

Chromone derivatives as potential modulators of inflammatory response through their effects on human neutrophils function

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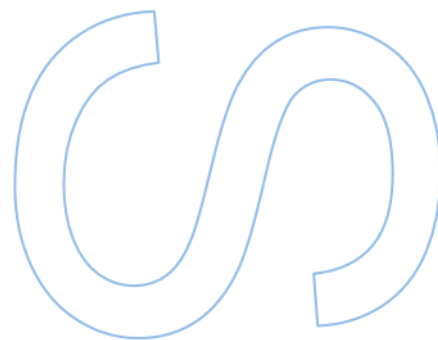
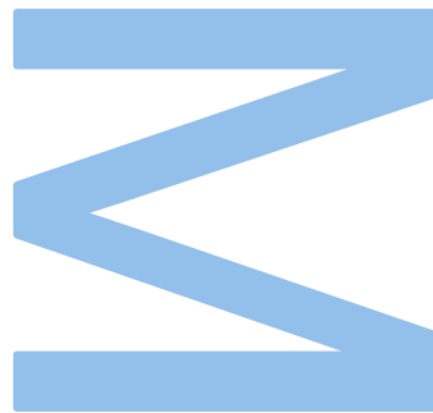
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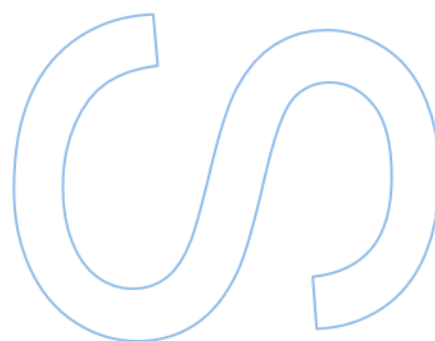
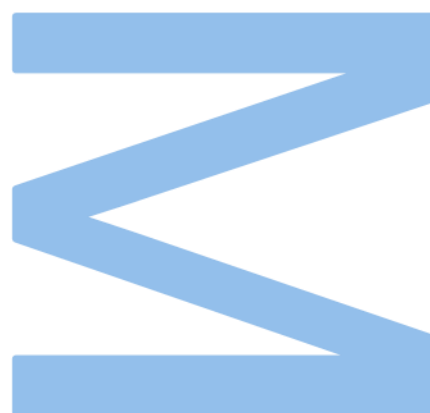
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Resumo

A inflamação é um processo fundamental para a manutenção da homeostasia do organismo, defendendo-o de agressões, quer seja por lesão ou infeção. Os neutrófilos são algumas das células efetoras mais importantes durante a inflamação, pois são as primeiras a serem mobilizadas e estão equipadas com vários mecanismos para eliminar células danificadas e patógenos invasores, como a geração e libertação de grandes quantidades de espécies reativas e armadilhas extracelulares de neutrófilos (NETs). Estes mecanismos, essenciais à resposta inflamatória, podem sair do controlo do organismo, resultando em condições de *stress*, devido a uma resposta desmedida e desequilibrada, levando a uma maior extensão dos danos sobre os tecidos e a uma potencial inflamação persistente. Destas condições exacerbadas, pode resultar o desenvolvimento de determinadas patologias, como é o caso da aterosclerose e da artrite reumatoide, entre outras. Assim, não sendo o organismo capaz de recuperar a homeostasia, será necessário apoio externo, nomeadamente através de fármacos anti-inflamatórios, que irão neutralizar as moléculas nocivas e regular a inflamação.

As calconas são derivados naturais de cromonas, estruturalmente relacionadas com os flavonoides, e possuem diversas atividades biológicas já descritas na literatura, tais como atividade antioxidante e anti-inflamatória. Esta proximidade com os flavonoides e a sua interessante estrutura química, compreendendo um núcleo facilmente substituível, têm despertado o interesse da comunidade científica para o desenvolvimento de fármacos capazes de modular a resposta inflamatória, permitindo um melhor resultado terapêutico. Assim, foi avaliado o potencial antioxidante e anti-inflamatório de sete calconas estruturalmente relacionadas. Para tal, foram realizados ensaios *in vitro* não celulares, avaliando a captação direta de espécies reativas fisiologicamente relevantes, como o peróxido de hidrogénio (H_2O_2), anião radical superóxido ($O_2^{\cdot-}$), ácido hipocloroso (HOCl) e radical óxido nítrico ($\cdot NO$), bem como ensaios *in vitro* celulares, avaliando-se a capacidade das calconas estudadas em modular a função de neutrófilos humanos durante a inflamação, nomeadamente os mecanismos de *burst* oxidativo e de produção de NETs. Sempre que possível, foram estabelecidas relações de estrutura-atividade das calconas estudadas, de modo a identificar possíveis características estruturais que favoreçam as atividades biológicas observadas.

Nos ensaios *in vitro* não celulares de captação de espécies reativas, as calconas C1 e C2 foram capazes de captar $O_2^{\cdot-}$, HOCl e $\cdot NO$, e as calconas C4, C5 e C6 foram capazes de captar HOCl. Estes resultados mostram a importância da presença de um grupo catecol no anel B (em C1 e C2) das calconas na captação das espécies reativas radicalares $O_2^{\cdot-}$ e $\cdot NO$. Além disso, a presença de grupos hidroxilo livres, principalmente no anel A, parece ser importante para a atividade de captação de HOCl. Foi também observada uma possível seletividade na atividade antioxidante das calconas C4, C5 e C6, uma vez que estas apenas foram capazes de neutralizar uma das quatro espécies reativas fisiologicamente relevantes em estudo, HOCl.

Nos ensaios *in vitro* celulares, as calconas C0, C1 e C2 foram capazes de inibir o *burst* oxidativo, quando avaliado com a sonda inespecífica luminol. As calconas C1, C2 e C6 foram capazes de inibir o *burst* oxidativo, quando avaliadas com a sonda específica 3'-(*p*-aminofenil) fluoresceína (APF), demonstrando uma capacidade modulatória das vias oxidativas associadas ao HOCl. Atendendo aos resultados dos ensaios não celulares, é possível afirmar que um dos mecanismos através dos quais as calconas ativas, C1 e C2, são capazes de modular o mecanismo de *burst* oxidativo, será através da captação direta das espécies reativas geradas após a estimulação dos neutrófilos. As calconas C1, C2 e C6 foram então escolhidas para avaliar a sua capacidade para modular a produção de NETs, utilizando a sonda SYTOX™ *green* ligada às NETs. As calconas C1 e C2 foram capazes de inibir totalmente a produção de NETs. De forma interessante, verificou-se que a atividade destas calconas foi dependente do tempo, com uma diminuição perceptível da atividade ao longo do tempo após a estimulação dos neutrófilos.

Este trabalho reforça o interesse das calconas no contexto da inflamação. Verificou-se que as calconas C1 e C2 apresentam uma modulação mais alargada da função dos neutrófilos estimulados, apresentando assim as características estruturais mais relevantes para o desenvolvimento de novos anti-inflamatórios.

Palavras-chave: Calconas, Inflamação, Espécies reativas pró-oxidantes, *Burst* oxidativo, NETs

Abstract

Inflammation is a fundamental process for the maintenance of the organism's homeostasis, defending it from aggressions, whether through injury or infection. Neutrophils are some of the most important effector cells during inflammation, as they are the first to be mobilized and are equipped with various mechanisms to eliminate damaged cells and invading pathogens, such as the generation and release of large amounts of reactive species and neutrophil extracellular traps (NETs). These essential mechanisms of the inflammatory response may become uncontrolled, which results in stressful conditions, due to an unmeasured and unbalanced response, often leading to more extensive tissue damage and a potential persistent inflammation. From these exacerbated conditions, can result the development of certain pathologies, such as atherosclerosis and rheumatoid arthritis, among many others. Therefore, when the body is not capable of recovering homeostasis, it requires external support, namely through anti-inflammatory drugs, that will neutralize damaging molecules and regulate inflammation.

Chalcones are naturally occurring chromone derivatives, structurally related to flavonoids, and have several biological activities described in the literature, such as antioxidant and anti-inflammatory activities. This proximity to flavonoids and their interesting chemical structure, comprising an easily substitutable scaffold, have been raising the interest of the scientific community in the development of drugs capable of modulating the inflammatory response, improving therapeutic outcomes. Therefore, it was evaluated the potential antioxidant and anti-inflammatory properties of seven structurally related chalcones. For this purpose, *in vitro* non-cellular assays were conducted, assessing the direct scavenging of physiologically relevant reactive species, such as hydrogen peroxide (H_2O_2), superoxide anion radical ($\text{O}_2^{\cdot-}$), hypochlorous acid (HOCl) and nitric oxide radical ($\cdot\text{NO}$), as well as *in vitro* cellular assays, evaluating the ability of the studied chalcones to modulate human neutrophils' function during inflammation, namely oxidative burst and NETs production mechanisms. Structure-activity relationships between the studied chalcones were established whenever possible, to identify possible structural characteristics favouring the observed biological activities.

In the *in vitro* non-cellular scavenging assays, chalcones C1 and C2 were able to scavenge $O_2^{\cdot-}$, HOCl and $\cdot NO$, and chalcones C4, C5 and C6 were able to scavenge HOCl. These results showed the importance of the presence of a catechol group in the B-ring (in C1 and C2) of chalcones in the scavenging of the radical reactive species $O_2^{\cdot-}$ and $\cdot NO$. Also, the presence of free hydroxy groups, mainly in the A-ring, seems to be important for the HOCl scavenging activity. It was also observed a possible selective antioxidant activity for chalcones C4, C5 and C6, as they were just able to scavenge one of the four selected physiologically relevant reactive species under study, HOCl.

In the *in vitro* cellular assays, chalcones C0, C1 and C2 were able to inhibit the oxidative burst when evaluated using the non-specific probe luminol. Chalcones C1, C2 and C6 were able to inhibit the oxidative burst, when evaluated using the specific probe 3'-(*p*-aminophenyl) fluorescein (APF), demonstrating their modulation of the HOCl-associated oxidative pathways. Considering the results of the non-cellular assays, it is possible to affirm that one of the mechanisms through which the active chalcones, C1 and C2, are able to modulate the oxidative burst mechanism is through the direct scavenging of the generated reactive species upon neutrophils stimulation. Chalcones C1, C2 and C6 were then chosen to evaluate their ability to modulate NETs production, using NET-bound SYTOX green. Chalcones C1 and C2 were able to fully inhibit NETs production. Interestingly, the activity of these chalcones was seen to be time-dependent, with a visible decrease in activity through time after neutrophils stimulation.

This work confirms chalcones as interesting compounds in the inflammatory context. Chalcones C1 and C2 were found to present a broader modulation of stimulated neutrophils function, presenting therefore the most relevant scaffold for the design of new anti-inflammatories.

Keywords: Chalcones, Inflammation, Prooxidant reactive species, Oxidative burst, NETs

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List of Abbreviations

4CL	4-coumarate:CoA ligase
APC	Antigen-presenting cells
APF	3'-(<i>p</i> -aminophenyl) fluorescein
C4H	Cinnamic acid 4-hydroxylase
CAMs	Cell adhesion molecules
CHI	Chalcone isomerase
CHS	Chalcone synthase
DAF-2	4,5-Diaminofluorescein
DAMPs	Damage-associated molecular patterns
DHR 123	Dihydrorhodamine 123
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium chloride
ELR-CXC	CXC chemokines containing the ELR motif
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GPCRs	G-protein-coupled receptors
GTP	Guanosine 5'-triphosphate
GTPase	Guanosine 5'-triphosphate hydrolase
H4B	(6R)-5,6,7,8-Tetrahydrobiopterin
IC ₅₀	Half maximal inhibitory concentration
IL-1 β	Interleukin-1 β
IL-8	Interleukin-8
IL-10	Interleukin-10
IM	Interference with the methodology
iNOS	Inducible nitric oxide synthase
JAMs	Junctional adhesion complexes
K ₃ EDTA	Tri-potassium ethylenediaminetetraacetic acid
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
NA	No activity
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium chloride
NETs	Neutrophil extracellular traps
NF- κ B	Nuclear factor κ B
NK cells	Natural killer cells
nNOS	Neuronal nitric oxide synthase
NOC-5	3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazinyl]-1-propanamine
PAL	Phenylalanine ammonia lyase
PAMPs	Pathogen-associated molecular patterns
PBS	Dulbecco's phosphate buffer saline without calcium chloride and magnesium chloride
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear cells
PMS	Phenazine methosulfate
R $^{\bullet}$	Alkyl radicals
RFU	Relative fluorescence units
RNS	Reactive nitrogen species
ROO $^{\bullet}$	Peroxyl radicals
ROOH	Alkyl peroxides
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
TGF- β	Transforming growth factor- β
TLR4	Toll-like receptor 4
TNF- α	Tumour necrosis factor- α
UV/vis	Ultraviolet-visible

1. Introduction

1.1. Inflammation

Inflammation is a physiological defence response of the body against harmful stimuli, being caused by external trauma, ischemic injury, pathogenic infection, and exposure to chemicals or radiation, among other causes. This highly regulated physiological process, conducted by the innate immune system, acts by attempting to eliminate the injurious stimulus, starting the healing of the affected tissues, and trying to re-establish homeostasis ¹⁻³.

The inflammatory process can be described by the five cardinal signs of inflammation, namely redness and heat (due to increased blood flow in the tissue), swelling (due to changes in vascular dilation and permeability to fluid and inflammatory cells), pain (due to the release of certain mediators, e.g., prostaglandins) and, finally, loss of function (a consequence of the previous signs and the tissue damage). However, the complexity of inflammation is much greater than this simple description, consisting of several cellular, molecular, and vascular events, to solve the disturbance to the body's homeostasis. This response involves several signalling pathways that highly regulate the unfolding events ^{4, 5}.

When a noxious stimulus is detected, an acute inflammatory response takes place (Figure 1). This happens through the recognition of microbial pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by tissue cell receptors (pattern-recognition receptors), mainly present in macrophages, dendritic cells, and mast cells. Specific inflammatory pathways are activated starting with the production and release, by the previously mentioned tissue sentinel cells, of inflammatory mediators, including cytokines, which mediate the inflammatory process, by regulating the function of several cell types and inducing biological responses; and chemokines, chemotactic molecules responsible for recruiting effector inflammatory cells to the affected site, to aid in the inflammatory response ⁶⁻⁸.

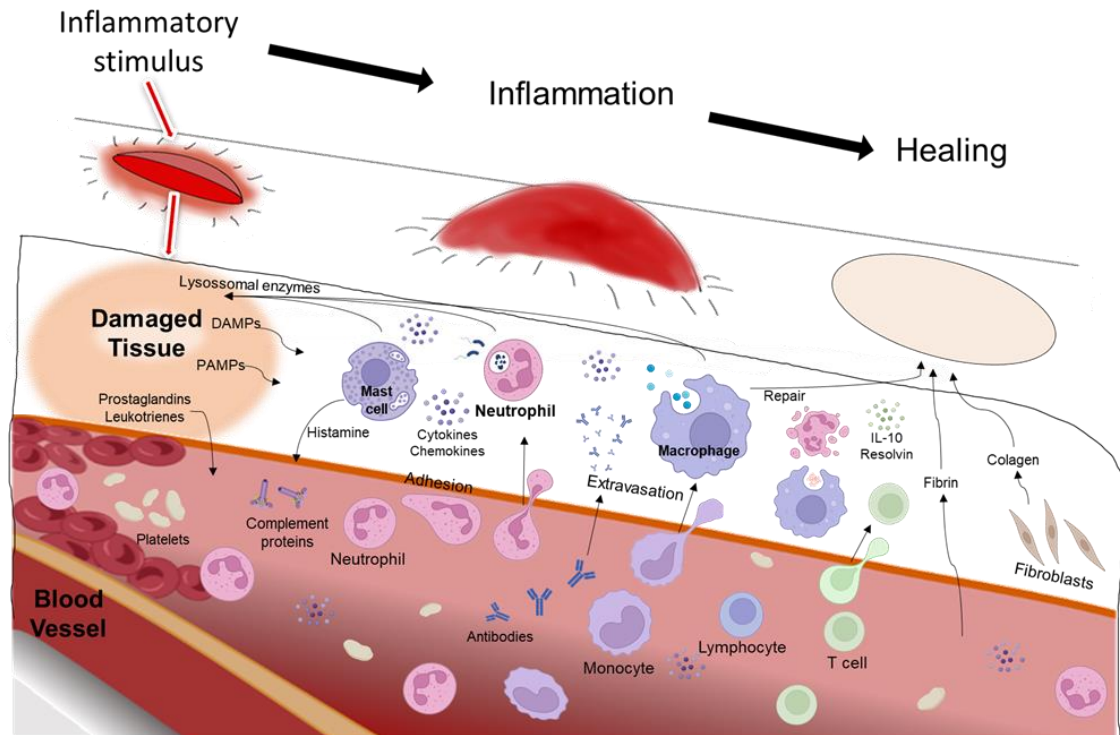


Figure 1. The process of acute inflammatory response, from injury until healing, including the cells and mediators involved. Adapted from ⁹. Created with BioRender.com. DAMPs, damage-associated molecular patterns; IL-10, interleukin 10; PAMPs, pathogen-associated molecular patterns.

In the beginning stages of this response, circulating leukocytes and plasma proteins, essential to the defence process, must be delivered to the affected area, therefore, resident mast cells release inflammatory mediators, such as histamine and prostaglandins, producing a change in vascular flow and permeability, leading to a higher supply of soluble molecules, and circulating effector cells, mainly neutrophils. These leukocytes are responsible for trying to eliminate pathogens and damaged cells, doing so through different mechanisms, like phagocytosis or the release of the content of their internal granules, containing several enzymes and microbiocidal substances. As leukocytes arrive at the targeted area, before they migrate into the damaged tissues, they interact with the vascular endothelium, allowing their adhesion to endothelial cells through the interaction between selectins and integrins and their ligands. Once this leukocyte-endothelial cell adhesion is completed, migration through the endothelium occurs, with the cells exiting the blood flow into the affected tissue, where they will perform their defence mechanisms ^{1-3, 10, 11}.

Simultaneously to the proinflammatory response, the organism attempts to maintain the balance through a dynamic production of anti-inflammatory mediators and, if the harmful stimulus ceases to exist or is eliminated by inflammatory cells, then a resolution and healing phase will follow. For the resolution of acute inflammation, proinflammatory signals and mediators need to be contained, to stop the defence process. Firstly, proinflammatory signalling pathways are inhibited to minimize the response amplification. Then, proinflammatory mediators, such as chemokines and cytokines, and inflammatory cells, mainly neutrophils, need to be catabolized, to avoid the persistence of the inflammatory state and the possible evolution into chronic inflammation, through continuous aggression to the affected tissue. The elimination of the recruited leukocytes is performed by phagocytes such as macrophages ^{12, 13}.

As macrophages phagocytize damaged and dead cells, as well as inflammatory cells, the production of anti-inflammatory mediators, such as interleukin (IL)-10 and pro-resolving, like resolvins, is maximized. These signalize the inhibition of leukocytes' recruitment and recruit other macrophages to aid in the repair of the damaged tissue, leading to the re-establishment of homeostasis ^{2, 12}.

When acute inflammation fails to remove the injurious stimulus or there is an inability of the body to repair and recover from the inflicted damage, there may be a progression to a long-term process, known as chronic inflammation (Figure 2).

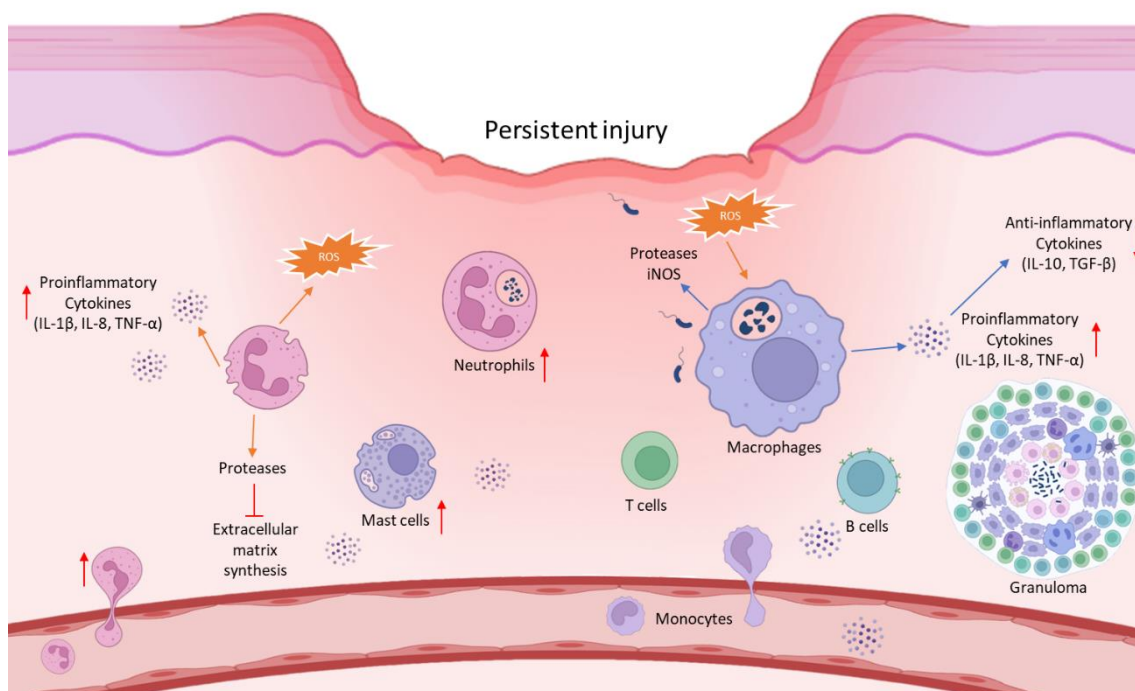


Figure 2. The chronic inflammatory process, including the cells and mediators involved. Adapted from ^{14, 15}. Created with BioRender.com. IL-1 β , interleukin-1 β ; IL-8, interleukin-8; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; TGF- β , transforming growth factor- β ; TNF- α , tumour necrosis factor- α .

