakov V, Jones BW, Perera P-Y, Thomas K, Cody MJ, Zhang S, et al. TLR4, but not TLR2, mediates IFN-β–induced STAT1α/β-dependent gene expression in macrophages. Nature immunology. 2002;3(4):392.

81. Molteni M, Bosi A, Rossetti C. Natural products with toll-like receptor 4 antagonist activity. International journal of inflammation. 2018;2018.

82. He X, Jing Z, Cheng G. MicroRNAs: new regulators of Toll-like receptor signalling pathways. BioMed research international. 2014;2014.

83. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010;466(7308):835-40.

84. Penalva LOF. Untranslated Region. In: Dubitzky W, Wolkenhauer O, Cho K-H, Yokota H, editors. Encyclopedia of Systems Biology. New York, NY: Springer New York; 2013. p. 2324-.

85. Quinn SR, O'Neill LA. A trio of microRNAs that control Toll-like receptor signalling. Int Immunol. 2011;23(7):421-5.

86. O'neill LA, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nature Reviews Immunology. 2011;11(3):163.

87. Li Y, Shi X. MicroRNAs in the regulation of TLR and RIG-I pathways. Cellular & molecular immunology. 2013;10(1):65.

88. Taganov KD, Boldin MP, Chang K-J, Baltimore D. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proceedings of the National Academy of Sciences. 2006;103(33):12481-6.

89. Jurkin J, Schichl YM, Koeffel R, Bauer T, Richter S, Konradi S, et al. miR-146a is differentially expressed by myeloid dendritic cell subsets and desensitizes cells to TLR2-dependent activation. The Journal of Immunology. 2010;184(9):4955-65.

90. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. Journal of Experimental Medicine. 2011;208(6):1189-201.

91. Doxaki C, Kampranis SC, Eliopoulos AG, Spilianakis C, Tsatsanis C. Coordinated regulation of miR-155 and miR-146a genes during induction of endotoxin tolerance in macrophages. The Journal of Immunology. 2015;195(12):5750-61.

92. Schulte LN, Westermann AJ, Vogel J. Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing. Nucleic acids research. 2012;41(1):542-53.

93. Asirvatham AJ, Gregorie CJ, Hu Z, Magner WJ, Tomasi TB. MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. Molecular immunology. 2008;45(7):1995-2006.

94. Asirvatham AJ, Magner WJ, Tomasi TB. miRNA regulation of cytokine genes. Cytokine. 2009;45(2):58-69.

95. Liu X, Rennard SI. MicroRNA and cytokines. Mol Cell Pharmacol. 2011;3(3):143-51.

96. Bhaumik D, Scott G, Schokrpur S, Patil C, Campisi J, Benz C. Expression of microRNA-146 suppresses NF-κB activity with reduction of metastatic potential in breast cancer cells. Oncogene. 2008;27(42):5643.

97. Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, et al. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. Arthritis & Rheumatism. 2008;58(5):1284-92.

98. Sonkoly E, Ståhle M, Pivarcsi A. MicroRNAs and immunity: novel players in the regulation of normal immune function and inflammation. Seminars in cancer biology. 2008;18(2):131-40.

99. Chatzikyriakidou A, Voulgari PV, Georgiou I, Drosos AA. A polymorphism in the 3'-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with rheumatoid arthritis susceptibility. Joint Bone Spine. 2010;77(5):411-3.

100. Yang K, He YS, Wang XQ, Lu L, Chen QJ, Liu J, et al. MiR-146a inhibits oxidized low-density lipoprotein-induced lipid accumulation and inflammatory response via targeting toll-like receptor 4. FEBS letters. 2011;585(6):854-60.

101. Quinn EM, Wang JH, O'Callaghan G, Redmond HP. MicroRNA-146a is upregulated by and negatively regulates TLR2 signaling. PloS one. 2013;8(4):e62232.

102. Benakanakere MR, Li Q, Eskan MA, Singh AV, Zhao J, Galicia JC, et al. Modulation of TLR2 protein expression by miR-105 in human oral keratinocytes. Journal of Biological Chemistry. 2009;284(34):23107-15.

103. Tang Y, Luo X, Cui H, Ni X, Yuan M, Guo Y, et al. MicroRNA-146a contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2009;60(4):1065-75.

104. Hou J, Wang P, Lin L, Liu X, Ma F, An H, et al. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. The journal of immunology. 2009;183(3):2150-8.

105. Nahid MA, Satoh M, Chan EK. MicroRNA in TLR signaling and endotoxin tolerance. Cellular & molecular immunology. 2011;8(5):388.

106. Li S, Yue Y, Xu W, Xiong S. MicroRNA-146a represses mycobacteria-induced inflammatory response and facilitates bacterial replication via targeting IRAK-1 and TRAF-6. PloS one. 2013;8(12):e81438.

107. Lin L, Hou J, Ma F, Wang P, Liu X, Li N, et al. Type I IFN inhibits innate IL-10 production in macrophages through histone deacetylase 11 by downregulating microRNA-145. The Journal of Immunology. 2013;191(7):3896-904.

108. Park H, Huang X, Lu C, Cairo MS, Zhou X. MicroRNA-146a and microRNA-146b regulate human dendritic cell apoptosis and cytokine production by targeting TRAF6 and IRAK1 proteins. Journal of Biological Chemistry. 2015;290(5):2831-41.

109. Nahid MA, Yao B, Dominguez-Gutierrez PR, Kesavalu L, Satoh M, Chan EK. Regulation of TLR2-mediated tolerance and cross-tolerance through IRAK4 modulation by miR-132 and miR-212. The Journal of Immunology. 2013;190(3):1250-63.

110. Starczynowski DT, Kuchenbauer F, Argiropoulos B, Sung S, Morin R, Muranyi A, et al. Identification of miR-145 and miR-146a as mediators of the 5q– syndrome phenotype. Nature medicine. 2010;16(1):49.

111. Loubaki L, Chabot D, Paré I, Drouin M, Bazin R. MiR-146a potentially promotes IVIg-mediated inhibition of TLR4 signaling in LPS-activated human monocytes. Immunology letters. 2017;185:64-73.

112. Shin VY, Jin H, Ng EK, Cheng AS, Chong WW, Wong CY, et al. NF-κB targets miR-16 and miR-21 in gastric cancer: involvement of prostaglandin E receptors. Carcinogenesis. 2010;32(2):240-5.

113. Chen Y, Chen J, Wang H, Shi J, Wu K, Liu S, et al. HCV-induced miR-21 contributes to evasion of host immune system by targeting MyD88 and IRAK1. PLoS pathogens. 2013;9(4):e1003248.

114. Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'leary JJ, Ruan Q, et al. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. Nature immunology. 2010;11(2):141.

115. Cheung ST, So EY, Chang D, Ming-Lum A, Mui AL. Interleukin-10 inhibits lipopolysaccharide induced miR-155 precursor stability and maturation. PloS one. 2013;8(8).

116. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proceedings of the National Academy of Sciences. 2007;104(5):1604-9.

117. Tili E, Michaille J-J, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-α stimulation and their possible roles in regulating the response to endotoxin shock. The Journal of Immunology. 2007;179(8):5082-9.

118. Alivernini S, Gremese E, McSharry C, Tolusso B, Ferraccioli G, McInnes IB, et al. MicroRNA-155—at the critical interface of innate and adaptive immunity in arthritis. Frontiers in immunology. 2018;8:1932.

119. Strassheim D, Kim J-Y, Park J-S, Mitra S, Abraham E. Involvement of SHIP in TLR2-induced neutrophil activation and acute lung injury. The Journal of Immunology. 2005;174(12):8064-71.

120. Quinn SR, O'Neill LA. A trio of microRNAs that control Toll-like receptor signalling. International immunology. 2011;23(7):421-5.

121. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, et al. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proceedings of the national academy of sciences. 2009;106(8):2735-40.

122. Andreu N, Phelan J, Paola F, Cliff JM, Clark TG, Hibberd ML. Primary macrophages and J774 cells respond differently to infection with Mycobacterium tuberculosis. Scientific reports. 2017;7:42225.

123. Nguyen J, Nohe A. Factors that affect the osteoclastogenesis of RAW264. 7 cells. Journal of biochemistry and analytical studies. 2017;2(1).

124. Takahashi N, Udagawa N, Kobayashi Y, Suda T. Generation of osteoclasts in vitro, and assay of osteoclast activity. Arthritis Research: Springer; 2007. p. 285-301.

125. Johnson BK, Abramovitch RB. Macrophage infection models for Mycobacterium tuberculosis. Mycobacteria Protocols: Springer; 2015. p. 329-41.

126. Mendoza-Coronel E, Castañón-Arreola M. Comparative evaluation of in vitro human macrophage models for mycobacterial infection study. Pathogens and disease. 2016;74(6).

127. Burdall SE, Hanby AM, Lansdown MR, Speirs V. Breast cancer cell lines: friend or foe? Breast cancer research. 2003;5(2):89.

128. Pan C, Kumar C, Bohl S, Klingmueller U, Mann M. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. Molecular & Cellular Proteomics. 2009;8(3):443-50.

129. Frattini A, Fabbri M, Valli R, De Paoli E, Montalbano G, Gribaldo L, et al. High variability of genomic instability and gene expression profiling in different HeLa clones. Scientific reports. 2015;5:15377.

130. Shelley CS, Teodoridis JM, Park H, Farokhzad OC, Böttinger EP, Arnaout MA. During differentiation of the monocytic cell line U937, Purα mediates induction of the CD11c β2 integrin gene promoter. The Journal of Immunology. 2002;168(8):3887-93.