This chronic inflammatory response is carried out mainly by macrophages and lymphocytes, who invade the affected tissue, producing proinflammatory mediators and enzymes, further worsening the damage in the area and often leading to the formation of granulomas, a structure of layered macrophages to isolate the invading aggressor or foreign body ^{2, 16-18}. During this chronic process, acute phases can reoccur, with new moments of recruitment of neutrophils, which, due to their activity [e.g. production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)] lead to larger damage over the affected area ^{19, 20}. An example of a disease promoted by chronic and persistent inflammation is atherosclerosis, a vascular disease associated with the deposition of lipids, fibrotic and calcification processes. The deposition and accumulation of lipids results in an inflammatory response that induces monocyte recruitment and leads to the formation of an atheromatous plaque and to the dysfunction of the endothelium ²¹. The recruited monocytes differentiate into macrophages that, through the persistent accumulation of low-density lipids, are stimulated to produce proinflammatory mediators, further increasing the damage to the endothelium and the progression of the disease ²¹. Chronic inflammation is indeed a major health threat, contributing to the development of other serious diseases, such as diabetes and cancer, among many others ^{1, 13, 16, 20}.

1.2. Neutrophils

1.2.1. Leukocytes

Leukocytes are a group of blood cells, also known as white blood cells, which play crucial roles in immune responses, defending the organism. Within this group, there are several cells, with different functions and physiology. They can be divided into two groups based on whether or not they contain cytoplasmic granules, the granulocytes and the agranulocytes, respectively ²².

The agranulocytes group (Figure 3) includes lymphocytes, monocytes, macrophages, and dendritic cells. The lymphocytes are generally divided into B cells, T cells and natural killer (NK) cells. B and T lymphocytes are part of the adaptive immune system, the first are capable of antibody secretion and the latter are responsible for killing infected, cancerous, and other targeted cells ²³. Also responsible for the elimination of target cells, NK cells are part of a group of cells that fit in between adaptive and immune system, the innate-like lymphocytes, with a less specific action than lymphocytes with high-affinity antibodies ²⁴. Monocytes are circulating cells that, when they exit the bloodstream to damaged tissues, differentiate into macrophages, responsible for eliminating, through phagocytosis, microbes, dead cells and apoptotic bodies, as well as foreign particles. They also act as antigen-presenting cells (APC), as they present antigens to lymphocytes, aiding the adaptive immune response ^{18, 23}. Macrophages are also present in different tissues, as tissue-resident macrophages, and act as immune sentinels, reacting when a damaging stimulus is detected. These cells are also fundamental for tissue development and maintenance ²⁵. Lastly, dendritic cells are APC, responsible for presenting pathogenic antigens to other effector immune cells ²⁶.

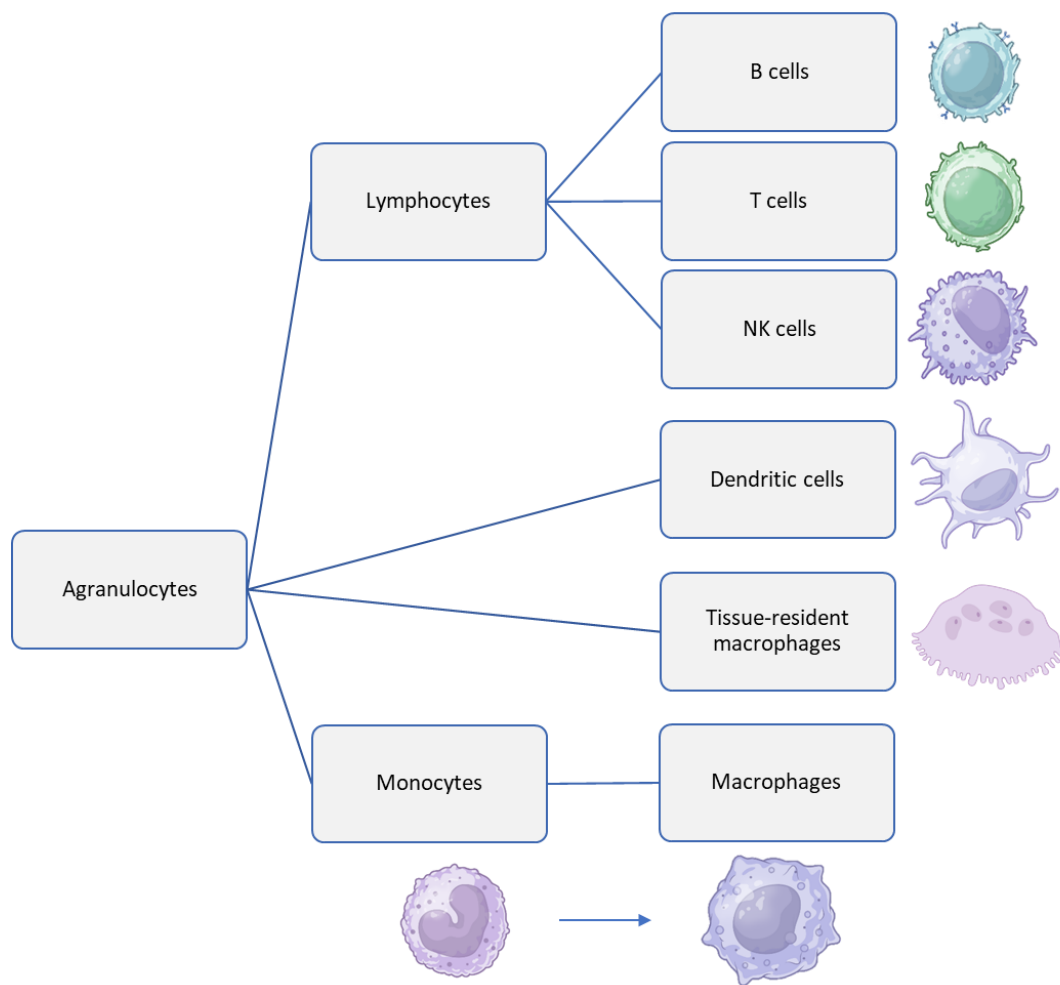


Figure 3. Types of agranulocytes. Created with BioRender.com.

The granulocytes group (Figure 4) includes cells that contain cytoplasmic granules and vesicles which keep several mediators and microbiocidal enzymes and proteins used for several purposes, e.g., cellular functions and elimination of microorganisms^{23, 27-29}. This group includes neutrophils, eosinophils, basophils, and mast cells. Neutrophils are the most abundant type of circulating leukocytes (around 70 % of total white blood cells)²⁷, the first responders to innate immune responses, mainly intending to eliminate pathogens. Neutrophils contain, in their cytoplasm, primary granules, containing the most cytotoxic molecules, such as myeloperoxidase (MPO), elastase, and defensins, and also contain secondary granules, with matrix metalloprotease and lactoferrin³⁰. Eosinophils are responsible for fighting parasitic infections. They contain a large number of secondary granules in their cytoplasm, storing cationic and acid proteins, such as elastase and eosinophil cationic protein, and also containing inflammatory mediators, such as chemokines and cytokines^{31, 32}. Due to the chemical properties of their granules' contents, eosinophils, when using the haematoxylin–eosin staining technique, are dyed

by eosin, hence the name of these cells³³. Basophils are often associated with allergic and hypersensitivity responses, expressing high-affinity immunoglobulin E, as well as containing histamine and heparin in cytoplasmic granules³⁴. These granules are dyed by haematoxylin, a basic stain, therefore presenting basophilic characteristics, hence the name of these cells³³. Mast cells are closely related to basophils, as both are rich in histamine and are involved in hypersensitivity reactions, mast cells are also involved in innate immune responses, degranulating upon activation, releasing histamine, responsible for leukocyte chemotaxis and vascular permeability increase²⁹.

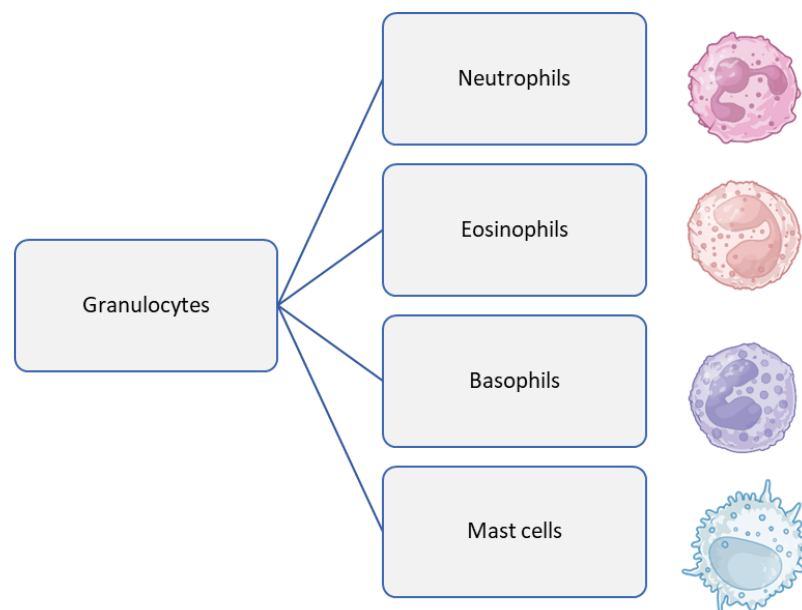


Figure 4. Types of granulocytes. Created with BioRender.com.

1.2.2. Neutrophils in the inflammatory context

As previously mentioned, neutrophils, also known as polymorphonuclear (PMN) cells, belong to the group of granulocytes. Neutrophils production begins in the bone marrow, where a process of maturation occurs, in which hematopoietic stem cells differentiate to mature neutrophils, acquiring the lobulated characteristic morphology of the nucleus and upregulating the expression of several receptors, like Toll-like receptor 4 (TLR4), an important receptor to microbial lipopolysaccharide (LPS) recognition. During this process, mainly in the later stages of neutrophils' maturation, these cells acquire cytoplasmic granules that store molecules used to fight foreign aggressors, including proteases, MPO, defensins, matrix metalloproteinases, among others^{2, 10, 28, 35, 36}.

In normal physiological conditions, neutrophils may reside for several days in the blood flow, as they roam the organism patrolling for possible invaders as well as acting as

important regulators of the immune system, producing cytokines and other inflammatory mediators and signalling molecules^{35, 37, 38}.

Upon detection of an invading agent or tissue damage, by cellular receptors, a large and quick response from neutrophils takes place, as explained in figure 5.

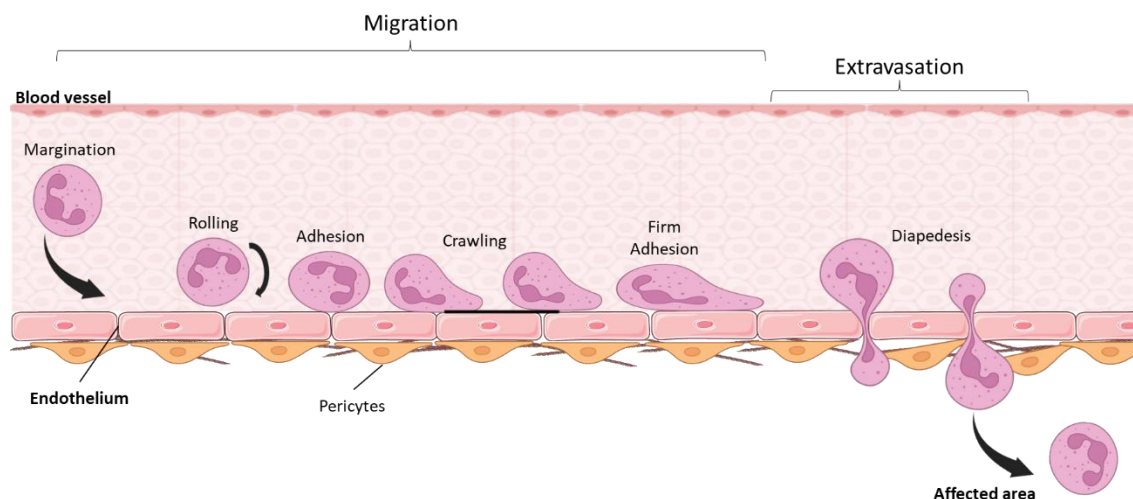


Figure 5. Vascular events during neutrophils recruitment and extravasation. Adapted from³⁹. Created with BioRender.com.

In this response, the production of large numbers of neutrophils in the blood marrow is stimulated, as well as the recruitment of these cells from the blood flow to the targeted area³⁵. Their recruiting is called chemotaxis, a process in which these leukocytes are attracted to the target area via cytokines, like IL-8, which enables fast neutrophils migration to the affected area. They are the first responders to attempt to control and eliminate said aggression^{40, 41}.

Upon arriving at the harmed tissue, through the bloodstream, the activation of neutrophils begins (Figure 5). Firstly, margination and adhesion to the vascular endothelium occur. These happen through the firm interactions between endothelial and neutrophils' surface molecules, such as selectins and integrins, followed by full activation of these leukocytes by endothelial CXC chemokines containing the ELR motif (ELR-CXC chemokines), e.g., IL-8²². These molecules bind to neutrophils' G-protein-coupled receptors (GPCRs), inducing a signalling cascade that results in conformational changes in integrins surface, producing a stronger binding to the endothelial wall¹¹. Then extravasation follows, as the PMN cells leave the bloodstream. This exit is called diapedesis, allowed by a selective permeability of the blood vessels to neutrophils, avoiding the exit of erythrocytes and other particles to the extravascular space. This process requires dynamic regulation of

adhesion proteins present in the vascular endothelium binding the neutrophils to the vascular wall [integrins and cell adhesion molecules (CAMs)], as well as junctional adhesion complexes (JAMs) that connect the endothelial cells^{2, 10, 11, 39}. Neutrophils transmigration occurs either paracellularly, through the space between two endothelial cells, or transcellularly, through an endothelial cell, either by transcellular pores or intracellular vesicles^{11, 42}.

Once out of the bloodstream, neutrophils arrive at the target tissue, following a chemokine gradient leading them to the targeted area, and become fully activated, by contact with either PAMPs or DAMPs and by proinflammatory cytokines. The recognition of these signals leads to the activation of major signalling pathways, starting with the activation of protein kinase C (PKC), resulting in functional and transcriptional changes^{11, 43}. Upon this activation, neutrophils will begin their function to attempt to resolve the existing aggression, being it by directly trying to kill any pathogen or by orchestrating the immune response by the production of several cytokines capable of modulating the activities of neighbouring inflammatory cells⁴⁴.

Neutrophils can eliminate pathogenic exogenous cells or damaged endogenous cells by three distinct mechanisms: degranulation, phagocytosis and the formation of NETs, through a process known as NETosis (Figure 6)³⁵. In degranulation, upon recognition of PAMPs or DAMPs, these granulocytes will release, via secretory vesicles, several cytotoxic molecules, such as enzymes (e.g. proteases) and prooxidant reactive species, either to the extracellular medium or into phagosomes^{27, 28}. Phagosomes are formed during phagocytosis, in which a targeted cell is encapsulated inside an invagination of the neutrophils' cell membrane, where nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent mechanisms (producing reactive species) and cytotoxic proteins (e.g., lysozyme) will eliminate it. The formation of the phagosome involves changes in lipid composition and remodelling of the actin cytoskeleton, as the cytoplasmic membrane encapsulates the aggressor⁴⁵. As the phagosome matures, it is internalised by the phagocyte and fused with internal vesicles, such as endosomes and lysosomes, finally forming a phagolysosome, the fully matured organelle, containing several degradative enzymes, such as lysozyme, proteases and lipases⁴⁵. Then, a third microbiocidal mechanism is available, the NETs. These are deoxyribonucleic acid (DNA) structures, coupled with proteins and enzymes, released to the extracellular space to capture the target cell, aiding its phagocytosis. This last mechanism always results in neutrophil death due to the loss of nuclear content^{2, 10, 11,}

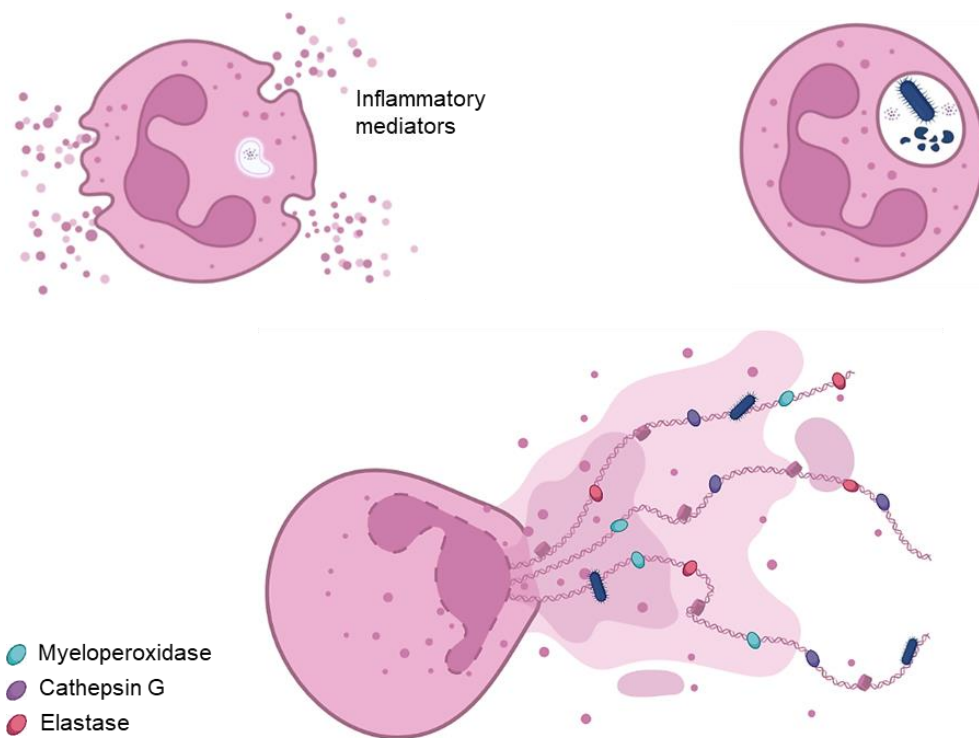


Figure 6. Neutrophils' mechanisms to eliminate pathogenic exogenous or damaged endogenous cells: (A) degranulation, (B) phagocytosis and (C) neutrophil extracellular traps (NETs) release. *Adapted from* ³⁵. *Created with BioRender.com.*

As the stimulus causing the inflammatory response is controlled or eliminated, a resolution stage begins, where neutrophils begin to aid in the repair of damaged tissue, by cleaning cellular debris and by releasing cytokines, modulating the activity of inflammatory cells, such as monocytes and macrophages ^{35, 44}. The recruitment of PMN cells is also reduced at this point, as anti-inflammatory cytokines, like IL-10, are released by other immune cells ^{2, 11}.

1.2.3. Prooxidant reactive species

Reactive species are molecules with oxidising capacity and can be either radicals or non-radicals, depending on whether or not they possess free unpaired electrons (and are able to exist independently), respectively ⁴⁶. These species are produced through different cellular metabolic and enzymatic processes, namely through mitochondrial respiration, which results in the release of free electrons, that may react with molecular oxygen; but also, during inflammation, by inflammatory cells, mainly neutrophils, that produce massive amounts of these reactive species as a mean to eliminate the aggression ⁴⁷⁻⁴⁹.

There are several types of reactive species, including reactive bromine species, reactive chlorine species, RNS, ROS, and reactive sulphur species, among others ^{50, 51}. However, in this study, the focus is given to the ROS and RNS, as these are the more physiologically relevant ones when talking about inflammation and neutrophils' functions ^{52, 53}.

Reactive species can be both beneficial and deleterious to the organism, being useful to physiological processes, but also inducing damage to cells and tissues. At normal levels, these molecules are essential for cellular processes, such as cell signalling, redox reactions, and for the defence of the organism, eliminating invaders and damaged cells through interactions with their biomolecules, like DNA, proteins and lipids, compromising their function ^{46, 48, 49, 54}. However, when there is an overproduction of these highly reactive species and the body's antioxidant defences are overwhelmed, a state of oxidative stress may occur, during which the prooxidant reactive species may lead to cell death and damage over tissues. These effects are due to the interactions of these species with biomolecules. For example, they promote lipid peroxidation, a process in which these oxidant molecules oxidise lipids containing carbon-carbon double bonds, resulting in the production of lipid peroxy radicals and hydroperoxides, often leading to the degradation of structural lipids, e.g., membrane phospholipids ⁵⁵⁻⁵⁷. This is also why inflammation and oxidative stress are often related to the development of various pathologies, such as rheumatoid arthritis, cardiovascular diseases, and cancer ^{10, 46, 52}.

As previously mentioned, during inflammation, after neutrophils are activated, one of the cellular events to take place is a respiratory or oxidative burst. In this process, neutrophils increase their consumption of molecular oxygen and consequently the production and release of prooxidant reactive species, ROS and RNS, as cytotoxic agents to fight invading microorganisms or eliminate damaged cells, a process of major importance for

the elimination of pathogens and other target cells ³⁵. However, this process may occur to an excessive extent, resulting in an oxidative stress state, a situation that often requires the administration of exogenous antioxidants or anti-inflammatory drugs, to control the process and the resulting damage to the organism ⁵⁸.

As neutrophils are recruited and activated, after recognition of deleterious stimuli, an important enzyme begins to be assembled, the NADPH oxidase, an enzyme present in neutrophils' membranes, including in the membranes of phagosomes. This enzyme is a complex of proteins, that before activation, are separated inside the cell ⁵². There are five subunits that, once assembled, constitute the NADPH oxidase (also known as phagocyte oxidase), these being: p22phox and gp91phox, two transmembrane proteins that bond, forming the flavocytochrome b558, the catalytic portion of the enzyme, present in neutrophils' membranes; and p40phox, p47phox and p67phox subunits, that together form the regulatory complex, present in the cytosol before activation ^{52, 59, 60}. For the translocation of these regulatory complexes and their binding to the transmembrane subunits to occur, the p47phox component has to be phosphorylated ⁶¹. The need for the assembly of the different subunits of the NADPH oxidase, in order to become active, serves as a regulatory mechanism, preventing the overproduction of reactive species under homeostatic conditions ⁵². There is still another fundamental component for NADPH oxidase full assembly and activation, which is a small guanosine 5'-triphosphate hydrolase (GTPase), the Rac2, present in the neutrophils' cytosol. This GTPase, once bound to guanosine 5'-triphosphate (GTP), is able to translocate to the membrane-bound complex, completing the active conformation of the NADPH oxidase ⁵².

Once assembled and active, the production of ROS by NADPH oxidase begins (Figure 7). The first event is an electron transfer from the electron donor, the NADPH molecule, to molecular oxygen (O_2), producing superoxide anion radical ($O_2^{\cdot-}$) ⁶². $O_2^{\cdot-}$ can also be intracellularly generated without catalysis by NADPH oxidase, being generated through enzymatic or non-enzymatic reactions every time molecular oxygen acts as an electron acceptor, which, for example, happens in the mitochondrial respiratory chain ⁶³. $O_2^{\cdot-}$ is quickly dismutated to hydrogen peroxide (H_2O_2), either spontaneously or through the activity of superoxide dismutase (SOD), both intracellularly and extracellularly ^{64, 65}. H_2O_2 , apart from being a ROS capable of reacting with biomolecules, may also act as a signalling molecule, mainly through oxidation of certain cysteine residues, resulting in the activation, for example, of transcriptional factors ^{63, 66}. Through two redox reactions, Fenton and Haber-Weiss, another ROS can be produced, the highly reactive and, thus, short-lived, hydroxyl radical ($HO^{\cdot}$). The reactivity of these specific ROS is due to the high

oxidation potential and nonselective interaction with biomolecules, easily degrading them⁶⁷. In the Fenton reaction, a molecule of iron (II) (Fe^{2+}) is oxidized to iron (III) (Fe^{3+}) by H_2O_2 , which, as a result, is converted into $\text{HO}^\bullet$ and hydroxide anion (HO^-)⁶⁸. In the Haber-Weiss reaction, $\text{O}_2^{\bullet-}$ reacts with H_2O_2 , producing $\text{HO}^\bullet$, HO^- and O_2 ⁶⁸. Another mechanism to produce $\text{HO}^\bullet$ is the reaction of $\text{O}_2^{\bullet-}$ with hypochlorous acid (HOCl). This latter ROS is formed as the result of the action of an enzyme present in the cytoplasmic granules of neutrophils, the MPO, that catalyses the reaction between a chloride (Cl^-) anion and H_2O_2 . HOCl is a powerful oxidant and a major bactericide produced by activated PMN cells^{69, 70}. This ROS is also a major cause of oxidative stress damage because it has a longer lifetime and is able to diffuse across membranes and tissues, expanding the scale of the damage caused by an uncontrolled oxidative environment⁷¹. Another powerful prooxidant ROS is singlet oxygen ($^1\text{O}_2$), produced by the reaction of H_2O_2 with hypochlorite anion (ClO^-). This ROS is an excited state of molecular oxygen and is capable of largely reacting with biomolecules, inflicting extensive damage to the target area of inflammation. Singlet oxygen has also been described as an important mediator for the formation of NETs^{70, 72}. Finally, there are the peroxy radicals ($\text{ROO}^\bullet$), produced by the reaction of oxygen with alkyl radicals ($\text{R}^\bullet$) or by the breakdown of alkyl peroxides (ROOH). These molecules are often involved in lipid peroxidation^{56, 70}. Phospholipid peroxidation may also be a contributing factor to the propagation of inflammation, as, from it, may result the production of inflammatory mediators, such as isoprostanes⁷³.

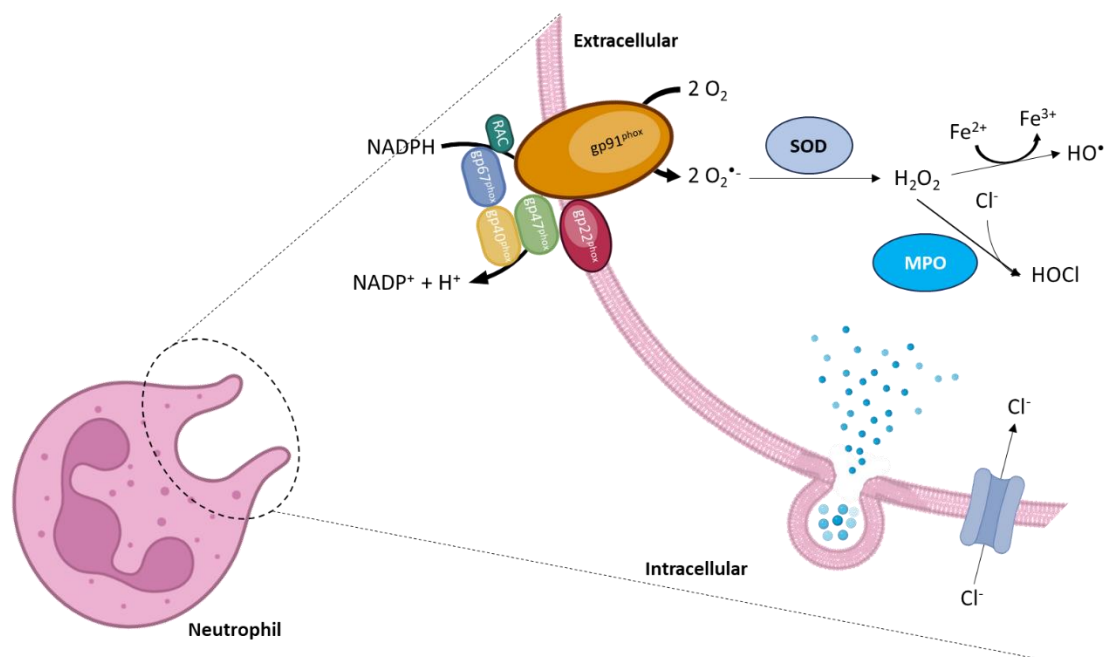


Figure 7. Generation of reactive oxygen species (ROS) by neutrophils^{62, 64, 69, 70}. Created with BioRender.com. MPO, myeloperoxidase; SOD, superoxide dismutase.

Simultaneously to ROS production, as neutrophils are activated, signalling pathways activate the production of RNS through the enzymatic activity of a cytoplasmic inducible enzyme, the inducible nitric oxide synthase (iNOS). iNOS belongs to a family of three heme-containing nitric oxide synthases, two of them being constitutively expressed, the neuronal nitric oxide synthase (nNOS) and the endothelial nitric oxide synthase (eNOS), and a third one whose expression is induced, the iNOS. This induction occurs after proinflammatory cytokines bind to neutrophils' cell surface receptors, thus activating receptor-associated kinases which lead to phosphorylation of intracellular proteins and activation of inflammation-related transcriptional factors, such as nuclear factor κ B (NF- κ B), which translocate into the nucleus, inducing the expression of the inducible synthase^{74, 75}.

iNOS catalyses the conversion of L-arginine to L-citrulline in the presence of O_2 (Figure 8), and, also, requires the intervention of several cofactors, such as NADPH (acting as an electron donor), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme groups, and (6R)-5,6,7,8-tetrahydrobiopterin (H4B)⁷⁴. The activity of this enzyme results in the production of important levels of nitric oxide radical ($^{\bullet}NO$), a reactive species important to innate immune responses, being used to eliminate pathogens, and, due to its high diffusion capacity, it is also of major importance in neurotransmission and

on vasodilation processes, as it induces relaxation of the vascular smooth muscle cells

74, 76-78.

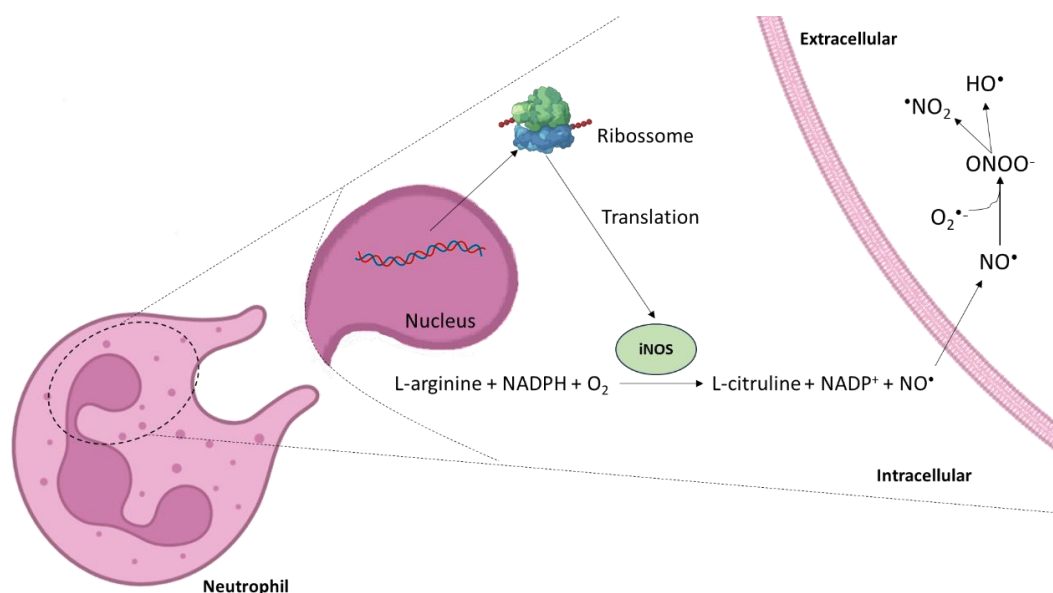


Figure 8. Generation of reactive nitrogen species (RNS) by neutrophils^{74-76, 79}. Created with BioRender.com. *iNOS*, inducible nitric oxide synthase.

ROS and RNS can interact with each other, as is the case of the reaction between $\cdot\text{NO}$ and $\text{O}_2^{\cdot-}$, resulting in the production of a short-lived RNS, peroxynitrite (ONOO^-), known as the most reactive of the RNS (Figure 8), because, once protonated at physiological pH, it undergoes homolytic cleavage, producing the reactive species $\text{HO}^\cdot$ and nitrogen dioxide ($\cdot\text{NO}_2$)^{76, 79, 80}. ONOO^- is also capable of reacting with amino acids, e.g. the nitration of tyrosine residues, and is also responsible for mitochondrial oxidative damage

80.

1.2.4. Neutrophil extracellular traps

As previously mentioned, one of the mechanisms that neutrophils possess to eliminate pathogens is the release of NETs. This mechanism is the most recently described for the elimination of pathogens by neutrophils, being discovered by Takei et al.⁸¹ in 1996, as the investigators identified a pathway for cell death distinct from regular apoptosis and necrosis, by detecting morphological changes⁸². These changes involved the compression of the multi-lobated nucleus of the neutrophils and the reduction of chromatin condensation⁸². NETosis is the process where neutrophils die by loss of DNA to the extracellular medium, through nuclear envelope disassembly and changes in cytoplasmic membrane permeability⁸³. This process differs from apoptosis, as in this new mechanism, neutrophils lose their nuclear envelope, leading to the mixing of nuclear and cytoplasmic contents, and loss of internal membranes and organelles, whereas none of these events are seen in apoptosis⁸⁴. This mechanism is quite similar to necrosis, as the integrity of membranes is lost, however, the organised release of nuclear material is distinct in NETosis⁸⁴.

The release of NETs is a highly regulated mechanism, not happening every time neutrophils are activated during an inflammatory response. This regulation is based on the assessment of the pathogen size, which, usually, is required to be of larger dimensions to induce this response and the expression of virulence factors⁸³. The need for this tight regulation is justified by the pathological results of uncontrolled production of NETs, which range from direct damage to cells and tissues; to vaso-occlusion, promoting thrombotic events in blood vessels; and tumour metastasis, as these may capture and transport tumoral cells^{11, 83}.

The production of NETs depends on two ROS-producing enzymes, the NADPH oxidase and MPO. Although this is known, the mechanism through which NETs production is mediated by ROS is not fully determined. It was already proven by inhibiting NADPH oxidase and MPO function, that, somehow, NETs production effectively depends on these enzymes' activity⁸⁵. Some studies provide information on how this may work. ROS may stimulate MPO release and activation, together with elastase release from azurophilic granules into the nucleus. Elastase can process histones, leading to chromatin decondensation⁸⁵. These events are believed to induce the rupture of the nucleus envelope, allowing the flowing out of the chromatin and then, in a small area of the cytoplasmic membrane, the bilipid layer is disrupted and the DNA is released to the extracellular space^{82, 83, 86}. Together with the release of DNA filaments, several antimicrobial proteins are also released from cytoplasmic granules to the extracellular

medium, such as elastase, MPO, and cathepsin G (Figure 9)⁸⁷. These networks of DNA filaments are able to capture and hold pathogens so the associated antimicrobial proteins can act, attacking the integrity of the pathogens and possibly eliminating them⁸³.

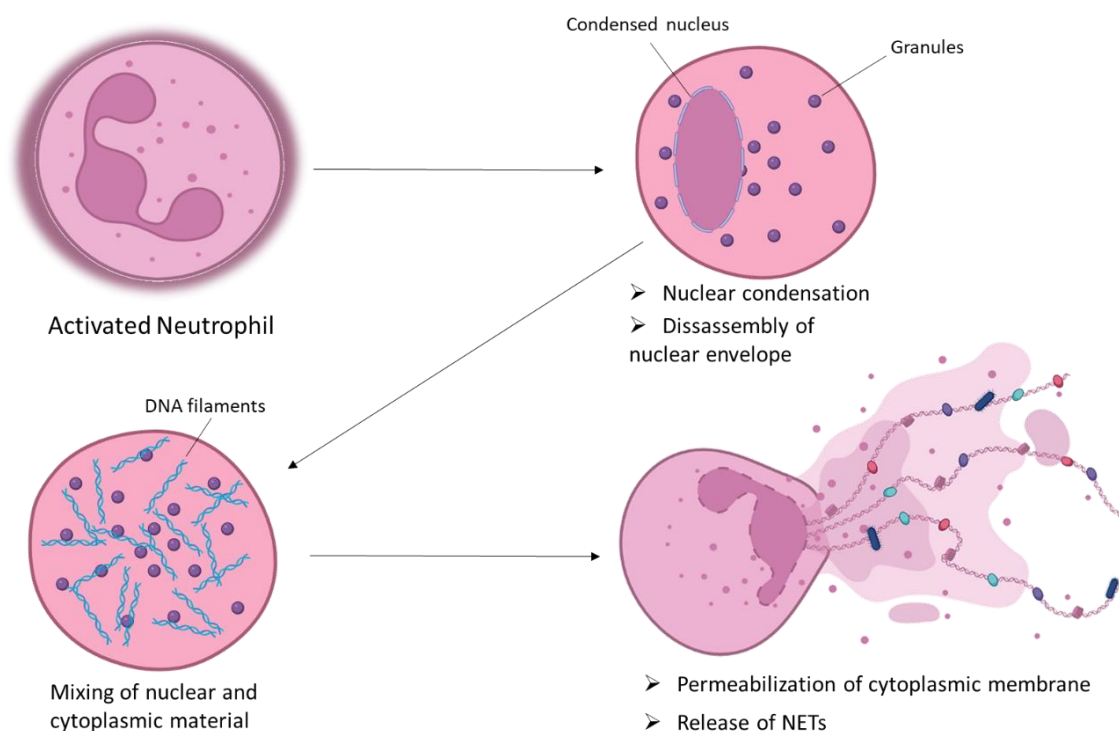


Figure 9. Neutrophil extracellular traps (NETs) production and release. Adapted from⁸³. Created with BioRender.com.

Although the previously described mechanisms, oxidative burst and NETs production, are essential tools for the role of neutrophils in innate immune responses, if uncontrolled, they can be highly deleterious for the organisms' homeostasis. Therefore, it is important to find compounds that are able to modulate, more than completely and drastically inhibit these mechanisms, in order to aid the body in regaining its balance, allowing for its effective recovery and healing.

1.3. Chalcones

1.3.1. Chemistry

Chalcones (Figure 10A) are bioprecursors of plant flavonoids and are considered by many authors to belong to the flavonoid (C6-C3-C6 system) family⁸⁸⁻⁹⁰. The basic structure of flavonoids is presented in figure 10B and they have a chromone structure as their core (Figure 10C). Indeed, chalcones have the skeleton of open-chain flavonoids in which the three-carbon aliphatic system is used as an adjunct between A- and B-rings (aromatic)⁹¹. This core structure is called 1,3-diaryl-2-propen-1-one, and consists of two aromatic rings (A and B, in either *cis* or *trans* isomers, the latter being the most thermodynamically stable) linked by a chain of three carbons, of which two are linked through a double bond and a third is a carbonyl group, forming a three-carbon α,β -unsaturated carbonyl system^{92, 93}. The chalconoid core is a promising scaffold for the development of several chalcone derivatives, as the presence of a large number of replaceable hydrogens allows the design of compounds with various biological activities⁹³.

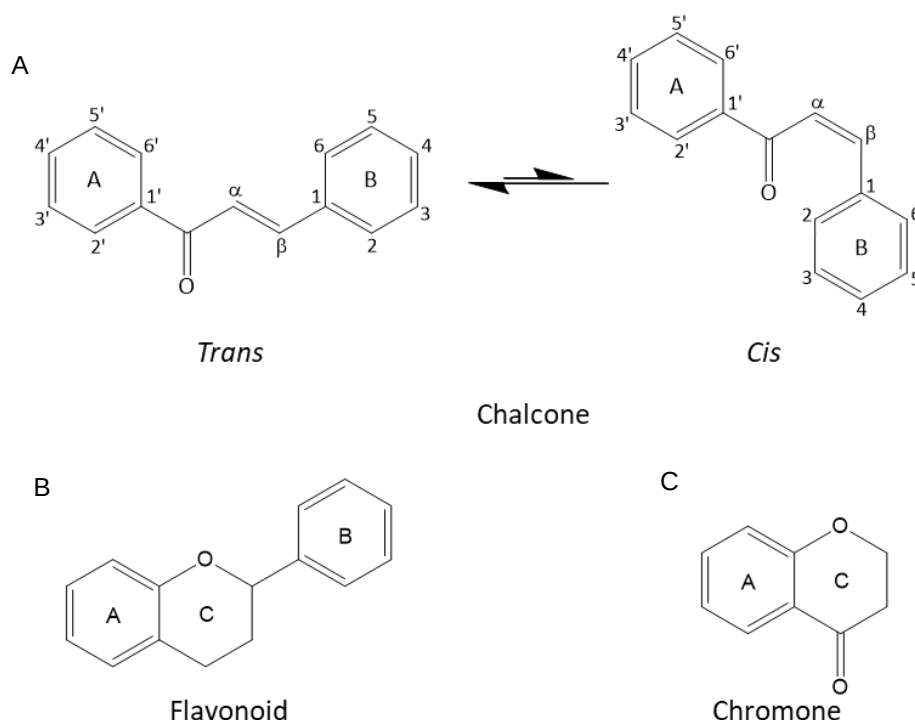


Figure 10. Chemical structures of (A) chalcones, (B) flavonoid and (C) chromone, with the identification of the rings, and the numeric system of the chalcones' isomers. Adapted from⁹³⁻⁹⁵.

1.3.2. Natural occurrence

Chalcones are naturally occurring compounds found throughout the whole plant kingdom, present in several medicinal herbs known to be used in traditional medicinal practice for centuries in various cultures, for treatment of ills and diseases, such as infections, inflammation, etc ⁹⁶. Chalcones are secondary plant metabolites, as they are not essential in plant metabolism, but are produced as defensive molecules, and are precursors of flavonoids in the biosynthetic pathway of these polyphenols, the phenylpropanoid pathway ⁹⁷. This pathway has, as a main precursor, a molecule of phenylalanine which, through a series of enzyme-catalysed reactions [one of these enzymes being the chalcone synthase (CHS)], is converted into a particular chalcone, the naringenin chalcone (Figure 11) ^{88, 96}.

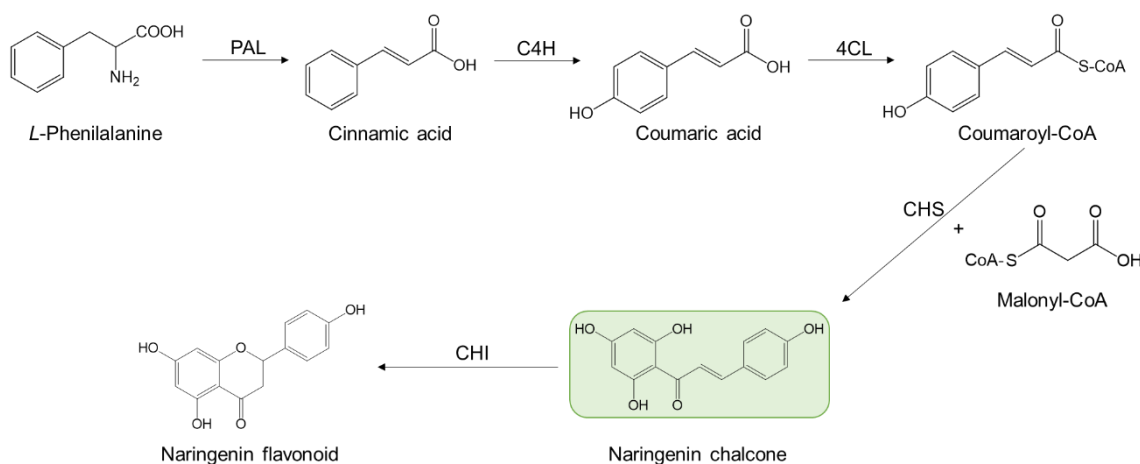


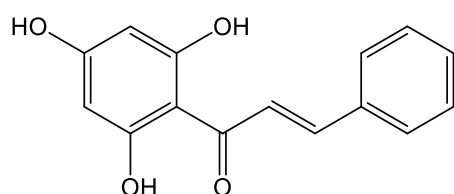
Figure 11. Simplified biosynthetic pathway of flavonoids. Adapted from ⁹⁸. **4CL**, 4-coumarate:CoA ligase; **C4H**, cinnamic acid 4-hydroxylase; **CHI**, chalcone isomerase; **CHS**, chalcone synthase; **PAL**, phenylalanine ammonia lyase.

Naringenin chalcone acts then as a biosynthetic precursor to produce several classes of polyphenolic compounds, such as flavanones, flavones, and flavanols, among others ^{88, 96}. Most of these molecules are polyhydroxylated, a contributing characteristic for their biological activities, namely the antioxidant activity, as this functional group is known to be able to transfer hydrogen atoms to free radicals, neutralizing them ^{88, 92, 99}. Chalcones can also be methoxylated through the transfer of methyl groups by the enzyme **S**-adenosyl-L-methionine-dependent-**O**-methyltransferase to chalcone derivatives ⁸⁸.