131. Whelan RD, Kiley SC, Parker PJ. Tetradecanoyl phorbol acetate-induced microtubule reorganization is required for sustained mitogen-activated protein kinase activation and morphological differentiation of U937 cells. Cell Growth and Differentiation-Publication American Association for Cancer Research. 1999;10(4):271-8.

132. Hadden EM, Sadlik JR, Coffey RG, Hadden JW. Effects of phorbol myristate acetate and a lymphokine on cyclic 3′: 5′-guanosine monophosphate levels and proliferation of macrophages. Cancer research. 1982;42(8):3064-9.

133. Merly L, Smith SL. Murine RAW 264.7 cell line as an immune target: are we missing something? Immunopharmacology and immunotoxicology. 2017;39(2):55-8.

134. Leenen PJ, de Bruijn MF, Voerman JS, Campbell PA, van Ewijk W. Markers of mouse macrophage development detected by monoclonal antibodies. Journal of immunological methods. 1994;174(1-2):5-19.

135. Duong LT, Rodan GA. PYK2 is an adhesion kinase in macrophages, localized in podosomes and activated by β2-integrin ligation. Cell motility and the cytoskeleton. 2000;47(3):174-88.

136. Fuentes A-L, Millis L, Vapenik J, Sigola L. Lipopolysaccharide-mediated enhancement of zymosan phagocytosis by RAW 264.7 macrophages is independent of opsonins, laminarin, mannan, and complement receptor 3. journal of surgical research. 2014;189(2):304-12.

137. Berghaus LJ, Moore JN, Hurley DJ, Vandenplas ML, Fortes BP, Wolfert MA, et al. Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. Comparative immunology, microbiology and infectious diseases. 2010;33(5):443-54.

138. Held TK, Weihua X, Yuan L, Kalvakolanu DV, Cross AS. Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. Infection and immunity. 1999;67(1):206-12.

139. Greenwell M, Rahman P. Medicinal plants: their use in anticancer treatment. International journal of pharmaceutical sciences and research. 2015;6(10):4103.

140. Amin A, Gali-Muhtasib H, Ocker M, Schneider-Stock R. Overview of major classes of plant-derived anticancer drugs. International journal of biomedical science: IJBS. 2009;5(1):1.

141. Espinosa E, Zamora P, Feliu J, Barón MG. Classification of anticancer drugs—a new system based on therapeutic targets. Cancer treatment reviews. 2003;29(6):515-23.

142. Bachur NR. Anthracycline antibiotic pharmacology and metabolism. Cancer Treatment Reports. 1979;63(8):7-820.

143. McGowan JV, Chung R, Maulik A, Piotrowska I, Walker JM, Yellon DM. Anthracycline chemotherapy and cardiotoxicity. Cardiovascular drugs and therapy. 2017;31(1):63-75.

144. Valcovici M, Andrica F, Serban C, Dragan S. Cardiotoxicity of anthracycline therapy: current perspectives. Archives of medical science: AMS. 2016;12(2):428.

145. Pugazhendhi A, Edison TNJI, Velmurugan BK, Jacob JA, Karuppusamy I. Toxicity of Doxorubicin (Dox) to different experimental organ systems. Life sciences. 2018;200:26-30.

146. Hortobagyi G. Anthracyclines in the treatment of cancer. Drugs. 1997;54(4):1-7.

147. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacological reviews. 2004;56(2):185-229.

148. Barrett-Lee P, Dixon J, Farrell C, Jones A, Leonard R, Murray N, et al. Expert opinion on the use of anthracyclines in patients with advanced breast cancer at cardiac risk. Annals of oncology. 2009;20(5):816-27.

149. Broxterman HJ, Gotink KJ, Verheul HM. Understanding the causes of multidrug resistance in cancer: a comparison of doxorubicin and sunitinib. Drug resistance updates. 2009;12(4-5):114-26.

150. Weiss RB, Sarosy G, Clagett-Carr K, Russo M, Leyland-Jones B. Anthracycline analogs the past, present, and future. Cancer chemotherapy and pharmacology. 1986;18(3):185-97.

151. Santos DS, Goldenberg RC. Doxorubicin-Induced Cardiotoxicity: From Mechanisms to Development of Efficient Therapy. Cardiotoxicity: IntechOpen; 2018. p. 3-24.

152. Shaul P, Frenkel M, Goldstein EB, Mittelman L, Grunwald A, Ebenstein Y, et al. The structure of anthracycline derivatives determines their subcellular localization and cytotoxic activity. ACS medicinal chemistry letters. 2013;4(3):323-8.

153. W Edwardson D, Narendrula R, Chewchuk S, Mispel-Beyer K, PJ Mapletoft J, M Parissenti A. Role of drug metabolism in the cytotoxicity and clinical efficacy of anthracyclines. Current drug metabolism. 2015;16(6):412-26.

154. Giordano SH, Lin Y-L, Kuo YF, Hortobagyi GN, Goodwin JS. Decline in the use of anthracyclines for breast cancer. Journal of clinical oncology. 2012;30(18):2232.

155. Nabhan C, Byrtek M, Rai A, Dawson K, Zhou X, Link BK, et al. Disease characteristics, treatment patterns, prognosis, outcomes and lymphoma-related mortality in elderly follicular lymphoma in the United States. British journal of haematology. 2015;170(1):85-95.

156. Chihara D, Westin JR, Oki Y, Ahmed MA, Do B, Fayad LE, et al. Management strategies and outcomes for very elderly patients with diffuse large B-cell lymphoma. Cancer. 2016;122(20):3145-51.

157. Smith LA, Cornelius VR, Plummer CJ, Levitt G, Verrill M, Canney P, et al. Cardiotoxicity of anthracycline agents for the treatment of cancer: systematic review and meta-analysis of randomised controlled trials. BMC cancer. 2010;10(1):337.

158. Bassan R, Lerede T, Rambaldi A, Di EB, Rossi G, Pogliani E, et al. The role of anthracyclines in adult acute lymphoblastic leukaemia. Leukemia. 1996;10:s58-61.

159. Abraham R, Basser RL, Green MD. A risk-benefit assessment of anthracycline antibiotics in antineoplastic therapy. Drug safety. 1996;15(6):406-29.

160. Senn H-J, Gelber RD, Goldhirsch A, Thürlimann B. Adjuvant therapy of primary breast cancer VI: Springer Science & Business Media; 2012.

161. Levine M, Bramwell V, Bowman D, Norris B, Findlay D, Warr K, editors. A clinical trial of intensive CEF versus CMF in premenopausal women with node positive breast cancer. Proc Am Soc Clin Oncol; 1995.

162. Twelves CJ. Oral idarubicin in solid tumour chemotherapy. Clinical Drug Investigation. 1995;9(2):39-54.

163. A'hern R, Gore M. Impact of doxorubicin on survival in advanced ovarian cancer. Journal of clinical oncology. 1995;13(3):726-32.

164. Meriwether WD, Bachur NR. Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukemia. Cancer research. 1972;32(6):1137-42.

165. Geisberg CA, Sawyer DB. Mechanisms of anthracycline cardiotoxicity and strategies to decrease cardiac damage. Current hypertension reports. 2010;12(6):404-10.

166. Reinert K. Anthracycline-binding induced Dna stiffenin, bending and elongation; stereochemical implications from viscometic investigations. Nucleic acids research. 1983;11(10):3411-30.

167. Sinha BK, Katki AG, Batist G, Cowan KH, Myers CE. Adriamycin-stimulated hydroxyl radical formation in human breast tumor cells. Biochemical pharmacology. 1987;36(6):793-6.

168. Dorr RT. Cytoprotective agents for anthracyclines. Seminars in oncology. 1996;23(4 Suppl 8):23-34.

169. Booser DJ, Hortobagyi GN. Anthracycline antibiotics in cancer therapy. Drugs. 1994;47(2):223-58.

170. Larsson R, Nygren P. Cytotoxic activity of topoisomerase II inhibitors in primary cultures of tumor cells from patients with human hematologic and solid tumors. Cancer. 1994;74(10):2857-62.

171. Efferth T, Osieka R. Clinical relevance of the MDR-1 gene and its gene product, P-glycoprotein, for cancer chemotherapy: a meta-analysis. Tumordiagnostik und Therapie. 1993;14(6):238-.

172. Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ, Moens AL. Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. Journal of molecular and cellular cardiology. 2012;52(6):1213-25.

173. Outomuro D, Grana DR, Azzato F, Milei J. Adriamycin-induced myocardial toxicity: new solutions for an old problem? International journal of cardiology. 2007;117(1):6-15.

174. Vivenza D, Feola M, Garrone O, Monteverde M, Merlano M, Lo Nigro C. Role of the renin-angiotensin-aldosterone system and the glutathione S-transferase Mu, Pi and Theta gene polymorphisms in cardiotoxicity after anthracycline chemotherapy for breast carcinoma. The International journal of biological markers. 2013;28(4):336-47.

175. Chatterjee K, Zhang J, Honbo N, Karliner JS. Doxorubicin cardiomyopathy. Cardiology. 2010;115(2):155-62.

176. Lipshultz SE, Alvarez JA, Scully RE. Anthracycline associated cardiotoxicity in survivors of childhood cancer. Heart. 2008;94(4):525-33.

177. Shan K, Lincoff AM, Young JB. Anthracycline-induced cardiotoxicity. Annals of internal medicine. 1996;125(1):47-58.

178. Umlauf J, Horký M. Molecular biology of doxorubicin-induced cardiomyopathy. Experimental & Clinical Cardiology. 2002;7(1):35.