Numerous natural chalcones have been identified with biological activities that have been used for medicinal purposes ⁹⁰. Among them, some examples can be given, such as the ones following. The first two examples are chalcones that have been selected for

this study, pinocembrin chalcone and butein, and the third and fourth examples are well-known structurally related chalcones, historically used in traditional medicine, which can be found in a plant that is commonly used, for example in making sweets, liquorice.

Pinocembrin chalcone can be found in extracts of shoots of *Helichrysum trilineatum*, a shrub endogenous from southern Africa (Figure 12) ¹⁰⁰.

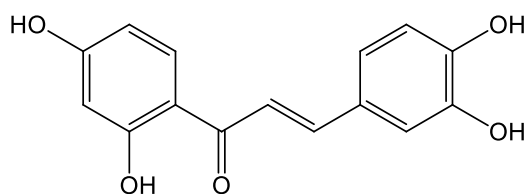


Pinocembrin chalcone



Figure 12. (A) Chemical structure of pinocembrin chalcone and (B) the plant species where it can be found, *Helichrysum trilineatum* ¹⁰¹.

Another example is butein, isolated as extracts from several parts of various plants, such as *Butea frondosa* or *Toxicodendron vernicifluum* (Figure 13), two trees endogenous from the Asian continent ¹⁰²⁻¹⁰⁴.

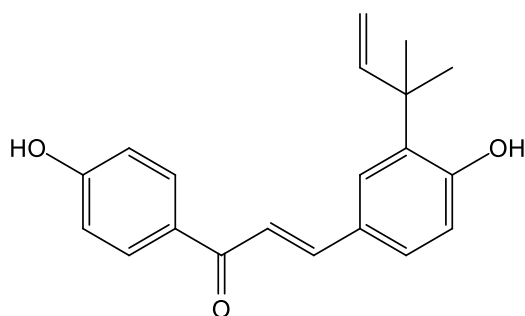


Butein chalcone

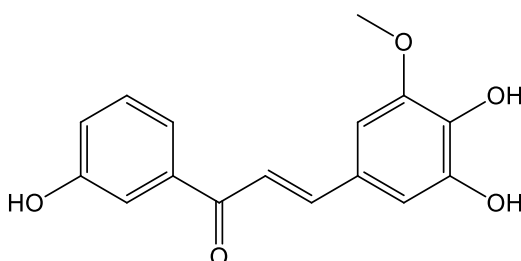


Figure 13. (A) Chemical structure of butein and (B and C) the plant species where it can be found, *Butea frondosa* and *Toxicodendron vernicifluum* ^{105, 106}.

Other examples of naturally occurring chalcones are licochalcones A and B. These chalcones can be isolated from the roots of the liquorice herb (*Glycyrrhiza glabra*) (Figure 14). This plant occurs mainly in northern India, northern Africa, western and southwestern Europe, with some occurrence in the Iberian Peninsula ^{107, 108}



Licochalcone A



Licochalcone B



Figure 14. (A and B) Chemical structures of licochalcone A and licochalcone B and (C) the plant species where they can be found, *Glycyrrhiza glabra* ^{109, 110}.

1.3.3. Chemical synthesis

Although chalcones are present in plants, extracting them from natural sources can be complex, expensive and it may be difficult to find the plant containing the desired compounds. Therefore, chalcones can also be obtained by chemical synthesis, as it allows the production of the desired chalcone from usually simple, cheap, and well-known methodologies ¹¹¹. Also, it allows for manipulation of the core structure, producing derivatives from natural products with optimized biological activities and improved metabolism characteristics ^{92, 93}.

There are several synthetic pathways available to produce chalcones, the main ones being the Claisen-Schmidt condensation (Figure 15A), cross-coupling reactions (consisting of several variations of the same principle, e.g., Suzuki coupling (Figure 15B), Heck coupling, Wittig coupling) and Friedel-Crafts acylation (Figure 15C) ⁹².

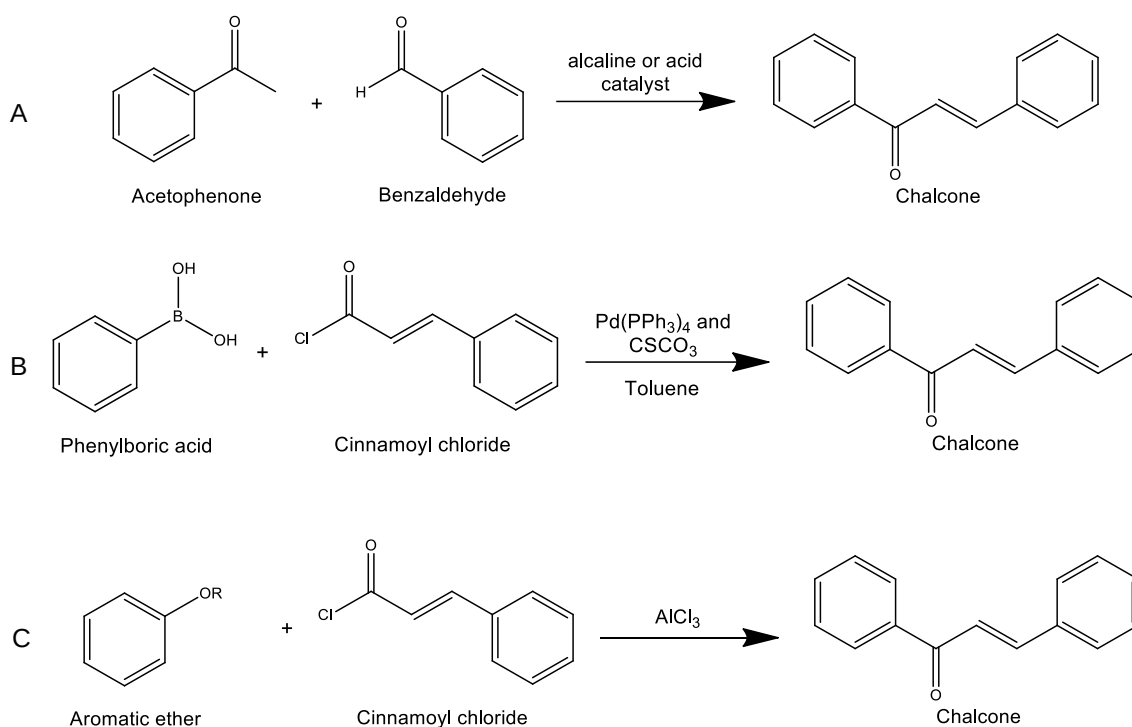


Figure 15. The most common chemical synthesis methods for chalcone obtention: (A) Claisen-Schmidt reaction, (B) Suzuki variation of cross-coupling reaction, and (C) Friedel-Crafts acylation reaction. Adapted from ^{92, 93}.

The most used method is the Claisen-Schmidt condensation (Figure 15A), where a benzaldehyde is condensed with a methyl ketone, utilizing either a strong alkaline or acid catalyst. This reaction is often preferred for its simplicity and high efficiency, allowing for high yields of the desired product ⁹².

Although the Claisen-Schmidt condensation has its advantages, it also has some drawbacks, such as being a slow process that may produce a mixture difficult to purify. Therefore, other reactions, among which are included in the previously mentioned methods, have been applied in chalcone chemical synthesis ⁹².

1.3.4. Biological activities

In a similar manner to flavonoids and other chromone derivatives, chalcones have been used for medicinal purposes for a long time, presenting several biological activities (Figure 16) ^{92, 96, 112-114}.

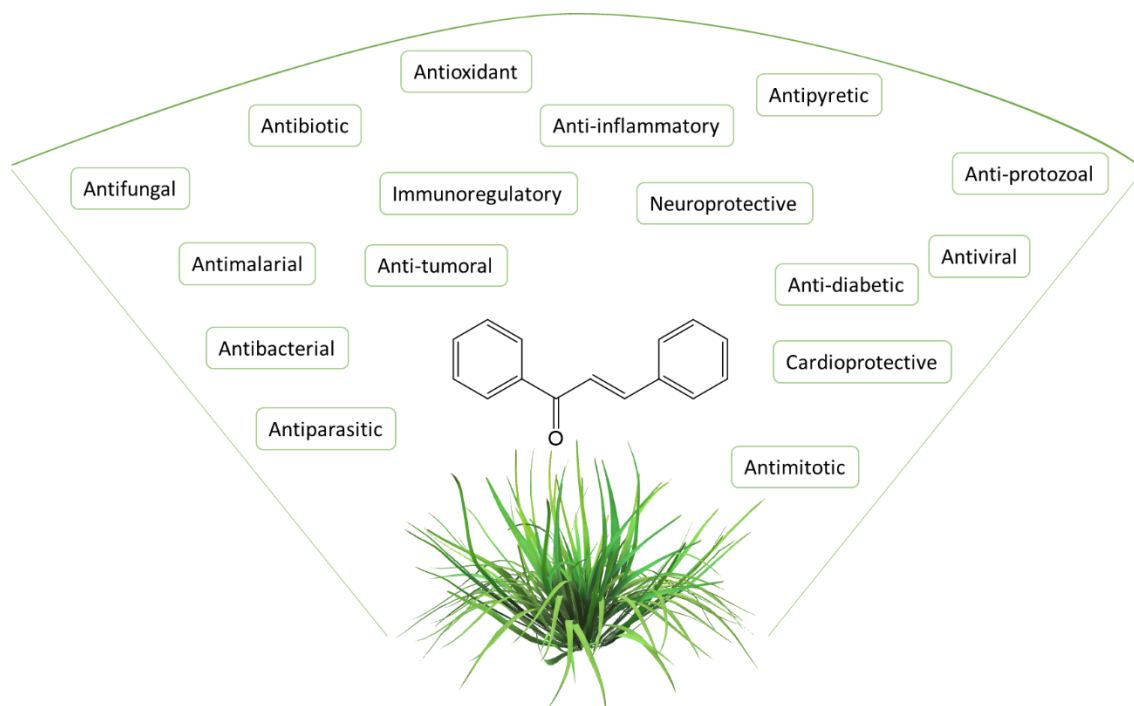


Figure 16. Some of the biological activities described for chalcones.

Using the previously cited naturally occurring chalcone examples, in the table 1 are compiled some of the described biological activities of these compounds.

Chalcone	Biological activities	Ref.
Pinocembrin chalcone	Antibacterial, antifungal, antimutagenic	96, 100, 115, 116
Butein	Anticancer, anti-inflammatory, antioxidant, antiangiogenic, anti-fibrogenic, anti-adipogenic	102, 103, 104, 117, 118, 119, 120, 121
Licochalcone A	Antimalarial, anti-inflammatory, antioxidant, anticancer, anti-adipogenic, anti-arthritis, hepatoprotective, neuroprotective	88, 107, 122, 123, 124, 125
Licochalcone B	Antitumoral, antioxidant, anti-inflammatory, hepatoprotective, neuroprotective	88, 107, 126, 125

Table 1. Described biological activities for some examples of naturally occurring chalcones.

Chalcones with beneficial health properties can also be found in commercially available health supplements, often in the form of pills containing mixes of extracts of chalcone-containing plants or isolated chalcones. An example is hesperidin methyl chalcone, a hesperidin chalcone modified for improved metabolization, used to treat chronic venous diseases ¹²⁷. Analysing the database of the North American FDA department, one chalcone has been approved as a food additive, the Ashitaba chalcone. This chalcone is obtained from the sap of the herb *Angelica keskei*, native to Japan and eastern Asia. There are several benefits in the use of this chalcone in dietary products, as it proved to have various healthy properties, such as antioxidant and anti-inflammatory activities ¹²⁸.

The interest in chalcones' bioactivities and their promising use in medicinal chemistry for the development of drugs with therapeutic potential is jeopardised by their poor bioavailability, poor dissolvability, poor absorption, and metabolism. Therefore, chemical manipulation may be needed to improve the compounds to avoid these limitations ⁸⁸.

2. Aim

This dissertation aimed to evaluate the potential of seven structurally related chalcones to interfere with human neutrophils' function. To assess the mechanisms through which chalcones exerted this effect, their antioxidant and anti-inflammatory activities were evaluated.

Therefore, the following goals were established:

- Evaluation of the ability of the chalcones to directly scavenge physiologically relevant ROS (H_2O_2 , $\text{O}_2^{\cdot-}$, HOCl) and RNS ($\cdot\text{NO}$), in *in vitro* non-cellular models, using chemiluminometric, colourimetric and fluorimetric microanalysis methodologies.
- Assessment of the cytotoxicity of the chalcones against human neutrophils, using a vital stain, trypan blue.
- Evaluation of the ability of the chalcones to modulate human neutrophils' oxidative burst, using a non-specific chemiluminometric probe and a specific fluorimetric probe.
- Evaluation of the ability of the chalcones to interfere with the production of NETs, using a fluorimetric probe.

3. Studied chalcones

The chalcones studied in this work are listed in table 2. The chalcone codes presented, from C0 to C6, will be used hereafter to refer to each one of the chalcones. All of them are commercially available. C0, C3 and C4 were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ, USA). C1 was purchased from Biosynth (Staad, Switzerland). The studied chalcones C2, C5 and C6 were purchased from Extrasynthese (Genay, France).

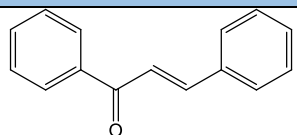
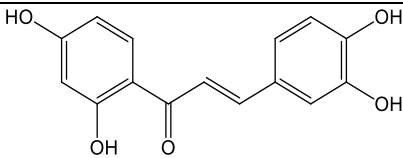
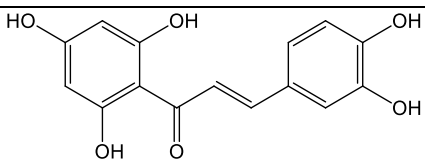
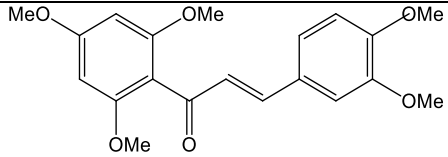
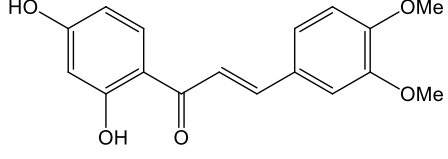
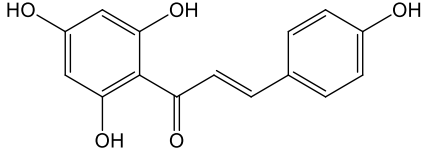
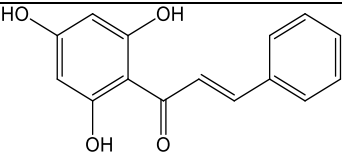
Chemical structure	Chalcone	Chalcone code
	Chalcone	C0
	2',4',3,4-Tetrahydroxychalcone (Butein)	C1
	2',4',6',3,4-Pentahydroxychalcone (Eriodictyol chalcone)	C2
	2',4',6',3,4-Pentamethoxychalcone	C3
	2',4'-Dihydroxy-3,4-dimethoxychalcone	C4
	2',4',6',4-Tetrahydroxychalcone (Naringenin chalcone)	C5
	2',4',6'-Trihydroxychalcone (Pinocembrin chalcone)	C6

Table 2. Chemical structures, names, and attributed codes of the studied chalcones.

4. Materials and methods

4.1. Chemicals

The following reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA): Trizma® base, hydrochloric acid (HCl), potassium phosphate monobasic (KH₂PO₄), potassium phosphate dibasic (Na₂HPO₄), lucigenin, nitroblue tetrazolium chloride (NBT), nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), dihydrorhodamine 123 (DHR 123), dimethylformamide (DMF), quercetin, histopaque 1077 and histopaque 1119, Dulbecco's phosphate buffer saline without calcium chloride (CaCl₂) and magnesium chloride (MgCl₂) (PBS), D-(+)-glucose, trypan blue solution, calcium chloride (CaCl₂), magnesium sulfate (MgSO₄), luminol, phorbol 12-myristate 13-acetate (PMA) and diphenyleneiodonium chloride (DPI). H₂O₂ was purchased from LABKEM Ltd. (Dublin, Ireland). Ascorbic acid was purchased from Alfa Aesar (Ward Hill, MA, USA). Sodium hypochlorite (NaOCl) was purchased from Acros Organics (Geel, Belgium). From Fisher Scientific (New Jersey, NJ, USA), the following reagents were purchased: dimethyl sulfoxide (DMSO), ethanol absolute and sodium chloride (NaCl). Tiron was purchased from Honeywell Fluka (Charlotte, NC, USA). 4,5-Diaminofluorescein (DAF-2) and sodium carbonate (Na₂CO₃) were purchased from EMD Millipore Corporation (Burlington, MA, USA). 3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazinyl]-1-propanamine (NOC-5) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Potassium chloride (KCl) was purchased from Pronalab (Lisboa, Portugal). The fluorescence probes 3'-(*p*-aminophenyl) fluorescein (APF) and SYTOX™ green were purchased from Invitrogen Molecular Probes (Waltham, MA, USA).

4.2. Equipment

The luminescence, absorbance and fluorescence readings were performed in a BioTek Synergy HT microplate reader, with a multi-detection system and temperature control. The fluorescence interference assays were performed in a BioTek Cytation 5 cell imaging multimode reader, equipped with a monochromator. The isolation of fresh human neutrophils through the density gradient centrifugation method was performed using a Beckman Coulter Alegria X-30R centrifuge. The cell viability assays were performed using a hemocytometer and a Nikon Eclipse E200 binocular microscope,

equipped with an image capture camera, connected to an image acquisition software IC Capture 2.3. The NET-bound SYTOX™ green fluorescence readings were performed in a BioTek Cytation 3 cell imaging multimode reader, also equipped with a monochromator.

4.3. *In vitro* non-cellular evaluation of chalcones' scavenging activity of prooxidant reactive species

4.3.1. Preliminary tests

4.3.1.1. Solubility tests

The chalcones and positive controls under study present low or no solubility in aqueous solutions and, therefore, alternative solvents were selected: DMSO or absolute ethanol. The latter was specifically used in the HOCl scavenging methodology, as DMSO is known to scavenge this reactive species¹²⁹. Despite the use of these alternative solvents and since the reaction medium of the performed assays is mainly aqueous, it was necessary to study the solubility of the compounds at different concentrations. Thus, preliminary tests were conducted in transparent microtubes to mimic the experimental conditions of each one of the performed reactive species scavenging assays (each compound tested and the solvent of each component of the reaction mixture) and verify the solubility of the tested compounds at the various tested concentrations, i.e., no precipitation visible at the end of the global time of each assay (maximum of 30 minutes).

4.3.1.2. Chalcones' UV/visible spectra analysis

As all compounds are capable of absorbing energy, to avoid any possible interference with the methodologies used, the ultraviolet-visible (UV/Vis) absorption spectrum of each chalcone and positive controls tested was plotted in the range between 200 and 700 nm, at various concentrations, with the maximum concentration tested being ruled by the solubility assays. These assays were conducted in a 96-well transparent microplate to mimic the experimental conditions of each one of the performed reactive species scavenging assays (each compound tested and the solvent of each component of the reaction mixture).

Each resulting spectrum was analysed with particular attention being given to the relevant wavelengths of the used probes (Table 3). It was considered that there was no interference when the absorbance value at these wavelengths did not exceed the limit of 0.1 absorbance units. According to the Beer-Lambert Law, a value of absorbance equal to 1 means that 90 % of light is being absorbed ¹³⁰. Therefore, the imposed limit of 0.1 absorbance units would mean that only 20 % of the light was being absorbed. Readings above this limit are considered an interference, indicating that the compounds are absorbing a significant amount of energy, which may result in inaccurate readings and affect the validity of the results.

Probes	Wavelengths (nm)	
Chemiluminometric	Absorption	
Lucigenin	470	
Colorimetric	Absorption	
NBT	560	
Fluorimetric	Excitation	Emission
DHR 123	505	530
DAF-2	495	515

Table 3. Relevant wavelengths for each one of the probes used in the performed reactive species scavenging assays ^{70, 131}.

4.3.1.3. Chalcones' fluorescence emission spectra analysis

Chalcones, due to the presence of benzene rings in their chemical structure and depending on the type and location of the substituents present, may be fluorescent ⁹².

Thus, different fluorescence emission assays were performed in a microplate reader equipped with a monochromator, allowing the selection of any relevant excitation and emission wavelength, to ensure that none of the studied compounds interferes with the fluorescence readings of the used methodologies.

Firstly, the UV/Vis spectra were analysed, and emission spectra were plotted in the range of " $\lambda_{\text{maximum absorbance}} + 45 \text{ nm}$ " to 700 nm, with excitation at the wavelength of maximum absorption for each compound. This ensures that at this wavelength, where the compound absorbs the greatest energy, it does not emit fluorescence that would interfere with the readings. Thus, the fluorimetric methodology was mimicked (each compound tested and the solvent of each component of the reaction mixture) in a 96-well microplate appropriate for fluorescence readings, and the emission spectra were analysed at the relevant excitation and emission wavelengths for the probe used (DAF-2 or DHR 123,

Table 2). Interference with the fluorescence readings was considered to exist when the relative fluorescence units (RFU) were significantly higher than the blank value (without the compound and with the solvent of each component of the reaction mixture).

Next, to ensure that the compounds under study do not emit fluorescence when excited at the excitation wavelength of the specific probe used (DAF-2 or DHR 123, Table 2), which would create false positives in the fluorescence readings, the emission spectra were plotted in the range between 528 nm and 700 nm, by exciting the compounds at the probe's excitation wavelength. Thus, each fluorimetric methodology was again mimicked (each compound tested and the solvent of each component of the reaction mixture) in a 96-well microplate, and the emission spectrum was analysed at the relevant emission wavelengths of the used probes. Interference with the fluorescence readings was considered to exist when the RFU were significantly higher than the blank value (without the compound and with the solvent of each component of the reaction mixture).

Finally, to ensure that at no time during the period of the reactive species scavenging assays the compounds under study emit fluorescence when excited at 485 ± 20 nm (excitation interval of the used reader filter), each fluorimetric methodology was mimicked (each compound tested and the solvent of each component of the reaction mixture) in the microplate reader using a filter with excitation at 485 ± 20 nm and emission at 528 ± 20 nm. Then, the RFU were analysed, and interference with the fluorescence readings was considered to exist when the RFU were significantly higher than the blank value (without the compound and with the solvent of each component of the reaction mixture).

4.3.2. Hydrogen peroxide scavenging activity

The scavenging of H_2O_2 , by the studied chalcones, was evaluated by a luminescence assay. In this method, H_2O_2 reacts with the luminescence probe lucigenin, leading to its oxidation. This oxidation reaction produces *N*-methylacridone in an unstable excited state, which, upon transition to the ground state, releases energy by emitting photons, a phenomenon called chemiluminescence (Figure 17) ¹³². The H_2O_2 scavenging activity, by the studied compounds, was assessed through the detection of the luminescence signal in a microplate reader, at 37 °C.

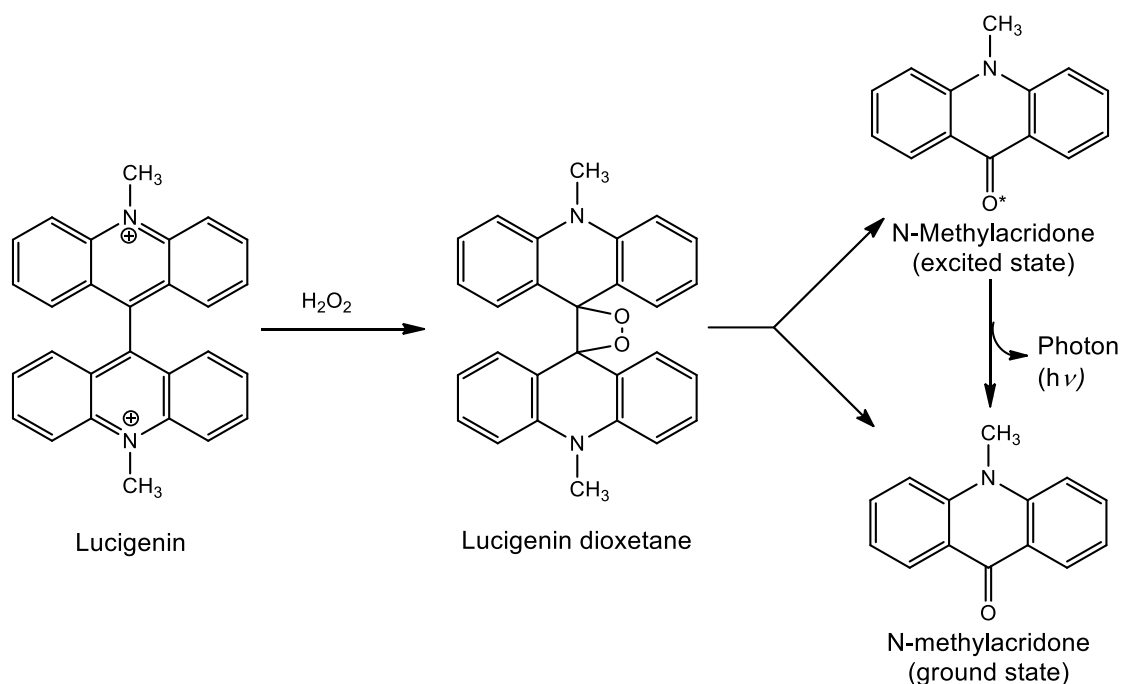


Figure 17. H_2O_2 -induced oxidation of lucigenin with the consequent emission of light ¹³².

The determination of H_2O_2 scavenging activity was performed by evaluating the H_2O_2 -induced oxidation of the luminescence probe lucigenin, using a previously described methodology ¹³³. To each well of a 96-well microplate, appropriate for luminescence readings, the following reagents were added, in the order and final concentrations indicated, totalling a final volume of 250 μL : 91.5 μL of tris-HCl buffer solution (Trizma® base 50 mM at pH 7.4, adjusted with HCl); 50 μL of the compounds under study, at different concentrations ($\leq 2000 \mu\text{M}$); 100 μL of lucigenin (800 μM) and finally, 8.5 μL of H_2O_2 (1 %). Readings were then taken at physiological temperature (37 °C), in a microplate reader. Arbitrary luminescence units were recorded 1 minute after the start of the reading. Ascorbic acid was used as a positive control. Each assay was performed in triplicate in, at least, four independent assays for each studied compound.

4.3.3. Superoxide anion radical scavenging activity

The scavenging of $O_2^{\cdot-}$, by the studied chalcones, was evaluated by a colorimetric assay. In this method, $O_2^{\cdot-}$ is generated through a redox reaction, by the non-enzymatic NADH/PMS system, where PMS acts as an electron transfer catalyst, promoting the O_2 oxidation to $O_2^{\cdot-}$, being reduced by NADH (Figure 18) ¹³⁴.

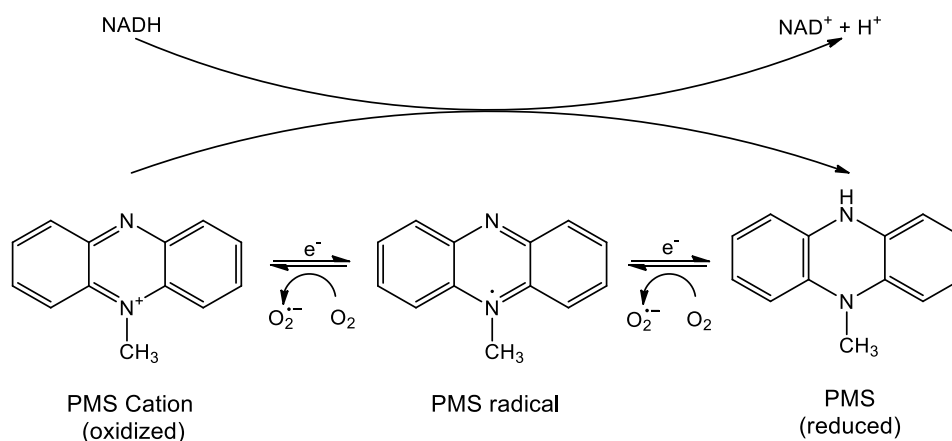


Figure 18. $O_2^{\cdot-}$ generation through the non-enzymatic nicotinamide adenine dinucleotide (NADH) / phenazine methosulfate (PMS) redox system. Adapted from ^{134, 135}.

As $O_2^{\cdot-}$ is generated, by the system mentioned above, it immediately reacts with the colourless probe NBT, leading to its reduction. This reduction reaction produces purple-coloured formazan, that absorbs radiation at 560 nm (Figure 19) ⁷⁰. The $O_2^{\cdot-}$ scavenging activity, by the studied compounds, was assessed through the detection of the absorbance signal in a microplate reader, at 37 °C.

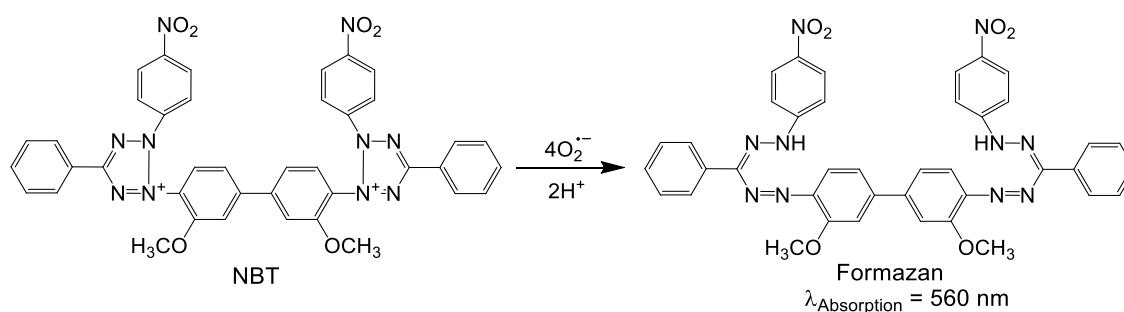
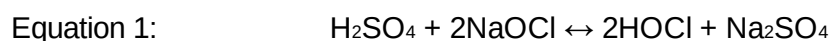


Figure 19. $O_2^{\cdot-}$ -induced reduction of nitroblue tetrazolium chloride (NBT) to formazan. Adapted from ⁷⁰.

The determination of $O_2^{\cdot-}$ scavenging activity was performed by evaluating the $O_2^{\cdot-}$ -induced reduction of the colorimetric probe NBT, using a previously described methodology¹³³. To each well of a 96-well microplate, appropriate for colorimetric readings, the following reagents were added, in the order and final concentrations indicated, totalling a final volume of 300 μ L: 50 μ L of the compounds under study, at different concentrations (≤ 2000 μ M); 50 μ L of NADH (166 μ M); 150 μ L of NBT (43.3 μ M) and finally, 50 μ L of PMS (2.7 μ M). Readings were then taken at physiological temperature (37 $^{\circ}$ C), in a microplate reader. Mean slopes of the colorimetric reaction kinetics, plotted through the measurement of absorbance values at 560 nm, between minutes 1 and 6, after the start of the reading, were recorded. Tiron was used as a positive control. Each assay was performed in triplicate in, at least, four independent assays for each studied compound.

4.3.4. Hypochlorous acid scavenging activity

The scavenging of HOCl, by the studied chalcones, was evaluated by a fluorescence assay¹³⁶. In this method, the reactive species were produced through the adjustment of the pH of a solution of NaOCl (1 %) to 6.2, by the addition of H_2SO_4 (Equation 1).



Upon addition to the reaction medium, HOCl reacts with the fluorescence probe DHR 123, leading to its oxidation. This oxidation reaction produces rhodamine 123, a fluorescent product ($\lambda_{excitation} = 505$ nm and $\lambda_{emission} = 529$ nm) (Figure 20)⁷⁰. The HOCl scavenging activity, by the studied compounds, was assessed through the detection of the fluorescence signal in a microplate reader, at 37 $^{\circ}$ C.

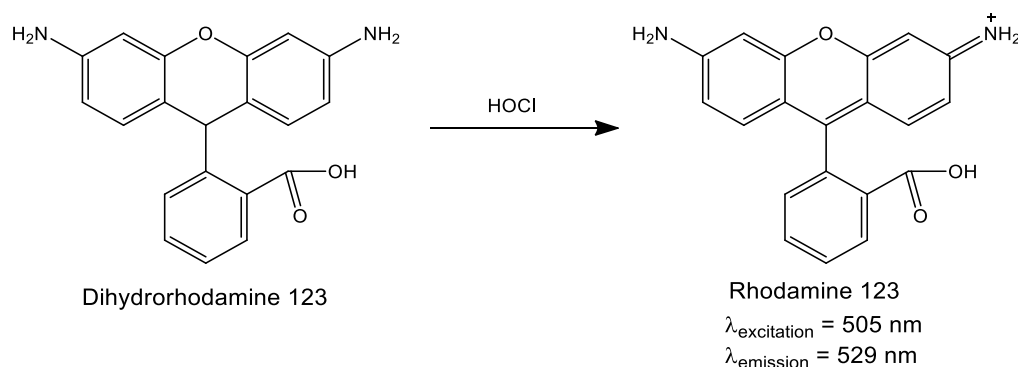


Figure 20. HOCl-induced oxidation of dihydrorhodamine 123 to rhodamine 123¹³⁷.

The determination of HOCl scavenging activity was performed by evaluating the HOCl-induced oxidation of the fluorescence probe DHR 123, using a previously described methodology ¹³⁶. To each well of a 96-well microplate, appropriate for fluorescence readings, the following reagents were added, in the order and final concentrations indicated, totalling a final volume of 300 μ L: 150 μ L of dibasic phosphate buffer solution (100 mM); 50 μ L of the compounds under study, at different concentrations ($\leq$ 300 μ M); 50 μ L of DHR (5 μ M) and finally, 50 μ L of HOCl (5 μ M). Readings were then taken at physiological temperature (37 $^{\circ}$ C), in a microplate reader. RFU, with excitation at 485 $\pm$ 20 nm and emission at 528 $\pm$ 20 nm, were recorded 1 minute after the start of the reading. Quercetin was used as a positive control. Each assay was performed in triplicate in, at least, four independent assays for each studied compound.

4.3.5. Nitric oxide radical scavenging activity

The scavenging of $\cdot\text{NO}$, by the studied chalcones, was evaluated by a fluorescence assay. In this method, the reagent NOC-5 generates $\cdot\text{NO}$, releasing it under physiological conditions (37 °C and pH 7.4) (Figure 21) ¹³⁸.

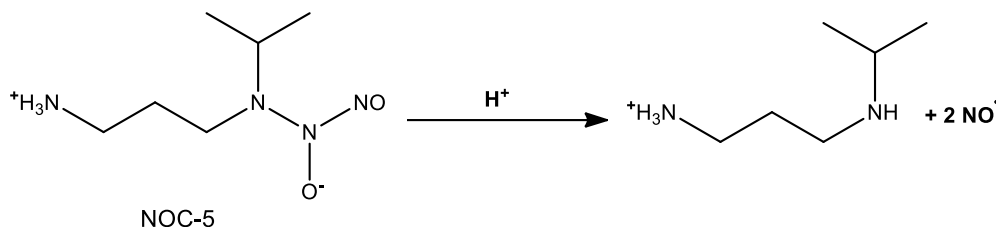


Figure 21. ¹NO-release from 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazinyl]-1-propanamine (NOC-5). Adapted from

Upon its production, $\cdot\text{NO}$ reacts with the fluorescence probe DAF-2, leading to its oxidation. This oxidation reaction produces 4,5-diaminofluorescein triazole (DAF-2T), a highly fluorescent molecule ($\lambda_{\text{excitation}} = 495 \text{ nm}$ and $\lambda_{\text{emission}} = 515 \text{ nm}$) (Figure 22)⁷⁰. The $\cdot\text{NO}$ scavenging activity, by the studied compounds, was assessed through the detection of the fluorescence signal in a microplate reader, at 37 °C.

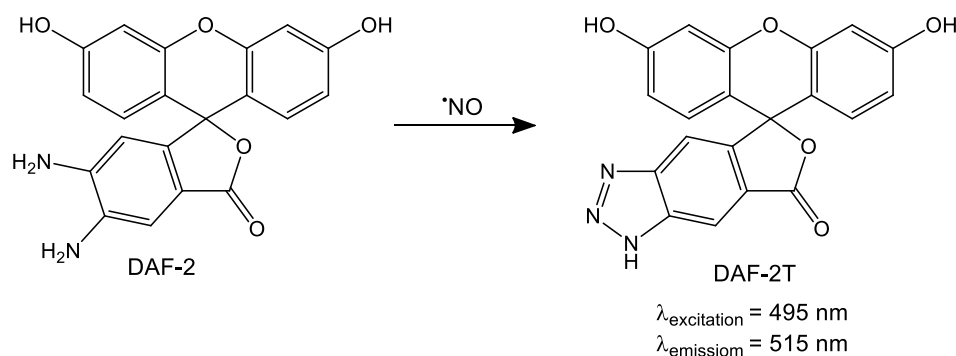


Figure 22. NO -induced oxidation of DAF-2 to DAF-2T. Adapted from ¹⁴⁰.

The determination of NO scavenging activity was performed by evaluating the NO -induced oxidation of the fluorescence probe DAF-2, using a previously described methodology ¹³³. To each well of a 96-well microplate, appropriate for fluorescence readings, the following reagents were added, in the order and final concentrations indicated, totalling a final volume of 300 μL : 200 μL of DAF-2 (5 μM); 50 μL of the compounds under study, at different concentrations ($\leq 800 \mu\text{M}$) and finally, 50 μL of NOC-5 (10 μM). Readings were then taken at physiological temperature (37 $^{\circ}\text{C}$), in a microplate reader. Mean slopes of the fluorescence reaction kinetics, plotted through the measurement of RFU, with excitation at $485 \pm 20 \text{ nm}$ and emission at $528 \pm 20 \text{ nm}$, between minutes 15 and 30 after the start of the reading, were recorded. Ascorbic acid was used as a positive control. Each assay was performed in triplicate in, at least, four independent assays for each studied compound.

4.4. *In vitro* cellular evaluation of chalcones modulation on human neutrophil activity

4.4.1. Human neutrophils isolation

Neutrophils were isolated daily, through a density gradient methodology, as previously reported ¹⁴¹, from whole blood donated by healthy human donors who were previously informed of the purpose of the donations and who gave consent for their use.

Human venous blood was daily collected by antecubital venipuncture, into K₃EDTA vacuum tubes to avoid its coagulation, and the neutrophils were isolated using two different density reagents to perform the separation of the blood constituents. Firstly, in a 15 mL centrifuge tube, 3 mL of histopaque 1077 (density of 1.077 g/mL) were carefully poured over the higher density reagent, histopaque 1119 (density of 1.199 g/mL). Then, 3 to 4 mL of the collected blood were deposited over the cell separation reagents, followed by a centrifugation at 900 relative centrifugal force units (g) for 30 minutes at 20 °C.

After this centrifugation, the different constituents of the blood should be clearly layered. The first three visible layers must be discarded and only the following two, corresponding to the layer of neutrophils and the sub-adjacent layer, which may also contain neutrophils, were to be collected into a new centrifuge tube, always avoiding collecting any content of the lowermost layer, composed of erythrocytes. This tube was then filled with PBS, allowing the use of lower gravitational forces by reducing the viscosity of the histopaque-neutrophil suspension. Then, followed a centrifugation at 850 g for 5 minutes at 4 °C.

After this centrifugation, the supernatant was discarded and 1250 µL of PBS were added to the pellet for resuspension. Then, 4 mL of sterilised water were added to the suspension and the tube was gently inverted for 2 to 3 minutes, to allow for the lysis of erythrocytes present in the cellular suspension. At the end of the lysis time, 2200 µL of NaCl (3 %) were added to the cell suspension, in order to reestablish the isotonicity. Then, followed centrifugation at 850 g for 5 minutes at 4 °C, after which the supernatant was discarded, the pellet resuspended using tris-glucose buffer solution (solution containing NaCl 140 mM, Trizma® base 25 mM, D-(+)-glucose 5.5 mM, KCl 5.37 mM, CaCl₂ 1.26 mM, and MgSO₄ 0.81 mM at pH 7.4, adjusted with HCl) and then the centrifuge tube was placed in ice.

After the isolation of neutrophils, the cellular density of the cell suspension must be determined and adjusted to the density required for the studies to be performed. Therefore, the number of cells per millilitre of neutrophil suspension is determined through the use of a hemocytometer and the trypan blue exclusion method ¹⁴¹. This dye is able to enter the permeable membranes of dead cells, binding to internal proteins and staining them with a blue colour ¹⁴².

Firstly, in a microtube, 20 μ L of neutrophil suspension were added to 10 μ L of trypan blue solution (0.4 %) and resuspended. This preparation was then applied to the hemocytometer (or Neubauer chamber), the cells were counted under a bright field microscope and the cellular density was adjusted to the desired quantity.

4.4.2. Preliminary tests

4.4.2.1. Solubility tests

The chalcones and positive controls under study are insoluble in aqueous solution and, therefore, alternative solvents were selected: DMSO. For the methodology of the modulation of neutrophils oxidative burst evaluated using the fluorescence probe APF, specific for detection of HOCl, the solvent had to be adapted, because, as it is known and was previously mentioned, DMSO interferes with HOCl detection. Therefore, a study was conducted, testing the fluorescence signal of different final percentages of DMSO in the reaction medium, being concluded that the best alternative to be used as a solvent was a mixture of 1:10 (v/v) DMSO/tris-glucose buffer solution (data not shown). Contrary to what was chosen, in the non-cellular assays, as the solvent to avoid the interference of DMSO in HOCl detection, absolute ethanol was not an option, as this solvent would certainly be deleterious to the isolated neutrophils.

Despite the use of this alternative solvent and since the reaction medium of the performed assays is mainly aqueous, it was necessary to study the solubility of the compounds at different relevant concentrations.

Thus, preliminary tests were conducted in transparent microtubes to mimic the experimental conditions of each one of the performed methodologies (each compound tested and the solvent of each component of the reaction mixture) and verify the solubility of the tested compounds at the various tested concentrations, i.e., no precipitation visible at the end of the global time in which each assay (maximum of 4 hours).

4.4.2.2. Chalcones' UV/visible spectra analysis

The study of the chalcones' UV/visible spectra for the *in vitro* cellular studies followed the same principles and methodologies as described in section 4.3.1.2., differing only in the given importance of the absorbance values at the wavelengths of interest, relative to the probes used. Therefore, each resulting spectrum was analysed with particular attention being given to the relevant wavelengths of the used probes (Table 4).

Probes	Wavelengths (nm)	
Chemiluminometric	Absorption	
Luminol	430 (aqueous solution); 500 (DMSO)	
Fluorimetric	Excitation	Emission
APF	490	515
SYTOX™ green	504	523

Table 4. Relevant wavelengths for the probes used in the different performed methodologies ^{70, 131}.

4.4.2.3. Chalcones' fluorescence emission spectra analysis

The study of the chalcones' fluorescence emission spectra for the *in vitro* cellular studies followed the same principles and methodologies as described in section 4.3.1.3., differing only in the given importance of the RFU at the wavelengths of interest, relative to the probes used, APF and SYTOX™ green.

4.4.2.4. Cell viability

To evaluate if the studied chalcones would affect the viability of human neutrophils, the trypan blue exclusion assay was used to determine the cell viability after incubation with the compounds at the maximum testable concentrations (defined by the prior preliminary tests).

The cells that lose their viability, after incubation with the compounds, go through a process in which the cytoplasmic membrane is compromised and the blue dye is then capable of entering the cells, staining internal proteins as it is negatively charged ¹⁴².

The evaluation of the cell viability of human neutrophils after incubation with the studied compounds was performed by counting cells in a hemocytometer using the trypan blue exclusion assay, using a previously described methodology, with adaptations ¹⁴¹. The cellular *in vitro* methodologies were mimicked (each compound tested and the solvent of

each component of the reaction mixture), including the incubation times (maximum of 4 hours).

After the incubation period, 20 μL of neutrophil suspension, in contact with the compound, were added to 10 μL of trypan blue solution (0.4 %) and resuspended. This preparation was then applied to the hemocytometer, the cells were counted under a bright field microscope and determined the cell viability, in percentage.

Each assay was performed in triplicate in, at least, four independent assays for each studied compound ($\leq 100 \mu\text{M}$).