179. Wang L, Chen Q, Qi H, Wang C, Wang C, Zhang J, et al. Doxorubicin-induced systemic inflammation is driven by upregulation of toll-like receptor TLR4 and endotoxin leakage. Cancer research. 2016;76(22):6631-42.

180. Singh P, Sharma R, McElhanon K, Allen CD, Megyesi JK, Beneš H, et al. Sulforaphane protects the heart from doxorubicin-induced toxicity. Free Radical Biology and Medicine. 2015;86:90-101.

181. Carvalho FS, Burgeiro A, Garcia R, Moreno AJ, Carvalho RA, Oliveira PJ. Doxorubicin-induced cardiotoxicity: from bioenergetic failure and cell death to cardiomyopathy. Medicinal research reviews. 2014;34(1):106-35.

182. Štěrba M, Popelová O, Vávrová A, Jirkovský E, Kovaříková P, Geršl V, et al. Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. Antioxidants & redox signaling. 2013;18(8):899-929.

183. Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. New England Journal of Medicine. 1998;339(13):900-5.

184. Davies K, Doroshow J. Redox cycling of anthracyclines by cardiac mitochondria. I. Anthracycline radical formation by NADH dehydrogenase. Journal of Biological Chemistry. 1986;261(7):3060-7.

185. Berthiaume J, Wallace KB. Adriamycin-induced oxidative mitochondrial cardiotoxicity. Cell biology and toxicology. 2007;23(1):15-25.

186. Raj S, Franco VI, Lipshultz SE. Anthracycline-induced cardiotoxicity: a review of pathophysiology, diagnosis, and treatment. Current treatment options in cardiovascular medicine. 2014;16(6):315.

187. Ventura-Clapier R, Garnier A, Veksler V. Energy metabolism in heart failure. The Journal of physiology. 2004;555(1):1-13.

188. Sawyer DB, Fukazawa R, Arstall MA, Kelly RA. Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane. Circulation research. 1999;84(3):257-65.

189. Horenstein MS, Vander Heide RS, L'Ecuyer TJ. Molecular basis of anthracycline-induced cardiotoxicity and its prevention. Molecular genetics and metabolism. 2000;71(1-2):436-44.

190. Ichikawa Y, Ghanefar M, Bayeva M, Wu R, Khechaduri A, Prasad SVN, et al. Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. The Journal of clinical investigation. 2014;124(2):617-30.

191. Salazar-Mendiguchía J, González-Costello J, Roca J, Ariza-Solé A, Manito N, Cequier A. Anthracycline-mediated cardiomyopathy: basic molecular knowledge for the cardiologist. Arch Cardiol Mex. 2014;84(3):218-23.

192. Ghigo A, Li M, Hirsch E. New signal transduction paradigms in anthracycline-induced cardiotoxicity. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2016;1863(7):1916-25.

193. Hahn VS, Lenihan DJ, Ky B. Cancer therapy–induced cardiotoxicity: basic mechanisms and potential cardioprotective therapies. Journal of the American Heart Association. 2014;3(2):e000665.

194. Moody BF, Calvert JW. Emergent role of gasotransmitters in ischemia-reperfusion injury. Medical gas research. 2011;1(1):3.

195. Octavia Y, Kararigas G, de Boer M, Chrifi I, Kietadisorn R, Swinnen M, et al. Folic acid reduces doxorubicin-induced cardiomyopathy by modulating endothelial nitric oxide synthase. Journal of cellular and molecular medicine. 2017;21(12):3277-87.

196. Neilan T, Blake S, Ichinose F, Raher M, Buys E, Jassal D, et al. Perez-743 Sanz TM, Graveline A, Janssens SP, Picard MH, Scherrer-Crosbie M, Bloch KD. 744 Disruption of nitric oxide synthase 3 protects against the cardiac injury, dysfunction, and 745 mortality induced by doxorubicin. Circulation. 2007;116(506-514):746.

197. Ye Y, Martinez JD, Perez-Polo RJ, Lin Y, Uretsky BF, Birnbaum Y. The role of eNOS, iNOS, and NF-κB in upregulation and activation of cyclooxygenase-2 and infarct size reduction by atorvastatin. American Journal of Physiology-Heart and Circulatory Physiology. 2008;295(1):H343-H51.

198. Mukhopadhyay P, Rajesh M, Bátkai S, Kashiwaya Y, Hasko G, Liaudet L, et al. Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. American Journal of Physiology-Heart and Circulatory Physiology. 2009;296(5):H1466-H83.

199. Liu B, Li H, Qu H, Sun B. Nitric oxide synthase expressions in ADR-induced cardiomyopathy in rats. Journal of biochemistry and molecular biology. 2006;39(6):759.

200. Champoux JJ. DNA topoisomerases: structure, function, and mechanism. Annual review of biochemistry. 2001;70(1):369-413.

201. Vejpongsa P, Yeh E. Topoisomerase 2β: a promising molecular target for primary prevention of anthracycline-induced cardiotoxicity. Clinical Pharmacology & Therapeutics. 2014;95(1):45-52.

202. Carpenter AJ, Porter AC. Construction, characterization, and complementation of a conditional-lethal DNA topoisomerase IIα mutant human cell line. Molecular biology of the cell. 2004;15(12):5700-11.

203. Chen GL, Yang L, Rowe T, Halligan BD, Tewey KM, Liu LF. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. Journal of Biological Chemistry. 1984;259(21):13560-6.

204. Tewey K, Rowe T, Yang L, Halligan B, Liu L-F. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science. 1984;226(4673):466-8.

205. Finck BN, Kelly DP. Peroxisome proliferator–activated receptor γ coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. Circulation. 2007;115(19):2540-8.

206. Lyu YL, Kerrigan JE, Lin C-P, Azarova AM, Tsai Y-C, Ban Y, et al. Topoisomerase IIβ–mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane. Cancer research. 2007;67(18):8839-46.

207. Zhang S, Liu X, Bawa-Khalfe T, Lu L-S, Lyu YL, Liu LF, et al. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. Nature medicine. 2012;18(11):1639.

208. Guo R-M, Xu W-M, Lin J-C, Mo L-Q, Hua X-X, Chen P-X, et al. Activation of the p38 MAPK/NF-κB pathway contributes to doxorubicin-induced inflammation and cytotoxicity in H9c2 cardiac cells. Molecular medicine reports. 2013;8(2):603-8.

209. Nebigil CG, Désaubry L. Updates in anthracycline-mediated cardiotoxicity. Frontiers in pharmacology. 2018;9:1262.

210. Krack A, Sharma R, Figulla HR, Anker SD. The importance of the gastrointestinal system in the pathogenesis of heart failure. European heart journal. 2005;26(22):2368-74.

211. Ding Z, Liu S, Wang X, Khaidakov M, Dai Y, Mehta JL. Oxidant stress in mitochondrial DNA damage, autophagy and inflammation in atherosclerosis. Scientific reports. 2013;3(1):1-6.

212. Okazaki S, Nishitani Y, Nagoya S, Kaya M, Yamashita T, Matsumoto H. Femoral head osteonecrosis can be caused by disruption of the systemic immune response via the toll-like receptor 4 signalling pathway. Rheumatology. 2009;48(3):227-32.

213. Chua W, Clarke SJ, Charles KA. Systemic inflammation and prediction of chemotherapy outcomes in patients receiving docetaxel for advanced cancer. Supportive Care in Cancer. 2012;20(8):1869-74.

214. Arshad S, Sharif M, Naseer A. A Mini Review on Cancer and Anticancer Drugs. Indo American Journal of Pharmaceutical Sciences. 2016;3(11):1383-8.

215. Vane J, Botting R. Anti-inflammatory drugs and their mechanism of action. Inflammation Research. 1998;47(2):78-87.

216. Flower RJ. Drugs which inhibit prostaglandin biosynthesis. Pharmacological Reviews. 1974;26(1):33-67.

217. Churchill L, Graham A, Shih C, Pauletti D, Farina P, Grob P. Selective inhibition of human cyclo-oxygenase-2 by meloxicam. Inflammopharmacology. 1996;4(2):125-35.

218. Thun MJ, Namboodiri MM, Heath Jr CW. Aspirin use and reduced risk of fatal colon cancer. New England Journal of Medicine. 1991;325(23):1593-6.

219. Luk G. Prevention of gastrointestinal cancer--the potential role of NSAIDs in colorectal cancer. Schweizerische Medizinische Wochenschrift. 1996;126(19):801-12.

220. Zhou W, Hashimoto K, Goleniewska K, O'Neal JF, Ji S, Blackwell TS, et al. Prostaglandin I2 analogs inhibit proinflammatory cytokine production and T cell stimulatory function of dendritic cells. The Journal of Immunology. 2007;178(2):702-10.

221. Proudfoot AE, Power CA, Schwarz MK. Anti-chemokine small molecule drugs: a promising future? Expert opinion on investigational drugs. 2010;19(3):345-55.

222. Marin V, Farnarier C, Gres S, Kaplanski S, Su MS-S, Dinarello CA, et al. The p38 mitogen-activated protein kinase pathway plays a critical role in thrombin-induced endothelial chemokine production and leukocyte recruitment. Blood, The Journal of the American Society of Hematology. 2001;98(3):667-73.

223. Huang Y, Qiao F, Atkinson C, Holers VM, Tomlinson S. A novel targeted inhibitor of the alternative pathway of complement and its therapeutic application in ischemia/reperfusion injury. The Journal of Immunology. 2008;181(11):8068-76.

224. Jin Y, Arita M, Zhang Q, Saban DR, Chauhan SK, Chiang N, et al. Anti-angiogenesis effect of the novel anti-inflammatory and pro-resolving lipid mediators. Investigative ophthalmology & visual science. 2009;50(10):4743-52.

225. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, Van Der Meer JW, et al. The effect of dietary supplementation with n—3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. New England Journal of Medicine. 1989;320(5):265-71.

226. Dasu MR, Park S, Devaraj S, Jialal I. Pioglitazone inhibits Toll-like receptor expression and activity in human monocytes and db/db mice. Endocrinology. 2009;150(8):3457-64.

227. Stafylas PC, Sarafidis PA, Lasaridis AN. The controversial effects of thiazolidinediones on cardiovascular morbidity and mortality. International journal of cardiology. 2009;131(3):298-304.

228. Tyler VE. Herbal medicine: from the past to the future. Public health nutrition. 2000;3(4a):447-52.

229. Chin Y-W, Balunas MJ, Chai HB, Kinghorn AD. Drug discovery from natural sources. The AAPS journal. 2006;8(2):E239-E53.

230. Paterson I, Anderson EA. The renaissance of natural products as drug candidates. Science. 2005;310(5747):451-3.

231. Bagul M, Srinivasa H, Kanaki N, Rajani M. Antiinflammatory activity of two Ayurvedic formulations containing guggul. Indian journal of pharmacology. 2005;37(6):399.

232. Gautam R, Jachak SM. Recent developments in anti-inflammatory natural products. Medicinal research reviews. 2009;29(5):767-820.

233. Schink A, Neumann J, Leifke AL, Ziegler K, Fröhlich-Nowoisky J, Cremer C, et al. Screening of herbal extracts for TLR2-and TLR4-dependent anti-inflammatory effects. PloS one. 2018;13(10).

234. Quideau S, Deffieux D, Douat-Casassus C, Pouységu L. Plant polyphenols: chemical properties, biological activities, and synthesis. Angewandte Chemie International Edition. 2011;50(3):586-621.

235. Mohammed MS, Osman WJ, Garelnabi EA, Osman Z, Osman B, Khalid HS, et al. Secondary metabolites as anti-inflammatory agents. J Phytopharmacol. 2014;3(4):275-85.

236. Fawole O, Ndhlala A, Amoo S, Finnie J, Van Staden J. Anti-inflammatory and phytochemical properties of twelve medicinal plants used for treating gastro-intestinal ailments in South Africa. Journal of ethnopharmacology. 2009;123(2):237-43.

237. Guabiraba R, Campanha-Rodrigues AL, Souza AL, Santiago HC, Lugnier C, Alvarez-Leite J, et al. The flavonoid dioclein reduces the production of pro-inflammatory mediators in vitro by inhibiting PDE4 activity and scavenging reactive oxygen species. European journal of pharmacology. 2010;633(1-3):85-92.

238. Bischoff KL. Glucosinolates. Nutraceuticals: Elsevier; 2016. p. 551-4.

239. Barba FJ, Nikmaram N, Roohinejad S, Khelfa A, Zhu Z, Koubaa M. Bioavailability of glucosinolates and their breakdown products: Impact of processing. Frontiers in nutrition. 2016;3:24.

240. Kang K-H, Kong C-S, Seo Y, Kim M-M, Kim S-K. Anti-inflammatory effect of coumarins isolated from Corydalis heterocarpa in HT-29 human colon carcinoma cells. Food and chemical toxicology. 2009;47(8):2129-34.

241. Silva J, Abebe W, Sousa S, Duarte V, Machado M, Matos F. Analgesic and anti-inflammatory effects of essential oils of Eucalyptus. Journal of ethnopharmacology. 2003;89(2-3):277-83.

242. Al-Reza SM, Yoon JI, Kim HJ, Kim J-S, Kang SC. Anti-inflammatory activity of seed essential oil from Zizyphus jujuba. Food and chemical toxicology. 2010;48(2):639-43.

243. Yamada M, Ichikawa T, Ii M, Sunamoto M, Itoh K, Tamura N, et al. Discovery of novel and potent small-molecule inhibitors of NO and cytokine production as antisepsis agents:

synthesis and biological activity of alkyl 6-(N-substituted sulfamoyl) cyclohex-1-ene-1-carboxylate. Journal of medicinal chemistry. 2005;48(23):7457-67.

244. Youn HS, Lee JY, Saitoh SI, Miyake K, Kang KW, Choi YJ, et al. Suppression of MyD88- and TRIF-dependent signaling pathways of Toll-like receptor by (−)-epigallocatechin-3-gallate, a polyphenol component of green tea. Biochemical pharmacology. 2006;72(7):850-9.

245. Park S-J, Lee M-Y, Son B-S, Youn H-S. TBK1-targeted suppression of TRIF-dependent signaling pathway of Toll-like receptors by 6-shogaol, an active component of ginger. Bioscience, biotechnology, and biochemistry. 2009;73(7):1474-8.

246. Tidswell M, Tillis W, LaRosa SP, Lynn M, Wittek AE, Kao R, et al. Phase 2 trial of eritoran tetrasodium (E5564), a toll-like receptor 4 antagonist, in patients with severe sepsis. Critical care medicine. 2010;38(1):72-83.

247. Peri F, Calabrese V. Toll-like receptor 4 (TLR4) modulation by synthetic and natural compounds: an update: miniperspective. Journal of medicinal chemistry. 2014;57(9):3612-22.

248. Houghton CA. Sulforaphane: Its "Coming of Age" as a Clinically Relevant Nutraceutical in the Prevention and Treatment of Chronic Disease. Oxidative medicine and cellular longevity. 2019;2019.

249. Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. Anticancer research. 2003;23(1/A):363-98.

250. Menon VP, Sudheer AR. Antioxidant and anti-inflammatory properties of curcumin. The molecular targets and therapeutic uses of curcumin in health and disease: Springer; 2007. p. 105-25.

251. Gupta SC, Patchva S, Aggarwal BB. Therapeutic roles of curcumin: lessons learned from clinical trials. The AAPS journal. 2013;15(1):195-218.

252. Perez G. Anti-inflammatory activity of compounds isolated from plants. The Scientific World Journal. 2001;1:713-84.

253. Shao-Ling W, Ying L, Ying W, Yan-Feng C, Li-Xin N, Song-Tao L, et al. Curcumin, a potential inhibitor of up-regulation of TNF-alpha and IL-6 induced by palmitate in 3T3-L1 adipocytes through NF-kappaB and JNK pathway. Biomedical and Environmental Sciences. 2009;22(1):32-9.

254. Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. The Journal of Immunology. 2003;171(7):3863-71.

255. Hussain AR, Ahmed M, Al-Jomah NA, Khan AS, Manogaran P, Sultana M, et al. Curcumin suppresses constitutive activation of nuclear factor-κB and requires functional Bax to induce apoptosis in Burkitt's lymphoma cell lines. Molecular cancer therapeutics. 2008;7(10):3318-29.

256. Jurenka JS. Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research. Alternative medicine review. 2009;14(2).

257. Fürst R, Zündorf I. Plant-derived anti-inflammatory compounds: hopes and disappointments regarding the translation of preclinical knowledge into clinical progress. Mediators of inflammation. 2014;2014.

258. Aggarwal BB, Harikumar KB. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. The international journal of biochemistry & cell biology. 2009;41(1):40-59.

259. Lichtenstein GR. Medical therapy of ulcerative colitis: Springer; 2014.

260. Rahmani AH, Alsahli MA, Aly SM, Khan MA, Aldebasi YH. Role of curcumin in disease prevention and treatment. Advanced biomedical research. 2018;7(38).

261. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Molecular pharmaceutics. 2007;4(6):807-18.

262. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas P. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta medica. 1998;64(04):353-6.

263. Basnet P, Skalko-Basnet N. Curcumin: an anti-inflammatory molecule from a curry spice on the path to cancer treatment. Molecules. 2011;16(6):4567-98.

264. IBRAHIM R, BARRON D. Phenylpropanoids. Methods in plant biochemistry. 1: Elsevier; 1989. p. 75-111.

265. Sahebkar A, Serban M-C, Ursoniu S, Banach M. Effect of curcuminoids on oxidative stress: A systematic review and meta-analysis of randomized controlled trials. Journal of functional foods. 2015;18:898-909.

266. Banach M, Serban C, Aronow WS, Rysz J, Dragan S, Lerma EV, et al. Lipid, blood pressure and kidney update 2013. International urology and nephrology. 2014;46(5):947-61.

267. Panahi Y, Alishiri GH, Parvin S, Sahebkar A. Mitigation of systemic oxidative stress by curcuminoids in osteoarthritis: results of a randomized controlled trial. Journal of dietary supplements. 2016;13(2):209-20.

268. Priyadarsini KI, Maity DK, Naik G, Kumar MS, Unnikrishnan M, Satav J, et al. Role of phenolic OH and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. Free Radical Biology and Medicine. 2003;35(5):475-84.

269. Dutartre P. Inflammasomes and natural ingredients towards new anti-inflammatory agents. Molecules. 2016;21(11):1492.

270. Lubbad A, Oriowo M, Khan I. Curcumin attenuates inflammation through inhibition of TLR-4 receptor in experimental colitis. Molecular and cellular biochemistry. 2009;322(1-2):127-35.

271. Guimarães MR, Leite FRM, Spolidorio LC, Kirkwood KL, Rossa Jr C. Curcumin abrogates LPS-induced pro-inflammatory cytokines in RAW 264.7 macrophages. Evidence for novel mechanisms involving SOCS-1,-3 and p38 MAPK. Archives of oral biology. 2013;58(10):1309-17.