4.4.3. Modulation of human neutrophils oxidative burst

4.4.3.1. Luminol oxidation assay

The modulation of human neutrophils' oxidative burst, by the studied chalcones, was, firstly, evaluated using a luminescence assay. In this method, a non-specific luminometric probe, luminol, was used. This probe is oxidized by both ROS, such as $\text{O}_2^{\cdot-}$, H_2O_2 , HOCl , $\text{HO}^{\cdot}$, and RNS, such as $\text{NO}^{\cdot}$ and ONOO^- , however, at the end time of the performed assays (about 30 minutes), the RNS did not have time to be generated, since the induction of iNOS transcription is required for their production. Also, if the duration of the performed assays was to be extended, to allow the generation of RNS, as these are produced in much inferior quantities, still the contribution to the observable oxidation of luminol would be mainly from ROS⁷⁰.

For these reactive species to be generated, the isolated neutrophils had to be stimulated, mimicking an inflammatory response. This was achieved through their stimulation with PMA, a molecule capable of activating the PKC, leading to the activation of inflammatory pathways, such as the NF- κB pathway, resulting in the stimulation of neutrophils and consequent activation of the NADPH oxidase complex and production of prooxidant reactive species (Figure 23)¹⁴³.

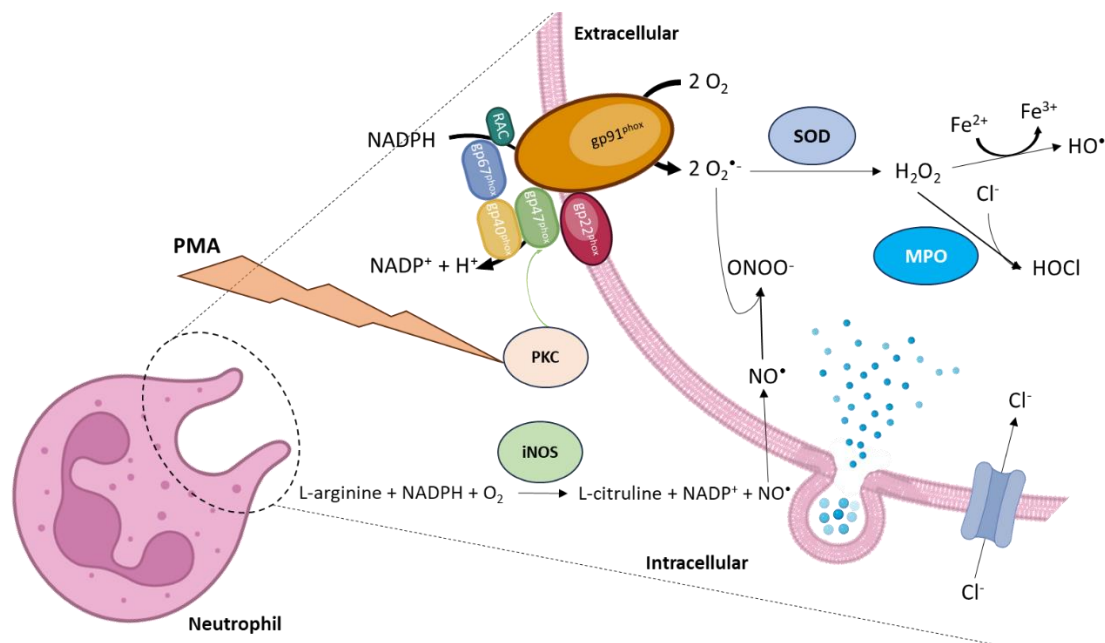


Figure 23. Oxidative burst induction by PMA stimulation. Adapted from ^{143, 144}. Created with BioRender.com. iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase.

The generated ROS react with the luminescence probe luminol, leading to its oxidation. This oxidation reaction produces aminophthalate anion in an excited state, which, upon transition to the ground state, releases energy by emitting photons, by chemiluminescence (Figure 24) ⁷⁰. The modulation of the human neutrophils' oxidative burst detected by luminol oxidation, was assessed through the detection of the luminescence signal in a microplate reader at 37 °C.

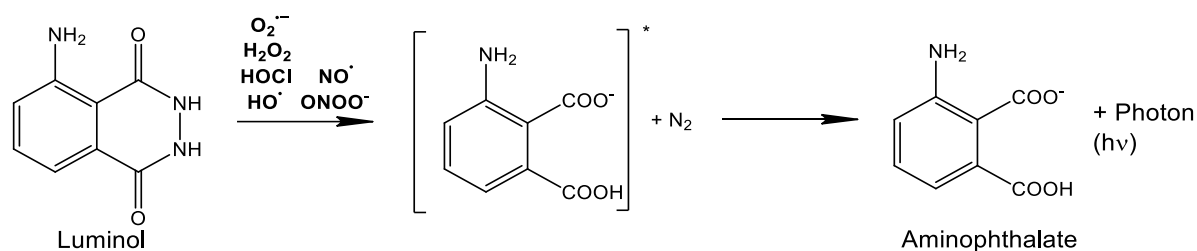


Figure 24. Reactive species-induced oxidation of luminol. Adapted from ⁷⁰.

The modulation of human neutrophils' oxidative burst, by the studied chalcones, was performed by evaluating the unspecific oxidation of the luminescence probe luminol, using a previously described methodology ¹⁴¹. To each well of a 96-well microplate, appropriate for luminescence readings, the following cell and reagents were added, in the order and final concentrations indicated, totalling a final volume of 250 μ L: 140 μ L of neutrophils suspension (1×10^6 cells/mL); 50 μ L of luminol (500 μ M); 10 μ L of the compounds under study, at different concentrations (≤ 100 μ M) and finally, 50 μ L of PMA (160 nM). The cells were pre-incubated with luminol and the tested compounds for 5 minutes at 37 °C before stimulation with PMA. Readings were then taken at physiological temperature (37 °C), in a microplate reader. Relative luminescence units were recorded at the moment in which the value of the control (without the tested compounds) reached its maximum. Quercetin was used as a positive control. Each assay was performed in triplicate in, at least, four independent assays for each studied compound.

4.4.3.2. APF oxidation assay

The modulation of human neutrophils' oxidative burst, by the studied chalcones, was also evaluated using a fluorescence assay. In this method, a specific fluorescence probe, APF, was used to detect HOCl. As in the previous method, HOCl generation was achieved through the stimulation of the neutrophils with PMA.

The generated HOCl reacts with the fluorescence probe APF, leading to its oxidation. This oxidation reaction produces a fluorescent molecule ($\lambda_{\text{excitation}} = 490$ nm and $\lambda_{\text{emission}} = 515$ nm) (Figure 25) ¹⁴⁵. The modulation of human neutrophils' oxidative burst was assessed through the detection of the fluorescence signal in a microplate reader at 37 °C.

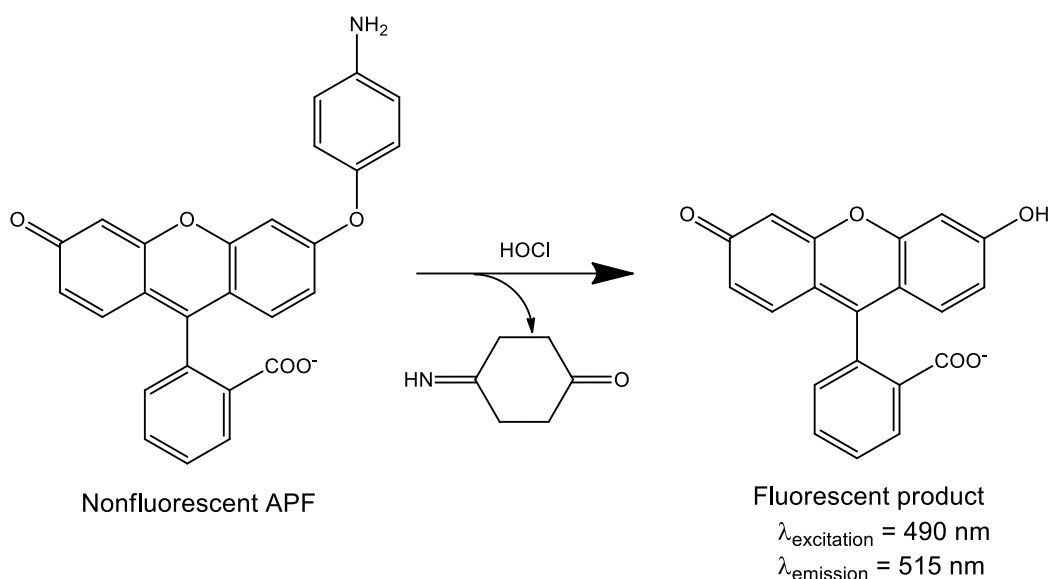


Figure 25. HOCl-induced oxidation of APF. Adapted from ¹⁴⁵.

The modulation of human neutrophils' oxidative burst, by the studied chalcones, was also performed by evaluating the HOCl-induced oxidation of the fluorescence probe APF, using a previously described methodology, with adaptations ¹⁴¹. To each well of a 96-well microplate, appropriate for fluorescence readings, the following cells and reagents were added, in the order and final concentrations indicated, totalling a final volume of 250 μL : 140 μL of neutrophils suspension (1×10^6 cells/mL); 50 μL of APF (5 μM); 10 μL of the compounds under study, at different concentrations ($\leq 40 \mu\text{M}$) and finally, 50 μL of PMA (160 nM). The cells were pre-incubated with APF and the tested compounds for 5 minutes at 37 °C before stimulation with PMA. Readings were then taken at physiological temperature (37 °C), in a microplate reader with soft shaking. Mean slopes of the fluorescence reaction kinetics, plotted through the measurement of RFU, with excitation at $485 \pm 20 \text{ nm}$ and emission at $528 \pm 20 \text{ nm}$, between minutes 8 and 15 after the start of the reading, were recorded. Quercetin was used as a positive control. Each assay was performed in triplicate in, at least, four independent assays for each studied compound.

4.4.4. Modulation of NETs production

The modulation of NETs production, by the studied chalcones, was evaluated by a fluorescence assay. In this method, a molecular fluorescence probe, SYTOX™ green, was used. As in the previous methods, NETs production was achieved through stimulation of the neutrophils with PMA. PMA leads to the generation of ROS, consequently, producing NETs by a ROS-dependent pathway ¹⁴⁶.

The produced NETs are bound to the fluorescence probe SYTOX™ green, a stain capable of binding to nucleic acid. Once bound, there is emission of fluorescence at 523 nm when the probe is excited at 504 nm (Figures 26 and 27) ¹⁴⁷. The modulation of the production of NETs was assessed through the detection of fluorescence in a microplate reader at 37 °C.

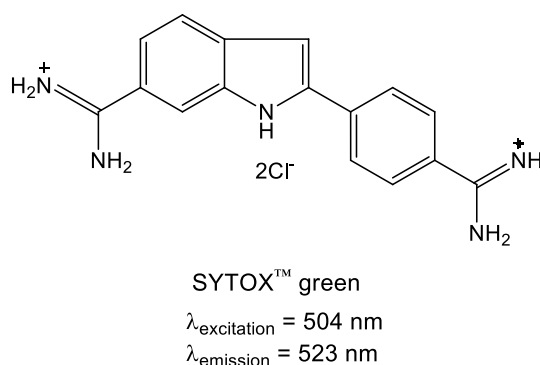


Figure 26. Chemical structure of SYTOX™ green. Adapted from ¹⁴⁸.

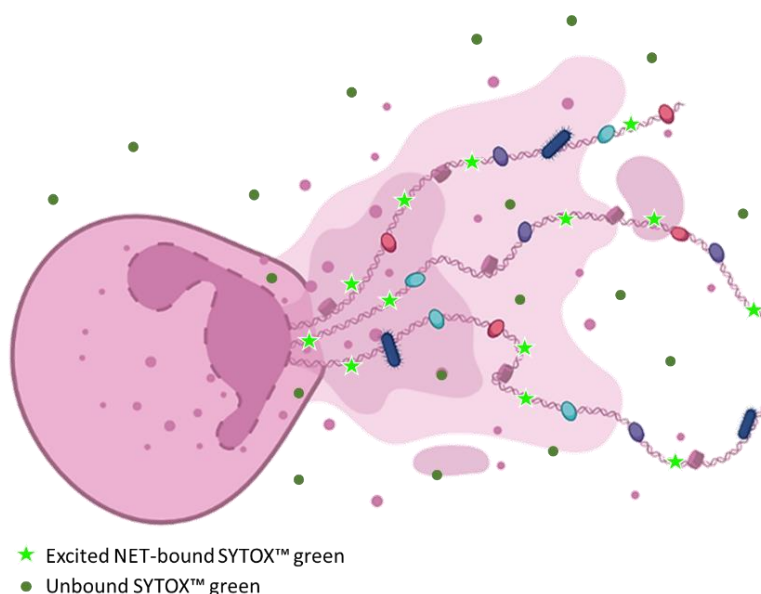


Figure 27. Emission of fluorescence from NET-bound SYTOX™ green, after stimulation with PMA ¹⁴⁷. Created with BioRender.com.

The modulation of NETs production, by the studied chalcones, was performed by the measurement of NET-bound SYTOX™ green fluorescence, using previously described methodologies with adaptations ¹⁴⁹⁻¹⁵¹. To each well of a 96-well microplate, appropriate for fluorescence readings, the following cells and reagents were added, in the order and final concentrations indicated, totalling a final volume of 250 µL: 140 µL of neutrophils suspension (1×10^6 cells/mL); 10 µL of the compounds under study, at different concentrations (≤ 40 µM); 50 µL of SYTOX™ green (5 µM); and, finally, 50 µL of PMA (160 nM). The cells were pre-incubated with the tested compounds for 5 minutes at 37 °C before the addition of SYTOX™ green and stimulation with PMA. Readings were then taken at physiological temperature (37 °C), in a microplate reader with soft shaking. RFU, with excitation at 504 nm and emission at 530 nm, were recorded every 5 minutes for 4 hours. RFU were analysed at 2.5, 3 and 4 hours after stimulation with PMA. Also, mean slopes of the fluorescence reaction kinetics, plotted through the measurement of RFU, with excitation at 504 nm and emission at 530 nm, between hours 3 and 4 after the start of the reading, were recorded. Quercetin and DPI were used as positive controls. Each assay was performed in triplicate in, at least, four independent assays for each studied compound.

4.4.5. Statistical analysis

The results of the performed assays, for the active compounds, are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis and comparison between the active compounds were determined by applying the one-way analysis of variance (ANOVA). Differences among the analysed groups, compared by the Tukey test, with a p -value < 0.05 were considered statistically significant. All the statistical analysis was performed using GraphPad Prism™ (version 8.0; GraphPad Software).

5. Results

5.1. General information

The results are expressed as the percentage of inhibition of the probe oxidation/reduction by the compounds under study, calculated using the following equation:

$$\text{Inhibition (\%)} = 100 - \left(\frac{A-B}{C-B} \times 100 \right)$$

A = mean of the values of the arbitrary signal of the duplicates or triplicates, in the presence of the tested compounds and of the reactive species (non-cellular assays) or with stimulation with PMA (cellular assays).

B = mean of the values of the arbitrary signal units of the duplicates or triplicates of the blank [absence of the tested compounds and of the reactive species (non-cellular assays) or without stimulation with PMA (cellular assays)].

C = mean of the values of the arbitrary signal units of the duplicates or triplicates of the control [absence of the tested compounds and presence of the reactive species (non-cellular assays) or with stimulation with PMA (cellular assays)].

For the most active compounds, the results are expressed as IC₅₀ values [mean ± SEM (standard error of the mean)], i.e., concentration for which 50 % of the effect is observed. For the less active compounds, for which it was not possible to calculate the IC₅₀ value, the results are presented as the mean value of the percentage of inhibition at the maximum tested concentration.

The neutrophils' viability immediately after the isolation procedure and after the incubation period with the tested compounds was calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Viable cells counted}}{\text{Total cells counted}} \times 100$$

Cellular density of the neutrophil's suspension is calculated by the following equation:

$$\text{Cell density (cells/mL)} = \frac{\text{Viable cells counted} / \text{Quadrants counted}}{\text{Neubauer chamber volume}} \times \text{Dilution factor}$$

5.2. *In vitro* non-cellular evaluation of chalcones' scavenging activity of prooxidant reactive species

5.2.1. Hydrogen peroxide scavenging activity

From the analysis of the solubility tests results and the obtained UV/Vis spectra for each of the tested compounds, the maximum or limit concentrations possible to be tested were established and they are displayed in table 5, as the highest concentration of the range of tested concentrations.

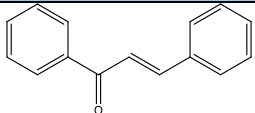
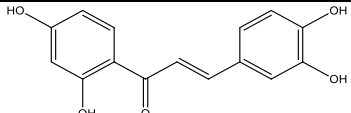
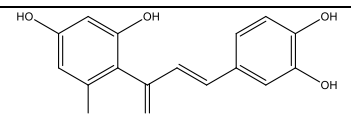
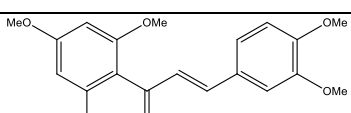
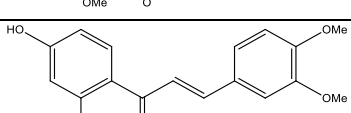
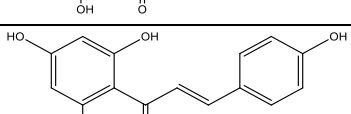
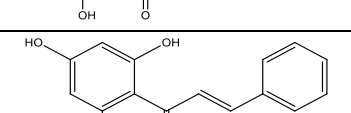
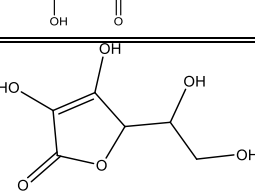
Studied compounds	Chemical structure	Range of tested concentrations (μM)
C0		200 - 700
C1		12.5 - 50
C2		25 - 100
C3		500 - 1400
C4		25 - 100
C5		12.5 - 100
C6		25 - 150
Ascorbic acid		250 - 2000

Table 5. Studied compounds, with the respective chemical structures and range of tested concentrations in the H₂O₂ scavenging assays.

None of the tested chalcones were able to appreciably inhibit the H_2O_2 -induced oxidation of lucigenin, *i.e.* the mean percentage of inhibition was equal or inferior to 30 %, up to the highest tested concentration. The tested positive control, ascorbic acid, presented a concentration-dependent scavenging activity and showed an IC_{50} value of $616 \pm 21 \mu\text{M}$ ($n=5$).

5.2.2. Superoxide anion radical scavenging activity

From the analysis of the solubility tests results and the obtained UV/Vis spectra for each of the tested compounds, the maximum or limit concentrations possible to be tested were established and are displayed in table 6, as the highest concentration of the range of tested concentrations.

Among all studied chalcones, C1 and C2 were able to inhibit, in a concentration-dependent manner (Figure 28), the $\text{O}_2^{\cdot-}$ -induced reduction of NBT, showing IC_{50} values of $53 \pm 2 \mu\text{M}$ and $46 \pm 3 \mu\text{M}$, respectively (Table 6). Chalcones C5 and C6 demonstrated, at the maximum tested concentrations (100 μM), some scavenging activity for $\text{O}_2^{\cdot-}$, with percentages of $50 \pm 3 \%$ and $38 \pm 3 \%$, respectively (Table 6). The tested positive control, tiron, showed an IC_{50} value of $316 \pm 25 \mu\text{M}$ (Table 6 and Figure 28). Chalcones C0, C3 and C4 were not active, up to the highest tested concentrations (400 μM , 800 μM and 200 μM , respectively).

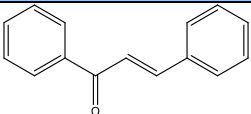
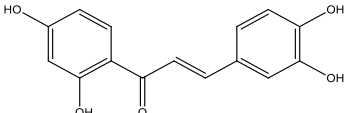
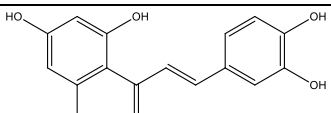
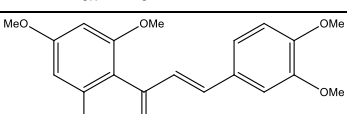
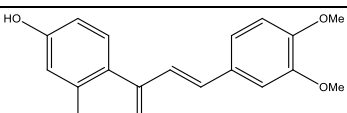
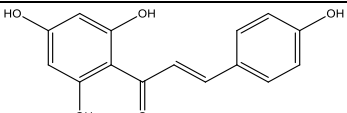
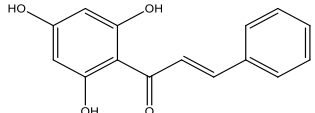
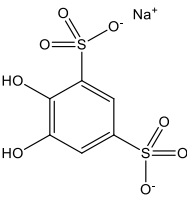
Studied compounds	Chemical structure	IC ₅₀ ± SEM (μM) or inhibition ± SEM (%)	Range of tested concentrations (μM)
C0		NA ^{400 μM}	25 - 400
C1		53 ± 2 μM ^a	25 - 200
C2		46 ± 3 μM ^a	25 - 200
C3		NA ^{800 μM}	6.25 - 800
C4		NA ^{200 μM}	6.25 - 200
C5		50 ± 3 % ^{100 μM}	12.5 - 100
C6		38 ± 3 % ^{100 μM}	25 - 100
Tiron		316 ± 25 μM ^b	125 - 2000

Table 6. O₂⁻ scavenging activity by the studied compounds (IC₅₀ ± SEM, μM, or mean inhibition at the maximum tested concentration, in superscript, ± SEM, %), with the respective chemical structures and range of tested concentrations. NA - No activity found (mean percentage of inhibition equal or inferior to 30 %) up to the highest tested concentration, indicated in superscript.

Similar letters, in superscript, indicate that the difference between the IC₅₀ values is not statistically significant and different letters, in superscript, indicate that the difference between the IC₅₀ values is statistically significant.