272. Panahi Y, Hosseini MS, Khalili N, Naimi E, Simental-Mendía LE, Majeed M, et al. Effects of curcumin on serum cytokine concentrations in subjects with metabolic syndrome: A post-hoc analysis of a randomized controlled trial. Biomedicine & pharmacotherapy. 2016;82:578-82.

273. Shah VO, Ferguson JE, Hunsaker LA, Deck LM, Vander Jagt DL. Natural products inhibit LPS-induced activation of pro-inflammatory cytokines in peripheral blood mononuclear cells. Natural product research. 2010;24(12):1177-88.

274. Kim G-Y, Kim K-H, Lee S-H, Yoon M-S, Lee H-J, Moon D-O, et al. Curcumin inhibits immunostimulatory function of dendritic cells: MAPKs and translocation of NF-κB as potential targets. The Journal of Immunology. 2005;174(12):8116-24.

275. Gradišar H, Keber MM, Pristovšek P, Jerala R. MD-2 as the target of curcumin in the inhibition of response to LPS. Journal of leukocyte biology. 2007;82(4):968-74.

276. Youn HS, Saitoh SI, Miyake K, Hwang DH. Inhibition of homodimerization of Toll-like receptor 4 by curcumin. Biochemical pharmacology. 2006;72(1):62-9.

277. Zhou Y, Zhang T, Wang X, Wei X, Chen Y, Guo L, et al. Curcumin modulates macrophage polarization through the inhibition of the toll-like receptor 4 expression and its signaling pathways. Cellular Physiology and Biochemistry. 2015;36(2):631-41.

278. Tu C-t, Han B, Yao Q-y, Zhang Y-a, Liu H-c, Zhang S-c. Curcumin attenuates Concanavalin A-induced liver injury in mice by inhibition of Toll-like receptor (TLR) 2, TLR4 and TLR9 expression. International immunopharmacology. 2012;12(1):151-7.

279. Kumar A, Dhawan S, Hardegen NJ, Aggarwal BB. Curcumin (diferuloylmethane) inhibition of tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppression of cell surface expression of adhesion molecules and of nuclear factor-κB activation. Biochemical pharmacology. 1998;55(6):775-83.

280. Tili E, Chiabai M, Palmieri D, Brown M, Cui R, Fernandes C, et al. Quaking and miR-155 interactions in inflammation and leukemogenesis. Oncotarget. 2015;6(28):24599.

281. Ma F, Liu F, Ding L, You M, Yue H, Zhou Y, et al. Anti-inflammatory effects of curcumin are associated with down regulating microRNA-155 in LPS-treated macrophages and mice. Pharmaceutical biology. 2017;55(1):1263-73.

282. Miller J, Thompson J, MacPherson M, Beuschel S, Westbom C, Sayan M, et al. Curcumin: a double hit on malignant mesothelioma. Cancer Prev Res (Phila) 2014; 7: 330–340. doi: 10.1158/1940-6207. CAPR-13-0259.[PMC free article][PubMed][Cross Ref].

283. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. Nature Reviews Microbiology. 2009;7(2):99-109.

284. Elmali N, Baysal O, Harma A, Esenkaya I, Mizrak B. Effects of resveratrol in inflammatory arthritis. Inflammation. 2007;30(1-2):1-6.

285. Hollman PC, Feskens EJ, Katan MB. Tea flavonols in cardiovascular disease and cancer epidemiology. Proceedings of the Society for Experimental Biology and Medicine. 1999;220(4):198-202.

286. Jancśo G, Király E, Jancsó-Gábor A. Direct evidence for an axonal site of action of capsaicin. Naunyn-Schmiedeberg's archives of pharmacology. 1980;313(1):91-4.

287. Malmstrom K, Daniels S, Kotey P, Seidenberg BC, Desjardins PJ. Comparison of rofecoxib and celecoxib, two cyclooxygenase-2 inhibitors, in postoperative dental pain: a randomized, placebo-and active-comparator-controlled clinical trial. Clinical therapeutics. 1999;21(10):1653-63.

288. Maroon JC, Bost JW. ω-3 Fatty acids (fish oil) as an anti-inflammatory: an alternative to nonsteroidal anti-inflammatory drugs for discogenic pain. Surgical neurology. 2006;65(4):326-31.

289. Stanbury RM, Graham EM. Systemic corticosteroid therapy—side effects and their management. British Journal of Ophthalmology. 1998;82(6):704-8.

290. Sumpio BE, Cordova AC, Berke-Schlessel DW, Qin F, Chen QH. Green tea, the "Asian paradox," and cardiovascular disease. Journal of the American College of Surgeons. 2006;202(5):813-25.

291. Amri A, Chaumeil J, Sfar S, Charrueau C. Administration of resveratrol: what formulation solutions to bioavailability limitations? Journal of controlled release. 2012;158(2):182-93.

292. Augustin MA, Sanguansri L, Lockett T. Nano-and micro-encapsulated systems for enhancing the delivery of resveratrol. Annals of the New York Academy of Sciences. 2013;1290(1):107-12.

293. R Neves A, Lucio M, LC Lima J, Reis S. Resveratrol in medicinal chemistry: a critical review of its pharmacokinetics, drug-delivery, and membrane interactions. Current medicinal chemistry. 2012;19(11):1663-81.

294. Santos AC, Veiga F, Ribeiro AJ. New delivery systems to improve the bioavailability of resveratrol. Expert Opinion on Drug Delivery. 2011;8(8):973-90.

295. Walle T. Bioavailability of resveratrol. Annals of the new York Academy of Sciences. 2011;1215(1):9-15.

296. Wenzel E, Somoza V. Metabolism and bioavailability of trans-resveratrol. Molecular nutrition & food research. 2005;49(5):472-81.

297. Singh AP, Singh R, Verma SS, Rai V, Kaschula CH, Maiti P, et al. Health benefits of resveratrol: Evidence from clinical studies. Medicinal research reviews. 2019;39(5):1851-91.

298. Bakker GC, Van Erk MJ, Pellis L, Wopereis S, Rubingh CM, Cnubben NH, et al. An antiinflammatory dietary mix modulates inflammation and oxidative and metabolic stress in overweight men: a nutrigenomics approach. The American journal of clinical nutrition. 2010;91(4):1044-59.

299. Bo S, Ciccone G, Castiglione A, Gambino R, De Michieli F, Villois P, et al. Anti-inflammatory and antioxidant effects of resveratrol in healthy smokers a randomized, double-blind, placebo-controlled, cross-over trial. Current medicinal chemistry. 2013;20(10):1323-31.

300. Militaru C, Donoiu I, Craciun A, Scorei ID, Bulearca AM, Scorei RI. Oral resveratrol and calcium fructoborate supplementation in subjects with stable angina pectoris: effects on lipid profiles, inflammation markers, and quality of life. Nutrition. 2013;29(1):178-83.

301. Tomé-Carneiro J, Gonzálvez M, Larrosa M, Yáñez-Gascón MJ, García-Almagro FJ, Ruiz-Ros JA, et al. One-year consumption of a grape nutraceutical containing resveratrol improves the inflammatory and fibrinolytic status of patients in primary prevention of cardiovascular disease. The American journal of cardiology. 2012;110(3):356-63.

302. Tomé-Carneiro J, Gonzálvez M, Larrosa M, Yáñez-Gascón MJ, García-Almagro FJ, Ruiz-Ros JA, et al. Grape resveratrol increases serum adiponectin and downregulates inflammatory genes in peripheral blood mononuclear cells: a triple-blind, placebo-controlled, one-year clinical trial in patients with stable coronary artery disease. Cardiovascular Drugs and Therapy. 2013;27(1):37-48.

303. Tomé-Carneiro J, Larrosa M, Yáñez-Gascón MJ, Dávalos A, Gil-Zamorano J, Gonzálvez M, et al. One-year supplementation with a grape extract containing resveratrol modulates inflammatory-related microRNAs and cytokines expression in peripheral blood mononuclear cells of type 2 diabetes and hypertensive patients with coronary artery disease. Pharmacological research. 2013;72:69-82.

304. Tili E, Michaille J-J. Resveratrol, microRNAs, inflammation, and cancer. Journal of nucleic acids. 2011;2011.

305. Yang Y, Li S, Yang Q, Shi Y, Zheng M, Liu Y, et al. Resveratrol reduces the proinflammatory effects and lipopolysaccharide-induced expression of HMGB1 and TLR4 in RAW264. 7 cells. Cellular Physiology and Biochemistry. 2014;33(5):1283-92.

306. Tong W, Chen X, Song X, Chen Y, Jia R, Zou Y, et al. Resveratrol inhibits LPS-induced inflammation through suppressing the signaling cascades of TLR4-NF-κB/MAPKs/IRF3. Experimental and Therapeutic Medicine. 2020;19(3):1824-34.

307. Alarcon De La Lastra C, Villegas I. Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. Molecular nutrition & food research. 2005;49(5):405-30.

308. Kundu JK, Shin YK, Surh Y-J. Resveratrol modulates phorbol ester-induced pro-inflammatory signal transduction pathways in mouse skin in vivo: NF-κB and AP-1 as prime targets. Biochemical pharmacology. 2006;72(11):1506-15.

309. Surh Y-J, Chun K-S, Cha H-H, Han SS, Keum Y-S, Park K-K, et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2001;480:243-68.

310. Huang T-T, Lai H-C, Chen Y-B, Chen L-G, Wu Y-H, Ko Y-F, et al. cis-Resveratrol produces anti-inflammatory effects by inhibiting canonical and non-canonical inflammasomes in macrophages. Innate immunity. 2014;20(7):735-50.

311. Karunaweera N, Raju R, Gyengesi E, Münch G. Plant polyphenols as inhibitors of NF-κB induced cytokine production—a potential anti-inflammatory treatment for Alzheimer's disease? Frontiers in molecular neuroscience. 2015;8:24.

312. Qureshi AA, Guan XQ, Reis JC, Papasian CJ, Jabre S, Morrison DC, et al. Inhibition of nitric oxide and inflammatory cytokines in LPS-stimulated murine macrophages by resveratrol, a potent proteasome inhibitor. Lipids in health and disease. 2012;11(1):76.

313. Li J, Xie C, Zhuang J, Li H, Yao Y, Shao C, et al. Resveratrol attenuates inflammation in the rat heart subjected to ischemia-reperfusion: Role of the TLR4/NF-κB signaling pathway. Molecular medicine reports. 2015;11(2):1120-6.

314. Arafa MH, Mohammad NS, Atteia HH, Abd-Elaziz HR. Protective effect of resveratrol against doxorubicin-induced cardiac toxicity and fibrosis in male experimental rats. Journal of physiology and biochemistry. 2014;70(3):701-11.

315. Tili E, Croce CM, Michaille J-J. miR-155: on the crosstalk between inflammation and cancer. International reviews of immunology. 2009;28(5):264-84.

316. Lin S-L, Chiang A, Chang D, Ying S-Y. Loss of mir-146a function in hormone-refractory prostate cancer. Rna. 2008;14(3):417-24.

317. Kim JK, Park SU. Current potential health benefits of sulforaphane. Experimental and Clinical Sciences. 2016;15:571-7.

318. Briones-Herrera A, Eugenio-Pérez D, Reyes-Ocampo JG, Rivera-Mancía S, Pedraza-Chaverri J. New highlights on the health-improving effects of sulforaphane. Food & function. 2018;9(5):2589-606.

319. Fahey JW, Holtzclaw WD, Wehage SL, Wade KL, Stephenson KK, Talalay P. Sulforaphane bioavailability from glucoraphanin-rich broccoli: Control by active endogenous myrosinase. PLoS One. 2015;10(11).

320. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry. 2001;56(1):5-51.

321. Sehrawat A, Roy R, Pore SK, Hahm E-R, Samanta SK, Singh KB, et al. Mitochondrial dysfunction in cancer chemoprevention by phytochemicals from dietary and medicinal plants. Seminars in cancer biology. 2017;47:147-53.

322. Mazarakis N, Snibson K, Licciardi PV, Karagiannis TC. The potential use of l-sulforaphane for the treatment of chronic inflammatory diseases: a review of the clinical evidence. Clinical Nutrition. 2020;39(3):664-75.

323. Hanlon N, Coldham N, Gielbert A, Kuhnert N, Sauer MJ, King LJ, et al. Absolute bioavailability and dose-dependent pharmacokinetic behaviour of dietary doses of the chemopreventive isothiocyanate sulforaphane in rat. British journal of nutrition. 2008;99(3):559-64.

324. Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. The American journal of clinical nutrition. 2005;81(1):230S-42S.

325. Cornblatt BS, Ye L, Dinkova-Kostova AT, Erb M, Fahey JW, Singh NK, et al. Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. Carcinogenesis. 2007;28(7):1485-90.

326. Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, Talalay P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clinica chimica acta. 2002;316(1-2):43-53.

327. Singh K, Connors SL, Macklin EA, Smith KD, Fahey JW, Talalay P, et al. Sulforaphane treatment of autism spectrum disorder (ASD). Proceedings of the National Academy of Sciences. 2014;111(43):15550-5.

328. Johansson NL, Pavia CS, Chiao JW. Growth inhibition of a spectrum of bacterial and fungal pathogens by sulforaphane, an isothiocyanate product found in broccoli and other cruciferous vegetables. Planta medica. 2008;74(07):747-50.

329. Fahey JW, Talalay P. Antioxidant functions of sulforaphane: a potent inducer of Phase II detoxication enzymes. Food and Chemical Toxicology. 1999;37(9-10):973-9.

330. Greaney AJ, Maier NK, Leppla SH, Moayeri M. Sulforaphane inhibits multiple inflammasomes through an Nrf2-independent mechanism. Journal of leukocyte biology. 2016;99(1):189-99.

331. Amjad AI, Parikh RA, Appleman LJ, Hahm E-R, Singh K, Singh SV. Broccoli-derived sulforaphane and chemoprevention of prostate cancer: from bench to bedside. Current pharmacology reports. 2015;1(6):382-90.

332. Sikdar S, Papadopoulou M, Dubois J. What do we know about sulforaphane protection against photoaging? Journal of cosmetic dermatology. 2016;15(1):72-7.

333. Yang L, Palliyaguru DL, Kensler TW. Frugal chemoprevention: targeting Nrf2 with foods rich in sulforaphane. Seminars in oncology. 2016;43(1):146-53.

334. Lee J-H, Moon M-H, Jeong J-K, Park Y-G, Lee Y-J, Seol J-W, et al. Sulforaphane induced adipolysis via hormone sensitive lipase activation, regulated by AMPK signaling pathway. Biochemical and biophysical research communications. 2012;426(4):492-7.

335. Pal S, Konkimalla VB. Sulforaphane regulates phenotypic and functional switching of both induced and spontaneously differentiating human monocytes. International immunopharmacology. 2016;35:85-98.

336. Zhao Z, Liao G, Zhou Q, Lv D, Holthfer H, Zou H. Sulforaphane attenuates contrast-induced nephropathy in rats via Nrf2/HO-1 pathway. Oxidative medicine and cellular longevity. 2016;2016.

337. Townsend BE, Johnson RW. Sulforaphane induces Nrf2 target genes and attenuates inflammatory gene expression in microglia from brain of young adult and aged mice. Experimental gerontology. 2016;73:42-8.

338. Wu J, Han J, Hou B, Deng C, Wu H, Shen L. Sulforaphane inhibits TGF-β-induced epithelial-mesenchymal transition of hepatocellular carcinoma cells via the reactive oxygen species-dependent pathway. Oncology reports. 2016;35(5):2977-83.

339. Jiang L-L, Zhou S-J, Zhang X-M, Chen H-Q, Liu W. Sulforaphane suppresses in vitro and in vivo lung tumorigenesis through downregulation of HDAC activity. Biomedicine & Pharmacotherapy. 2016;78:74-80.

340. Li B, Tian S, Liu X, He C, Ding Z, Shan Y. Sulforaphane protected the injury of human vascular endothelial cell induced by LPC through up-regulating endogenous antioxidants and phase II enzymes. Food & function. 2015;6(6):1984-91.

341. Li Y-p, Wang S-l, Liu B, Tang L, Kuang R-r, Wang X-b, et al. Sulforaphane prevents rat cardiomyocytes from hypoxia/reoxygenation injury in vitro via activating SIRT1 and subsequently inhibiting ER stress. Acta Pharmacologica Sinica. 2016;37(3):344-53.

342. Shehatou GS, Suddek GM. Sulforaphane attenuates the development of atherosclerosis and improves endothelial dysfunction in hypercholesterolemic rabbits. Experimental Biology and Medicine. 2016;241(4):426-36.

343. Kee HJ, Kim GR, Kim IK, Jeong MH. Sulforaphane suppresses cardiac hypertrophy by inhibiting GATA4/GATA6 expression and MAPK signaling pathways. Molecular nutrition & food research. 2015;59(2):221-30.

344. Ma L-L, Xing G-P, Yu Y, Liang H, Yu T-X, Zheng W-H, et al. Sulforaphane exerts neuroprotective effects via suppression of the inflammatory response in a rat model of focal cerebral ischemia. International journal of clinical and experimental medicine. 2015;8(10):17811.

345. Nguyen B, Luong L, Naase H, Vives M, Jakaj G, Finch J, et al. Sulforaphane pretreatment prevents systemic inflammation and renal injury in response to cardiopulmonary bypass. The Journal of thoracic and cardiovascular surgery. 2014;148(2):690-7.

346. Sayed RH, Khalil WK, Salem HA, El-Sayeh BM. Sulforaphane increases the survival rate in rats with fulminant hepatic failure induced by D-galactosamine and lipopolysaccharide. Nutrition research. 2014;34(11):982-9.

347. Wardyn JD, Ponsford AH, Sanderson CM. Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. Biochemical society transactions. 2015;43(4):621-6.

348. Gaona-Gaona L, Molina-Jijón E, Tapia E, Zazueta C, Hernández-Pando R, Calderón-Oliver M, et al. Protective effect of sulforaphane pretreatment against cisplatin-induced liver and mitochondrial oxidant damage in rats. Toxicology. 2011;286(1-3):20-7.

349. Mein JR, James DR, Lakkanna S. Induction of phase 2 antioxidant enzymes by broccoli sulforaphane: perspectives in maintaining the antioxidant activity of vitamins A, C, and E. Frontiers in genetics. 2012;3:7.

350. Riedl MA, Saxon A, Diaz-Sanchez D. Oral sulforaphane increases Phase II antioxidant enzymes in the human upper airway. Clinical immunology. 2009;130(3):244-51.

351. RK T. Mai KH. Srisuma S. Kensler TW. Yamamoto M. Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res. 2002;62:5196-203.

352. Guerrero-Beltrán CE, Calderón-Oliver M, Pedraza-Chaverri J, Chirino YI. Protective effect of sulforaphane against oxidative stress: recent advances. Experimental and Toxicologic Pathology. 2012;64(5):503-8.

353. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proceedings of the National Academy of Sciences. 2002;99(18):11908-13.

354. Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J, et al. Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. Journal of Biological Chemistry. 1999;274(39):27545-52.