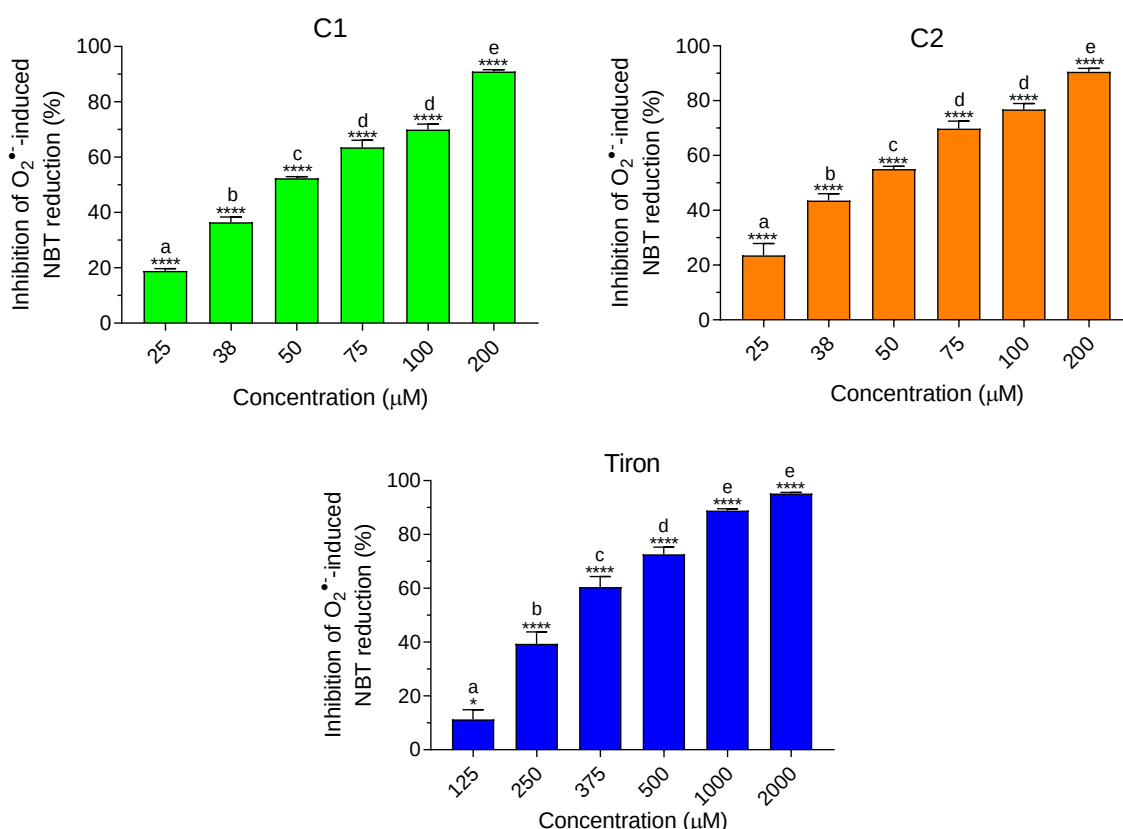


Figure 28. Inhibition of O₂•⁻-induced reduction of NBT by the active chalcones, C1 (*n*=4) and C2 (*n*=4), and the tested positive control, tiron (*n*=6). **p* ≤ 0.05 and *****p* ≤ 0.0001, compared with the control (without compound); similar letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is not statistically significant (*p* > 0.05) and different letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is statistically significant (*p* < 0.05).

5.2.3. Hypochlorous acid scavenging activity

From the analysis of the solubility tests results, the obtained UV/Vis spectra and fluorescence emission spectra for each of the tested compounds, the maximum or limit concentrations possible to be tested were established and they are displayed in table 7, as the highest concentration of the range of tested concentrations.

Among all studied chalcones, C2, C5 and C6 were the most active, being able to inhibit, in a concentration-dependent manner (Figure 29), the HOCl-induced oxidation of DHR 123, showing IC₅₀ values of 0.69 ± 0.05 μM, 0.59 ± 0.04 μM and 0.56 ± 0.05 μM, respectively (Table 7). Chalcone C1 and C4 also demonstrated a concentration-dependent activity (Figure 29), showing IC₅₀ values of 2.0 ± 0.2 μM and 6.9 ± 0.6 μM, respectively (Table 7). The tested positive control, ascorbic acid, showed an IC₅₀ value of 1.03 ± 0.07 μM (Table 7 and Figure 29). Chalcones C0 and C3 showed no scavenging activity, up to the highest tested concentration (300 μM).

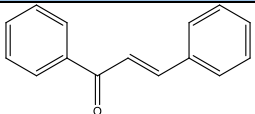
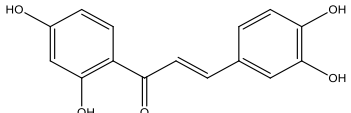
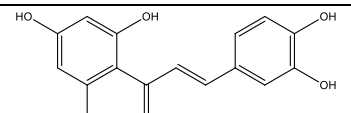
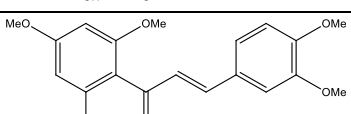
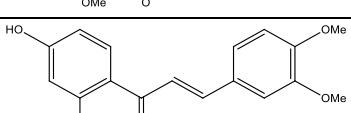
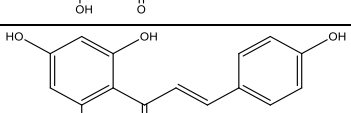
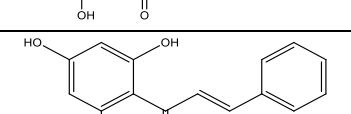
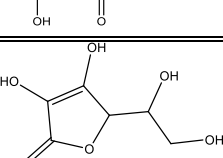
Studied compounds	Chemical structure	IC ₅₀ ± SEM (μM)	Range of tested concentrations (μM)
C0		NA ^{300 μM}	100 – 300
C1		2.0 ± 0.2 μM ^a	0.8 – 25
C2		0.69 ± 0.05 μM ^{a,b}	0.2 – 3.1
C3		NA ^{300 μM}	100 – 300
C4		6.9 ± 0.6 μM ^c	1.6 – 50
C5		0.59 ± 0.04 μM ^{a,b}	0.2 – 3.1
C6		0.56 ± 0.05 μM ^{a,b}	0.2 – 6.3
Ascorbic acid		1.8 ± 0.4 μM ^a	0.4 – 6.3

Table 7. HOCl scavenging activity by the studied compounds (IC₅₀ ± SEM, μM), with the respective chemical structures and range of tested concentrations.

NA - No activity found (mean percentage of inhibition equal or inferior to 30 %) up to the highest tested concentration, indicated in superscript.

Similar letters, in superscript, indicate that the difference between the IC₅₀ values is not statistically significant and different letters, in superscript, indicate that the difference between the IC₅₀ values is statistically significant.

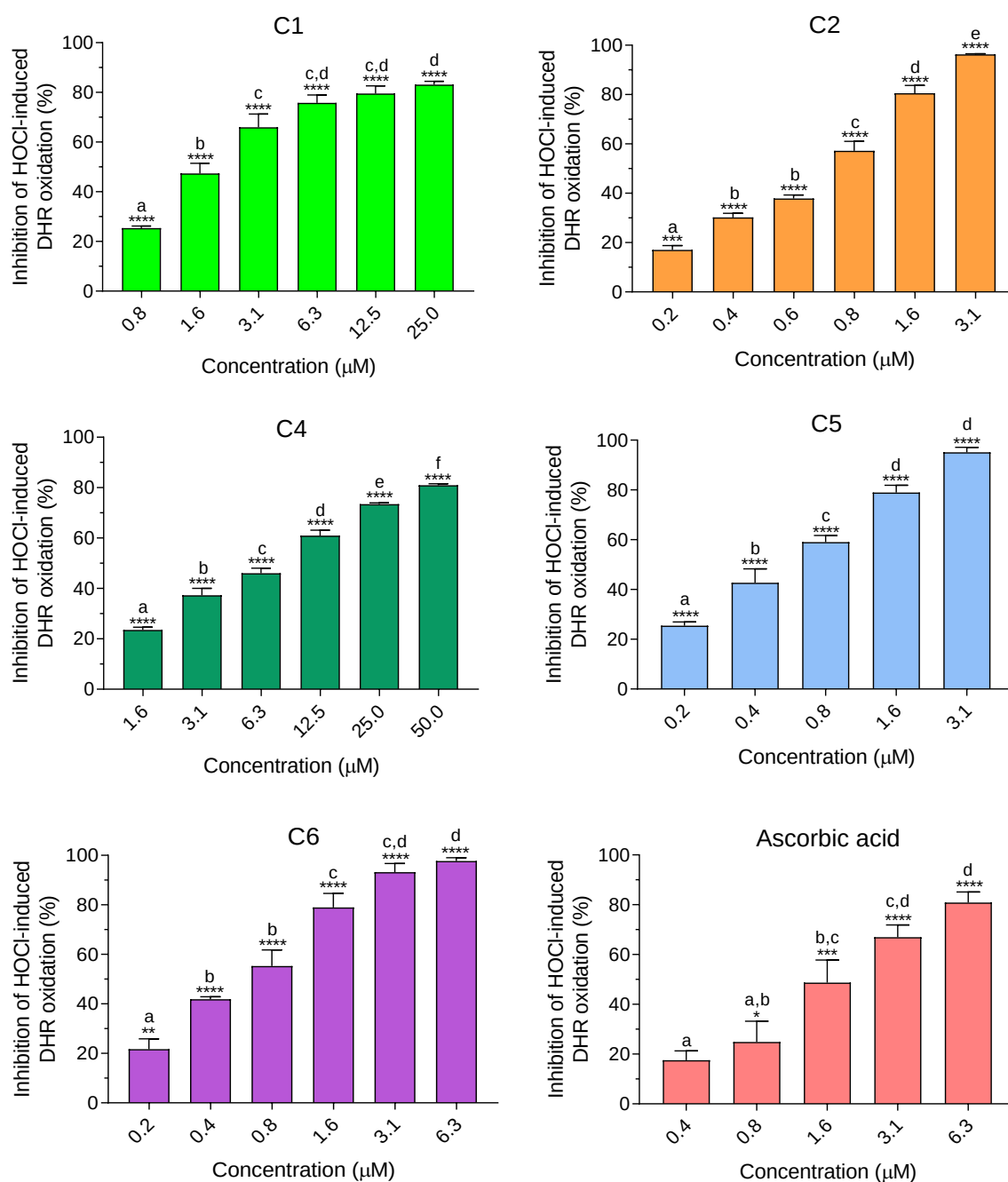


Figure 29. Inhibition of HOCl-induced oxidation of DHR 123 by the active chalcones, C1 ($n=4$), C2 ($n=4$), C4 ($n=5$), C5 ($n=4$), C6 ($n=4$), and the tested positive control, ascorbic acid ($n=4$). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$, compared with the control (without compound); similar letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is not statistically significant ($p > 0.05$) and different letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is statistically significant ($p < 0.05$).

5.2.4. Nitric oxide radical scavenging activity

From the analysis of the solubility tests results, the obtained UV/Vis spectra and fluorescence emission spectra for each of the tested compounds, the maximum or limit concentrations possible to be tested were established and they are displayed in table 8, as the highest concentration of the range of tested concentrations.

Among all the studied chalcones, just C1 and C2 were able to inhibit, in a concentration-dependent manner (Figure 30), the $\cdot\text{NO}$ -induced oxidation of DAF-2, showing IC_{50} values of $2.3 \pm 0.2 \mu\text{M}$ and $13.3 \pm 0.5 \mu\text{M}$, respectively (Table 8). The tested positive control, ascorbic acid, showed an IC_{50} value of $7.9 \pm 0.4 \mu\text{M}$ (Table 8 and Figure 30).

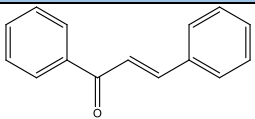
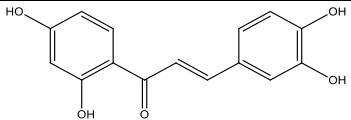
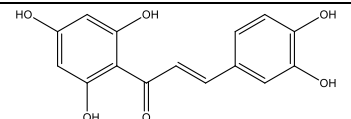
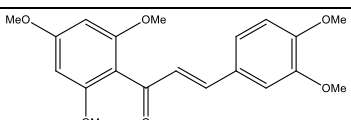
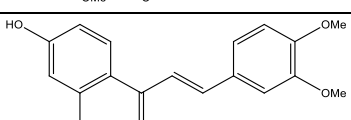
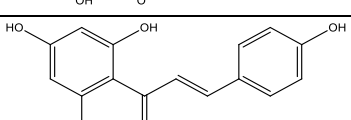
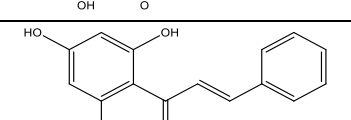
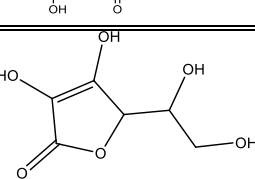
Studied compounds	Chemical structure	$\text{IC}_{50} \pm \text{SEM} (\mu\text{M})$	Range of tested concentrations (μM)
C0		NA ^{400 μM}	50 - 400
C1		$2.3 \pm 0.2 \mu\text{M}^a$	0.4 - 12.5
C2		$13.3 \pm 0.5 \mu\text{M}^b$	1.6 - 50
C3		NA ^{800 μM}	50 - 800
C4		NA ^{200 μM}	25 - 200
C5		NA ^{50 μM}	12.5 - 50
C6		NA ^{200 μM}	25 - 200
Ascorbic acid		$7.9 \pm 0.4 \mu\text{M}^c$	1.6 - 25

Table 8. $\cdot\text{NO}$ scavenging activity by the studied compounds ($\text{IC}_{50} \pm \text{SEM}$, μM), with the respective chemical structures and range of tested concentrations.

NA - No activity found (mean percentage of inhibition equal or inferior to 30 %) up to the highest tested concentration, indicated in superscript.

Similar letters, in superscript, indicate that the difference between the IC_{50} values is not statistically significant and different letters, in superscript, indicate that the difference between the IC_{50} values is statistically significant.

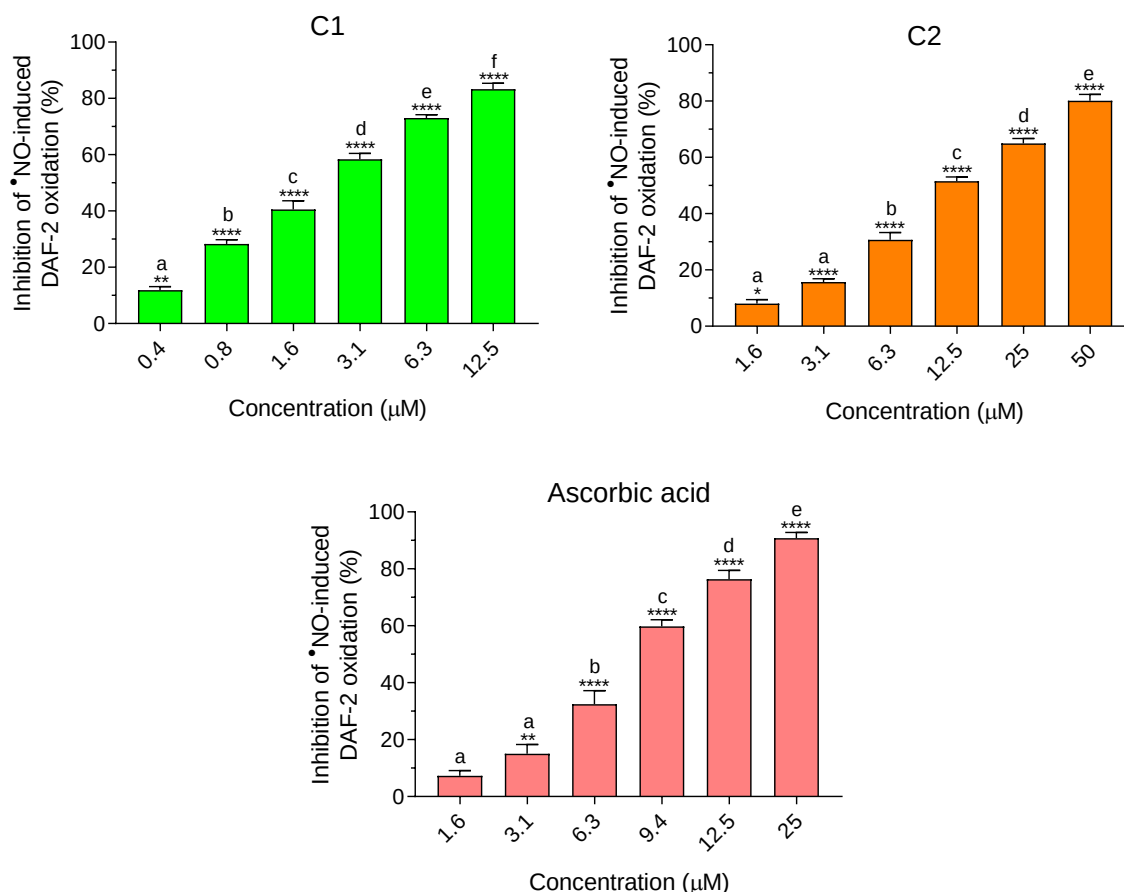


Figure 30. Inhibition of $\bullet$ NO-induced oxidation of DAF-2 by the active chalcones, C1 ($n=4$) and C2 ($n=4$), and the tested positive control, ascorbic acid ($n=6$). * $p \leq 0.05$, ** $p \leq 0.01$ and **** $p \leq 0.0001$, compared with the control (without compound); similar letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is not statistically significant ($p > 0.05$) and different letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is statistically significant ($p < 0.05$).

5.3. *In vitro* cellular evaluation of chalcones' modulation of human neutrophils function

5.3.1. Assessment of solvent cytotoxicity

To determine if the compounds' solvent generally used in the prior assays, DMSO interferes with the viability of human neutrophils, a study of the effect of increasing levels of DMSO on the viability was conducted. These studies were performed using the trypan blue exclusion assay (Figure 31).

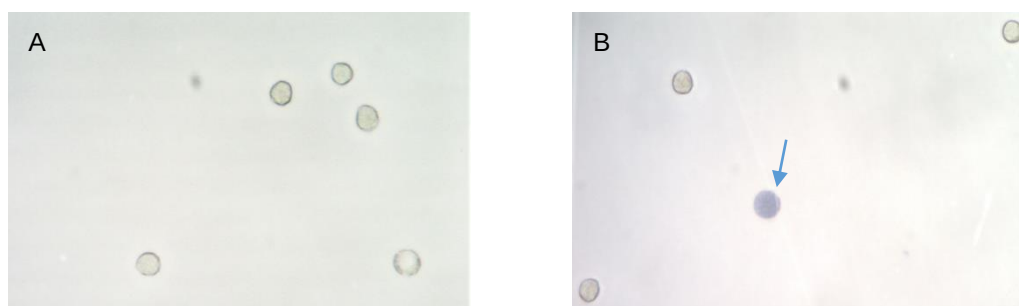


Figure 31. Representative photographs of microscope observation of neutrophils in the trypan blue exclusion assay (400x). (A) Viable cells. (B) Blue arrow points to a non-viable cell, stained by trypan blue.

Of the various increasing levels of DMSO (% , v/v) tested, there was a statistically significant loss of cellular viability, compared to the control (tris-glucose buffer solution instead of DMSO), only for DMSO percentages above and including 10 % (Figure 32).

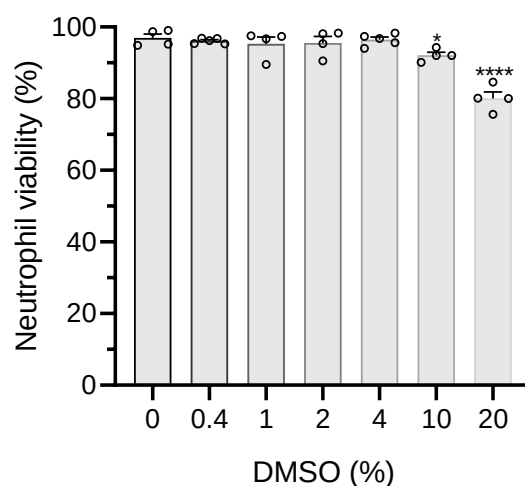


Figure 32. Effect of DMSO on human neutrophils' viability assessed by trypan blue assay. Freshly isolated neutrophils were incubated for 30 minutes with increasing levels of DMSO (% , v/v). * $p \leq 0.05$ and **** $p \leq 0.0001$, compared with the control (tris-glucose buffer, i.e. 0 % of DMSO).

5.3.2. Modulation of human neutrophils' oxidative burst

5.3.2.1. Luminol oxidation assay

From the analysis of the solubility tests results and the obtained UV/Vis spectra for each of the tested compounds, the maximum or limit concentrations possible to be tested were established and they are displayed in table 9.

Compounds	Maximum concentration possible to be tested (μM)
C0	100
C1	6.25
C2	12.5
C3	100
C4	12.5
C5	12.5
C6	25
Quercetin	25

Table 9. Maximum concentration possible to be tested for each compound, based on the preliminary tests.

Also, the effect of the studied chalcones on neutrophils' viability, after 30 minutes of incubation, was assessed testing the maximum concentration possible to be tested (Table 9). The only chalcone that caused a statistically significant loss of neutrophil viability was C0, at 100 μM (Figure 33). Therefore, the maximum concentration possible to be tested for C0 was 50 μM . The effect of the tested positive control, quercetin, on neutrophils' viability was also assessed, however, as supported by the vast known literature, this compound had no undesired effect (data not shown)¹⁵². A loss of viability was considered to exist if the difference between the viability of the tested concentration of the compound and the viability of the control (with the solvent, DMSO 4 % v/v, instead of the compound) was statistically significant.

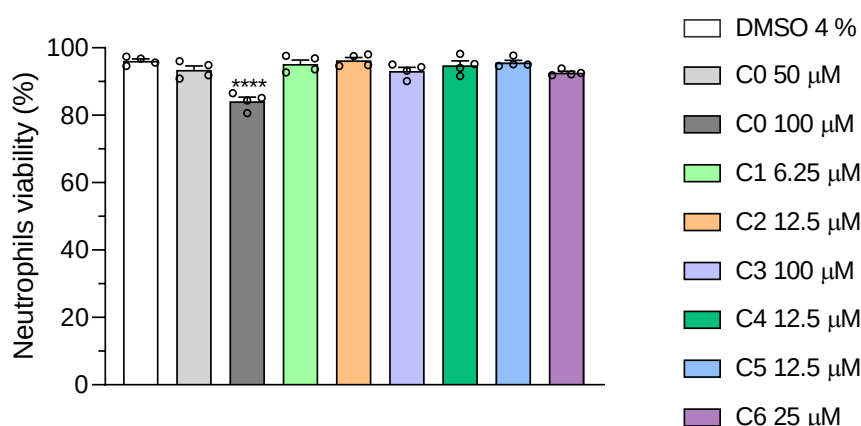


Figure 33. Effect of chalcones on human neutrophils' viability assessed by trypan blue assay under the conditions of luminol oxidation assay. Freshly isolated neutrophils were incubated for 30 minutes with the tested chalcones. **** $p \leq 0.0001$, compared with the control (solvent, DMSO 4 % v/v).