355. Hu R, Hebbar V, Kim B-R, Chen C, Winnik B, Buckley B, et al. In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. Journal of Pharmacology and Experimental Therapeutics. 2004;310(1):263-71.

356. Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. Pharmacological research. 2008;58(5-6):262-70.

357. Philbrook NA, Winn LM. Sub-chronic sulforaphane exposure in CD-1 pregnant mice enhances maternal NADPH quinone oxidoreductase 1 (NQO1) activity and mRNA expression of NQO1, glutathione S-transferase, and glutamate-cysteine ligase: potential implications for fetal protection against toxicant exposure. Reproductive Toxicology. 2014;43:30-7.

358. An YW, Jhang KA, Woo S-Y, Kang JL, Chong YH. Sulforaphane exerts its anti-inflammatory effect against amyloid-β peptide via STAT-1 dephosphorylation and activation of Nrf2/HO-1 cascade in human THP-1 macrophages. Neurobiology of aging. 2016;38:1-10.

359. Pan H, He M, Liu R, Brecha NC, Yu ACH, Pu M. Sulforaphane protects rodent retinas against ischemia-reperfusion injury through the activation of the Nrf2/HO-1 antioxidant pathway. PloS one. 2014;9(12).

360. Lee C, Park GH, Lee S-R, Jang J-H. Attenuation of-amyloid-induced oxidative cell death by sulforaphane via activation of NF-E2-related factor 2. Oxidative medicine and cellular longevity. 2013;2013.

361. Zhang Y, Tang L. Discovery and development of sulforaphane as a cancer chemopreventive phytochemical 1. Acta Pharmacologica Sinica. 2007;28(9):1343-54.

362. Myzak MC, Dashwood WM, Orner GA, Ho E, Dashwood RH. Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc min mice. The FASEB journal. 2006;20(3):506-8.

363. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhäuser C. Nuclear factor κB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. Journal of Biological Chemistry. 2001;276(34):32008-15.

364. Killeen ME, Englert JA, Stolz DB, Song M, Han Y, Delude RL, et al. The phase 2 enzyme inducers ethacrynic acid, DL-sulforaphane, and oltipraz inhibit lipopolysaccharide-induced high-mobility group box 1 secretion by RAW 264.7 cells. Journal of Pharmacology and Experimental Therapeutics. 2006;316(3):1070-9.

365. Wagner AE, Boesch-Saadatmandi C, Dose J, Schultheiss G, Rimbach G. Anti-inflammatory potential of allyl-isothiocyanate–role of Nrf2, NF-κB and microRNA-155. Journal of cellular and molecular medicine. 2012;16(4):836-43.

366. Checker R, Gambhir L, Thoh M, Sharma D, Sandur SK. Sulforaphane, a naturally occurring isothiocyanate, exhibits anti-inflammatory effects by targeting GSK3β/Nrf-2 and NF-κB pathways in T cells. Journal of Functional Foods. 2015;19:426-38.

367. Youn HS, Kim YS, Park ZY, Kim SY, Choi NY, Joung SM, et al. Sulforaphane suppresses oligomerization of TLR4 in a thiol-dependent manner. The Journal of Immunology. 2010;184(1):411-9.

368. Eren E, Tufekci KU, Isci KB, Tastan B, Genc K, Genc S. Sulforaphane inhibits lipopolysaccharide-induced inflammation, cytotoxicity, oxidative stress, and miR-155 expression and switches to Mox phenotype through activating extracellular signal-regulated kinase 1/2–nuclear factor erythroid 2-related factor 2/antioxidant response element pathway in murine microglial cells. Frontiers in immunology. 2018;9:36.

369. Subedi L, Lee JH, Yumnam S, Ji E, Kim SY. Anti-inflammatory effect of sulforaphane on LPS-activated microglia potentially through JNK/AP-1/NF-κB inhibition and Nrf2/HO-1 activation. Cells. 2019;8(2):194.

370. Koo JE, Park Z-Y, Kim ND, Lee JY. Sulforaphane inhibits the engagement of LPS with TLR4/MD2 complex by preferential binding to Cys133 in MD2. Biochemical and biophysical research communications. 2013;434(3):600-5.

371. Velloso LA, Folli F, Saad MJ. TLR4 at the crossroads of nutrients, gut microbiota, and metabolic inflammation. Endocrine reviews. 2015;36(3):245-71.

372. Kim Y, Kim K, Park D, Lee E, Lee H, Lee Y-S, et al. Histone deacetylase 3 mediates allergic skin inflammation by regulating expression of MCP1 protein. Journal of Biological Chemistry. 2012;287(31):25844-59.

373. Li B, Cui W, Liu J, Li R, Liu Q, Xie X-H, et al. Sulforaphane ameliorates the development of experimental autoimmune encephalomyelitis by antagonizing oxidative stress and Th17-related inflammation in mice. Experimental neurology. 2013;250:239-49.

374. Li Y, Paonessa JD, Zhang Y. Mechanism of chemical activation of Nrf2. PloS one. 2012;7(4).

375. Brandenburg L-O, Kipp M, Lucius R, Pufe T, Wruck CJ. Sulforaphane suppresses LPS-induced inflammation in primary rat microglia. Inflammation Research. 2010;59(6):443-50.

376. Hung C-N, Huang H-P, Wang C-J, Liu K-L, Lii C-K. Sulforaphane inhibits TNF-α-induced adhesion molecule expression through the Rho A/ROCK/NF-κB signaling pathway. Journal of medicinal food. 2014;17(10):1095-102.

377. Sun C-C, Li S-J, Yang C-L, Xue R-L, Xi Y-Y, Wang L, et al. Sulforaphane attenuates muscle inflammation in dystrophin-deficient mdx mice via NF-E2-related factor 2 (Nrf2)-mediated inhibition of NF-κB signaling pathway. Journal of Biological Chemistry. 2015;290(29):17784-95.

378. Deramaudt TB, Ali M, Vinit S, Bonay M. Sulforaphane reduces intracellular survival of Staphylococcus aureus in macrophages through inhibition of JNK and p38 MAPK-induced inflammation. International Journal of Molecular Medicine.45(6):1927-41.

379. Myzak MC, Karplus PA, Chung F-L, Dashwood RH. A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. Cancer research. 2004;64(16):5767-74.

380. Steinkellner H, Rabot S, Freywald C, Nobis E, Scharf G, Chabicovsky M, et al. Effects of cruciferous vegetables and their constituents on drug metabolizing enzymes involved in the bioactivation of DNA-reactive dietary carcinogens. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2001;480:285-97.

381. Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. Carcinogenesis. 2006;27(4):811-9.

382. RAW 264.7 (ATCC® TIB-71™). Available from: https://www.lgcstandards-atcc.org/Products/All/TIB-71.aspx?geo_country=eg.

383. Chiou W-F, Chou C-J, Chen C-F. Camptothecin suppresses nitric oxide biosynthesis in RAW 264.7 macrophages. Life sciences. 2001;69(6):625-35.

384. Wang C, Levis GBS, Lee EB, Levis WR, Lee DW, Kim BS, et al. Platycodin D and D3 isolated from the root of Platycodon grandiflorum modulate the production of nitric oxide and secretion of TNF-α in activated RAW 264.7 cells. International immunopharmacology. 2004;4(8):1039-49.

385. Hassan F, Islam S, Mu MM, Ito H, Koide N, Mori I, et al. Lipopolysaccharide prevents doxorubicin-induced apoptosis in RAW 264.7 macrophage cells by inhibiting p53 activation. Mol Cancer Res. 2005;3(7):373-9.

386. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983;65(1-2):55-63.

387. Vishwakarma NK, Patel VK, Hira SK, Ramesh K, Srivastava P, Mitra K, et al. Tadpole-shaped β-cyclodextrin-tagged poly (N-vinylpyrrolidone): synthesis, characterization and studies of its complexation with phenolphthalein and anti tumor activities. RSC Advances. 2015;5(20):15547-58.

388. Griess Reagent Kit for Nitrite Determination (G-7921). Available from: http://tools.thermofisher.com/content/sfs/manuals/mp07921.pdf.

389. QIAzol™ Lysis Reagent. Available from: https://www.qiagen.com/us/resources/resourcedetail?id=61c3ddbd-69c1-4b68-ab89-a428f14a9245&lang=en.

390. Thermo Scientific RevertAid First Strand cDNA Synthesis Kit Available from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012716_RevertAid_FirstStrand_cDNA_Syn_K1622_UG.pdf.

391. miScript PCR System Handbook. Available from: https://www.qiagen.com/us/resources/resourcedetail?id=7954ef25-3a39-4b0a-a27e-42689dbb4f5f&lang=en.

392. Chaudhari U, Nemade H, Gaspar JA, Hescheler J, Hengstler JG, Sachinidis A. MicroRNAs as early toxicity signatures of doxorubicin in human-induced pluripotent stem cell-derived cardiomyocytes. Arch Toxicol. 2016;90(12):3087-98.

393. Nahid MA, Satoh M, Chan EK. Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. J Immunol. 2011;186(3):1723-34.

394. Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X). Available from: https://www.thermofisher.com/order/catalog/product/K0253#/K0253.

395. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001;25(4):402-8.

396. User Bulletin #2 ABI PRISM 7700 Sequence Detection System. Available from: http://tools.thermofisher.com/content/sfs/manuals/cms_040980.pdf.

397. Ching-Hsiang L, Chiao-Wen H, Nan-Fu C, Wen-Sheng L, Ya-Fen H, Wen-Tung W. In vivo effects of Ginkgo biloba extract on interleukin-6 cytokine levels in patients with neurological disorders. Indian journal of pharmacology. 2012;44(1):118.

398. TNF-a elisa kit. Available from: https://www.caymanchem.com/pdfs/500850.pdf.

399. Interleukin-6 (mouse) ELISA Kit. Available from: https://www.caymanchem.com/pdfs/583371.pdf.

400. Kirpich IA, Parajuli D, McClain CJ. The gut microbiome in NAFLD and ALD. Clinical liver disease. 2015;6(3):55.

401. Lavine KJ, Pinto AR, Epelman S, Kopecky BJ, Clemente-Casares X, Godwin J, et al. The macrophage in cardiac homeostasis and disease: JACC macrophage in CVD series (Part 4). Journal of the American College of Cardiology. 2018;72(18):2213-30.

402. Sauter KA, Wood LJ, Wong J, Iordanov M, Magun BE. Doxorubicin and daunorubicin induce processing and release of interleukin-1β through activation of the NLRP3 inflammasome: Progress at a snail's pace. Cancer biology & therapy. 2011;11(12):1008-16.

403. Kota RS, Rutledge JC, Gohil K, Kumar A, Enelow RI, Ramana CV. Regulation of gene expression in RAW 264.7 macrophage cell line by interferon-γ. Biochemical and biophysical research communications. 2006;342(4):1137-46.

404. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14-20.

405. Reis J, Guan XQ, Kisselev AF, Papasian CJ, Qureshi AA, Morrison DC, et al. LPS-induced formation of immunoproteasomes: TNF-α and nitric oxide production are regulated by altered composition of proteasome-active sites. Cell Biochemistry and Biophysics. 2011;60(1-2):77-88.

406. Shi H, Guo Y, Liu Y, Shi B, Guo X, Jin L, et al. The in vitro effect of lipopolysaccharide on proliferation, inflammatory factors and antioxidant enzyme activity in bovine mammary epithelial cells. Animal Nutrition. 2016;2(2):99-104.

407. Liu L, Lu Y, Martinez J, Bi Y, Lian G, Wang T, et al. Proinflammatory signal suppresses proliferation and shifts macrophage metabolism from Myc-dependent to HIF1α-dependent. Proceedings of the National Academy of Sciences. 2016;113(6):1564-9.

408. Fan K. Regulatory effects of lipopolysaccharide in murine macrophage proliferation. World journal of gastroenterology. 1998;4(2):137.

409. El Chartouni C, Rehli M. Comprehensive analysis of TLR4-induced transcriptional responses in interleukin 4-primed mouse macrophages. Immunobiology. 2010;215(9-10):780-7.

410. Tsatsanis C, Androulidaki A, Alissafi T, Charalampopoulos I, Dermitzaki E, Roger T, et al. Corticotropin-releasing factor and the urocortins induce the expression of TLR4 in macrophages via activation of the transcription factors PU. 1 and AP-1. The Journal of Immunology. 2006;176(3):1869-77.

411. Fang W, Bi D, Zheng R, Cai N, Xu H, Zhou R, et al. Identification and activation of TLR4-mediated signalling pathways by alginate-derived guluronate oligosaccharide in RAW264. 7 macrophages. Scientific reports. 2017;7(1):1-13.

412. Hausmann M, Kiessling S, Mestermann S, Webb G, Spöttl T, Andus T, et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology. 2002;122(7):1987-2000.

413. Matsuguchi T, Musikacharoen T, Ogawa T, Yoshikai Y. Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. The Journal of Immunology. 2000;165(10):5767-72.

414. Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, et al. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. The Journal of Immunology. 2000;164(7):3476-9.

415. Moreno C, Merino J, Vazquez B, Ramirez N, Echeverria A, Pastor F, et al. Anti-inflammatory cytokines induce lipopolysaccharide tolerance in human monocytes without modifying Toll-like receptor 4 membrane expression. Scandinavian journal of immunology. 2004;59(6):553-8.

416. Kong X-N, Yan H-X, Chen L, Dong L-W, Yang W, Liu Q, et al. LPS-induced down-regulation of signal regulatory protein α contributes to innate immune activation in macrophages. The Journal of experimental medicine. 2007;204(11):2719-31.

417. Shih YL, Wu LY, Lee CH, Chen YL, Hsueh SC, Lu HF, et al. Sulforaphane promotes immune responses in a WEHI-3-induced leukemia mouse model through enhanced phagocytosis of macrophages and natural killer cell activities in vivo. Molecular medicine reports. 2016;13(5):4023-9.

418. Sharma J, Al-Omran A, Parvathy S. Role of nitric oxide in inflammatory diseases. Inflammopharmacology. 2007;15(6):252-9.

419. Ziche M. Role of nitric oxide in the angiogenesis of avascular tissue. Osteoarthritis and cartilage. 1999;7(4):403-5.

420. Archer S. Measurement of nitric oxide in biological models. The FASEB journal. 1993;7(2):349-60.

421. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proceedings of the National Academy of Sciences. 1987;84(24):9265-9.

422. Tracey WR, Linden J, Peach MJ, Johns RA. Comparison of spectrophotometric and biological assays for nitric oxide (NO) and endothelium-derived relaxing factor (EDRF): nonspecificity of the diazotization reaction for NO and failure to detect EDRF. Journal of Pharmacology and Experimental Therapeutics. 1990;252(3):922-8.

423. Stuehr DJ, Kwon NS, Gross SS, Thiel BA, Levi R, Nathan CF. Synthesis of nitrogen oxides from L-arginine by macrophage cytosol: requirement for inducible and constitutive components. Biochemical and biophysical research communications. 1989;161(2):420-6.

424. Stuehr D, Nathan C. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. The Journal of experimental medicine. 1989;169(5):1543-55.

425. Aktan F. iNOS-mediated nitric oxide production and its regulation. Life sciences. 2004;75(6):639-53.

426. Soromou LW, Zhang Z, Li R, Chen N, Guo W, Huo M, et al. Regulation of inflammatory cytokines in lipopolysaccharide-stimulated RAW 264.7 murine macrophage by 7-O-methyl-naringenin. Molecules. 2012;17(3):3574-85.

427. Ruhee RT, Ma S, Suzuki K. Sulforaphane protects cells against lipopolysaccharide-stimulated inflammation in murine macrophages. Antioxidants. 2019;8(12):577.

428. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Frontiers in immunology. 2014;5:461.

429. Youn HS, Lee JY, Fitzgerald KA, Young HA, Akira S, Hwang DH. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. The Journal of Immunology. 2005;175(5):3339-46.

430. Chen D, Nie M, Fan M-w, Bian Z. Anti-inflammatory activity of curcumin in macrophages stimulated by lipopolysaccharides from Porphyromonas gingivalis. Pharmacology. 2008;82(4):264-9.

431. Nelson KM, Dahlin JL, Bisson J, Graham J, Pauli GF, Walters MA. The essential medicinal chemistry of curcumin: miniperspective. Journal of medicinal chemistry. 2017;60(5):1620-37.

432. Curtale G, Rubino M, Locati M. MicroRNAs as molecular switches in macrophage activation. Frontiers in immunology. 2019;10:799.

433. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature reviews genetics. 2008;9(2):102-14.

434. Bai X, Zhang J, Cao M, Han S, Liu Y, Wang K, et al. MicroRNA-146a protects against LPS-induced organ damage by inhibiting Notch1 in macrophage. International immunopharmacology. 2018;63:220-6.

435. Huang C, Liu X-j, Xie J, Ma T-t, Meng X-m, Li J. MiR-146a modulates macrophage polarization by inhibiting Notch1 pathway in RAW264. 7 macrophages. International immunopharmacology. 2016;32:46-54.

436. Bala S, Marcos M, Kodys K, Csak T, Catalano D, Mandrekar P, et al. Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor α (TNFα) production via increased mRNA half-life in alcoholic liver disease. Journal of Biological Chemistry. 2011;286(2):1436-44.

437. Pfeiffer D, Roßmanith E, Lang I, Falkenhagen D. miR-146a, miR-146b, and miR-155 increase expression of IL-6 and IL-8 and support HSP10 in an in vitro sepsis model. PLoS One. 2017;12(6).

438. Feng J, Li A, Deng J, Yang Y, Dang L, Ye Y, et al. miR-21 attenuates lipopolysaccharide-induced lipid accumulation and inflammatory response: potential role in cerebrovascular disease. Lipids in health and disease. 2014;13(1):27.

439. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. The Journal of Immunology. 2009;182(8):4994-5002.

440. Wang Z, Brandt S, Medeiros A, Wang S, Wu H, Dent A, et al. MicroRNA 21 is a homeostatic regulator of macrophage polarization and prevents prostaglandin E2-mediated M2 generation. PloS one. 2015;10(2).

441. Horie T, Ono K, Nishi H, Nagao K, Kinoshita M, Watanabe S, et al. Acute doxorubicin cardiotoxicity is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. Cardiovascular research. 2010;87(4):656-64.

442. Griss K, Bertrams W, Sittka-Stark A, Seidel K, Stielow C, Hippenstiel S, et al. MicroRNAs constitute a negative feedback loop in Streptococcus pneumoniae–induced macrophage activation. The Journal of infectious diseases. 2016;214(2):288-99.

443. Lin W, Wu RT, Wu T, Khor T-O, Wang H, Kong A-N. Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. Biochemical pharmacology. 2008;76(8):967-73.

444. Cho JH, Kim YW, Keum YS. Sulforaphane Suppresses LPS-Induced or TPA-Induced Downregulation of PDCD4 in RAW 264.7 Cells. Phytotherapy research. 2014;28(11):1606-11.