Among all studied chalcones, C0, C1 and C2 were able to inhibit, in a concentration-dependent manner (Figure 34), the ROS-induced oxidation of luminol, showing IC_{50} values of $29 \pm 3 \mu M$, $0.9 \pm 0.1 \mu M$ and $1.60 \pm 0.06 \mu M$, respectively (Table 10). The remaining tested chalcones, C3, C4, C5 and C6, demonstrated, at the maximum tested concentrations (100 μM , 12.5 μM , 12.5 μM and 25 μM , respectively), some inhibition of the oxidation of luminol, with percentages of 37 ± 4 ($n=4$), 47 ± 4 ($n=4$), 39 ± 1 ($n=5$) and 45 ± 2 ($n=4$), respectively (Table 10). The tested positive control, quercetin, showed an IC_{50} value of $1.2 \pm 0.2 \mu M$ (Table 10 and Figure 34).

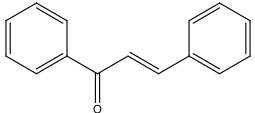
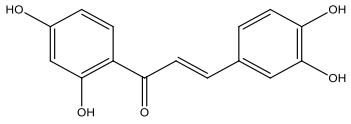
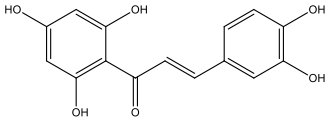
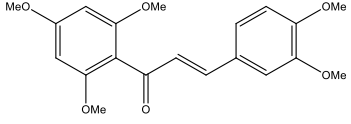
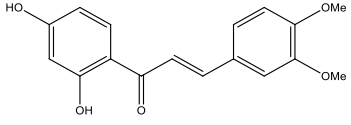
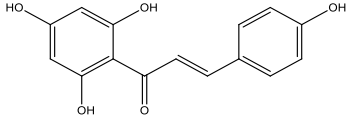
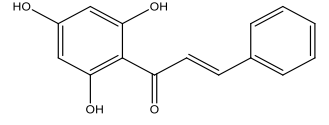
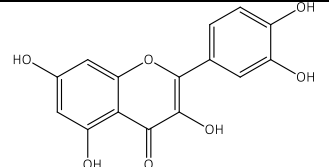
Studied compounds	Chemical structure	IC ₅₀ ± SEM (μM) or inhibition ± SEM (%)	Range of tested concentrations (μM)
C0		29 ± 3 μM ^a	6.3 – 50
C1		0.9 ± 0.1 μM ^b	0.2 – 6.3
C2		1.60 ± 0.06 μM ^b	0.4 – 12.5
C3		37 ± 4 % ¹⁰⁰ μM	25 – 100
C4		47 ± 4 % ^{12.5} μM	1.6 – 12.5
C5		39 ± 1 % ^{12.5} μM	1.6 – 12.5
C6		45 ± 2 % ²⁵ μM	6.3 – 25
Quercetin		1.2 ± 0.2 μM ^b	0.2 – 12.5

Table 10. Modulation of human neutrophils' oxidative burst, detected by the inhibition of luminol oxidation, (IC₅₀ ± SEM, μM or mean percentage of inhibition at the maximum tested concentration, in superscript, ± SEM), with the respective chemical structures and range of tested concentrations.

Similar letters, in superscript, indicate that the difference between the IC₅₀ values is not statistically significant and different letters, in superscript, indicate that the difference between the IC₅₀ values is statistically significant.

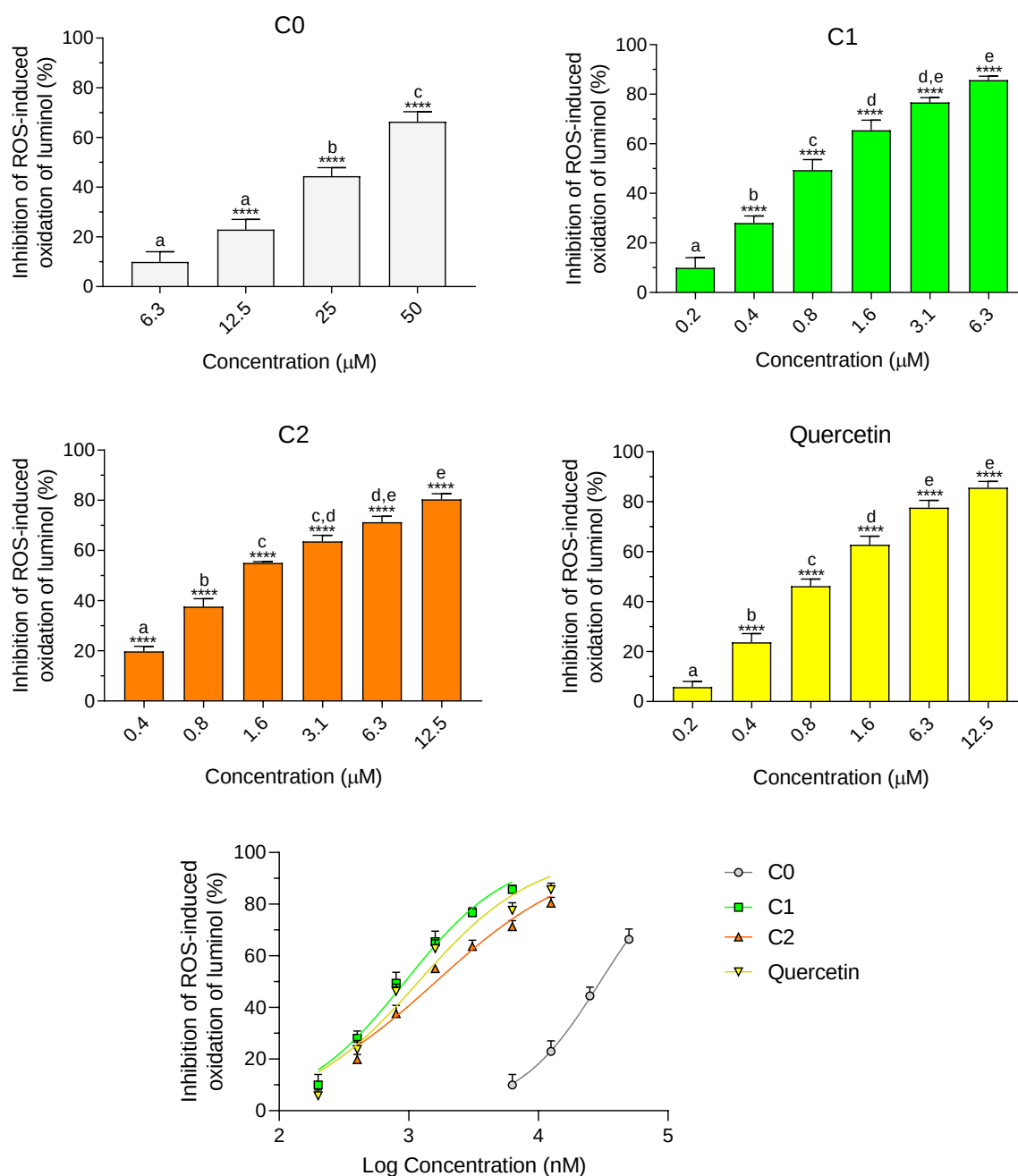


Figure 34. Inhibition of ROS-induced oxidation of luminol by the active chalcones, C0 ($n=6$), C1 ($n=6$) and C2 ($n=4$), and the tested positive control, quercetin ($n=5$) and comparison of the logarithmic concentration-response curve of the active chalcones and quercetin inhibition of ROS-induced oxidation of luminol on human neutrophils. **** $p \leq 0.0001$, compared with the control (without compound); similar letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is not statistically significant ($p > 0.05$) and different letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is statistically significant ($p < 0.05$).

5.3.2.2. APF oxidation assay

From the analysis of the solubility tests results, the obtained UV/Vis spectra and fluorescence emission spectra for each of the tested compounds, the maximum or limit concentrations possible to be tested were established and they are displayed in table 11, as the highest concentration of the range of tested concentrations.

Also, the effect of the studied chalcones on neutrophils' viability, after 30 minutes of incubation, was assessed by testing the maximum concentration possible to be tested (Table 11). None of the studied chalcones caused a statistically significant loss of neutrophils' viability (Figure 35). The effect of the tested positive control, quercetin, on neutrophils' viability was also assessed, however, as supported by the vast known literature, this compound had no undesired effect (data not shown)¹⁵². A loss of viability was considered to exist if the difference between the viability of the tested concentration for the compound and the viability of the control (with the solvent, DMSO 0.4 % v/v, instead of the compound) was statistically significant.

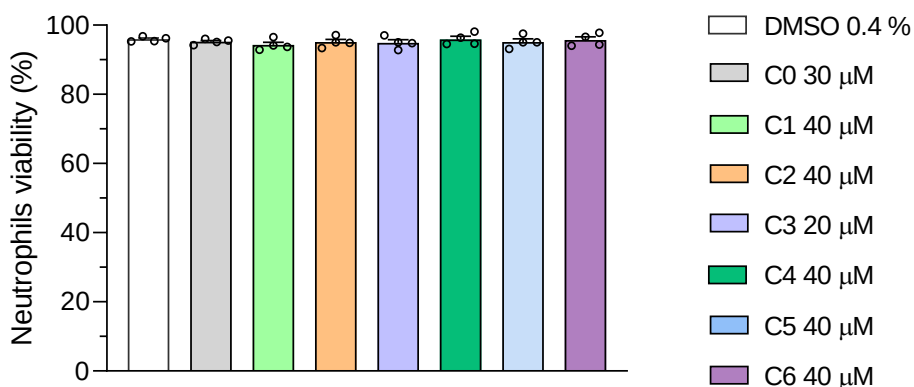


Figure 35. Effect of chalcones on human neutrophils' viability assessed by trypan blue assay under the conditions of the APF oxidation assay. Freshly isolated neutrophils were incubated for 30 minutes with the tested chalcones.

Among all the studied chalcones, C1, C2 and C6 were able to inhibit, in a concentration-dependent manner (Figure 36), the HOCl-induced oxidation of APF, showing IC_{50} values of $1.5 \pm 0.2 \mu M$, $3.7 \pm 0.4 \mu M$ and $4.3 \pm 0.5 \mu M$, respectively (Table 11). Chalcone C0 demonstrated, at the maximum tested concentration, some inhibition of the oxidation of APF, with a percentage of 30 ± 4 ($n=5$), at $30 \mu M$ (Table 11). Chalcone C0 was not active, up to the highest tested concentration, $20 \mu M$. The tested positive control, quercetin, showed an IC_{50} value of $1.07 \pm 0.09 \mu M$ (Table 11 and Figure 36).

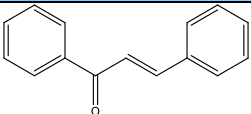
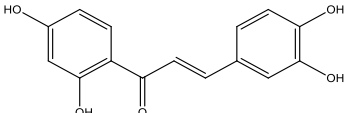
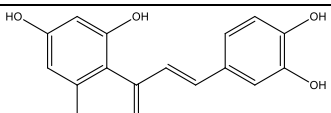
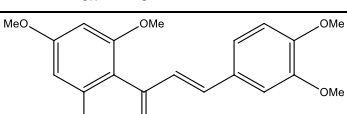
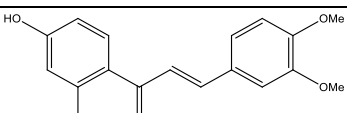
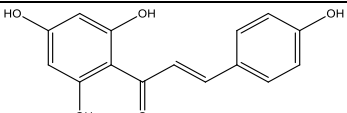
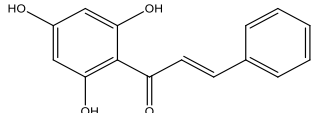
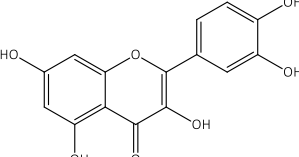
Studied compounds	Chemical structure	IC ₅₀ ± SEM (μM) or inhibition ± SEM (%)	Range of tested concentrations (μM)
C0		30 ± 4 % ³⁰ μM	6.3 – 30
C1		1.5 ± 0.2 μM ^a	0.2 – 40
C2		3.7 ± 0.4 μM ^b	0.4 – 40
C3		NA ²⁰ μM	6.3 – 20
C4		IM	-
C5		IM	-
C6		4.3 ± 0.5 μM ^b	0.1 – 40
Quercetin		1.07 ± 0.09 μM ^a	0.1 – 40

Table 11. Modulation of human neutrophils' oxidative burst, detected by the inhibition of APF oxidation, (IC₅₀ ± SEM, μM or mean percentage of inhibition at the maximum tested concentration, in superscript, ± SEM), with the respective chemical structures and range of tested concentrations.

NA - No activity found (mean percentage of inhibition equal or inferior to 30 %) up to the highest tested concentration, indicated in superscript.

IM - Not possible to be tested due to an interference with the methodology

Similar letters, in superscript, indicate that the difference between the IC₅₀ values is not statistically significant and different letters, in superscript, indicate that the difference between the IC₅₀ values is statistically significant.

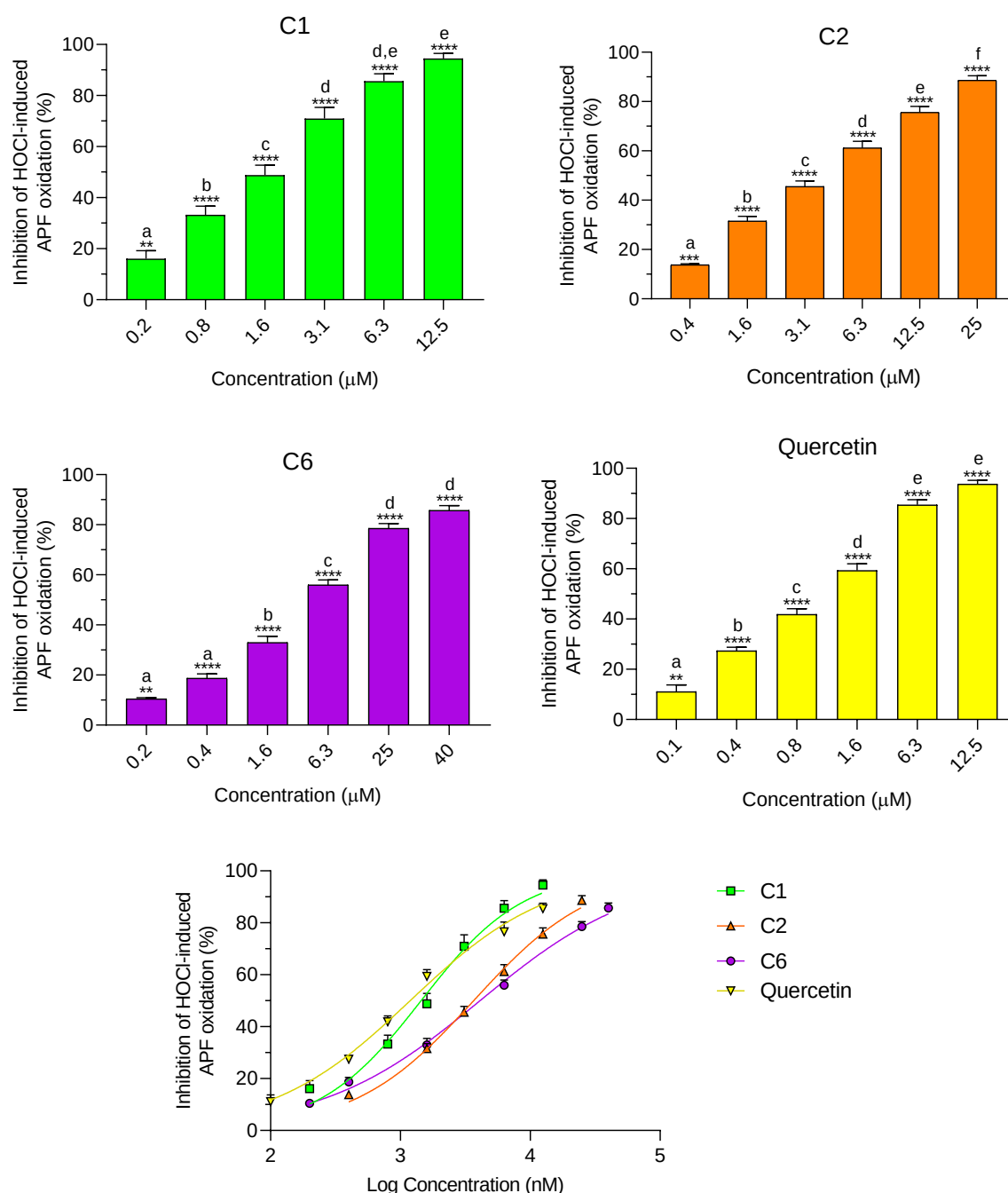


Figure 36. Inhibition of HOCl-induced oxidation of APF by the active chalcones, C1 ($n=6$), C2 ($n=5$) and C6 ($n=6$), and the tested positive control, quercetin ($n=7$) and comparison of the logarithmic concentration-response curve of active chalcones and quercetin inhibition of HOCl-induced oxidation of APF on human neutrophils. $**p \leq 0.01$, $***p \leq 0.001$ and $****p \leq 0.0001$, compared with the control (without compound); similar letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is not statistically significant ($p > 0.05$) and different letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is statistically significant ($p < 0.05$).

5.3.3. Modulation of NETs production

After the previous methodologies were performed, the chalcones showing the most promisor results were selected to be tested for their ability to modulate NETs production. Therefore, C1, C2 and C6 were selected. The ability of quercetin to modulate the production of NETs was studied, since this flavonoid was used in the previous *in vitro* cellular methodologies as a positive control. DPI was used as a positive control.

From the analysis of the solubility tests results, the obtained UV/Vis spectra and fluorescence emission spectra for each of the tested compounds, the maximum or limit concentrations possible to be tested were established and they are displayed in table 12, as the highest concentration of the range of tested concentrations.

The graph in figure 37 contains the results of the assessment of the tested chalcones cytotoxicity, after 4 hours of incubation. None of the studied chalcones or quercetin caused a statistically significant loss of neutrophils' viability (Figure 37). The effect of the tested positive control, DPI, on neutrophils' viability was also assessed, however, as supported by the known literature ^{152, 153}, these compounds had no undesired effect (data not shown). A loss of viability was considered to exist if the difference between the viability of the tested concentration for the compound and the viability of the control (with the solvent, DMSO 0.4 % v/v, instead of the compound) was statistically significant.

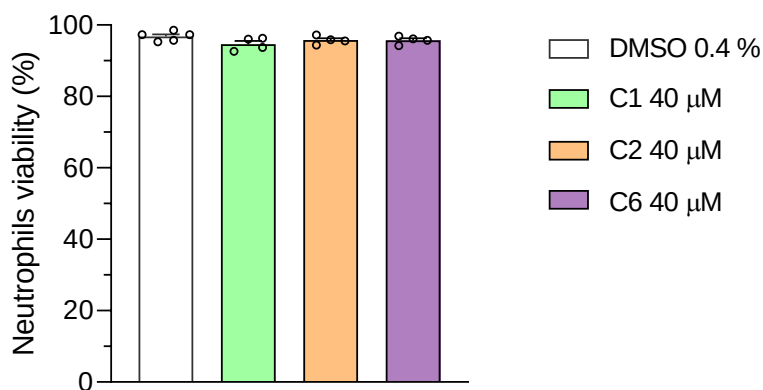


Figure 37. Effect of chalcones on human neutrophils' viability assessed by trypan blue assay under the conditions of the NET-bound SYTOX™ green assay. Freshly isolated neutrophils were incubated for 4 hours with the tested chalcones.

Chalcones C1 and C2 were able to inhibit, in a concentration-dependent manner (Figure 38), the production of NETs by human neutrophils, presenting IC_{50} values of $13.5 \pm 2.7 \mu M$ and $8.8 \pm 2.6 \mu M$, respectively (Table 12). Chalcone C6 demonstrated, at the maximum tested concentration ($40 \mu M$), some inhibition of the release of NETs, with a percentage of 40.9 ± 3.0 ($n=5$) (Table 12). Quercetin was only capable of partially inhibiting NETs production, at the maximum tested concentration ($40 \mu M$), with a percentage of 45.7 ± 12.0 (Table 12 and Figure 38). DPI was used as a positive control, as it is a NADPH oxidase inhibitor and is known as an indirect inhibitor of ROS-dependent NETs production¹⁵⁴. As expected, DPI inhibited NETs production by $91.5 \pm 3.3 \%$ at the maximum tested concentration ($1.6 \mu M$) (Table 12 and Figure 38). Generally, for the remaining tested compounds, at the concentration of $1.6 \mu M$, the inhibition of NETs production was inferior to 30 %. All the results mentioned above are the product of the analysis of the mean slope values of the reaction kinetics defined between the 3 and 4 hours after PMA stimulation of human neutrophils.

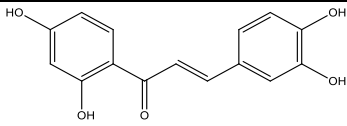
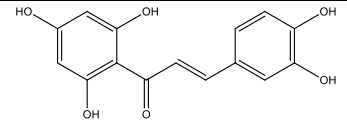
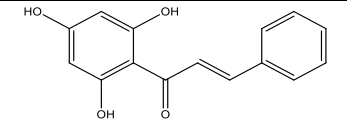
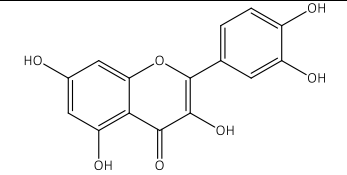
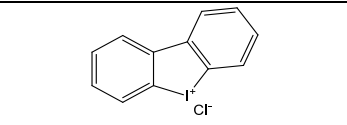
Studied compounds	Chemical structure	$IC_{50} \pm SEM (\mu M)$ or inhibition $\pm SEM$ (%)	Range of tested concentrations (μM)
C1		$13.5 \pm 2.7 \mu M^a$	1.6 – 40
C2		$8.8 \pm 2.6 \mu M^a$	1.6 – 40
C6		$40.9 \pm 3.0 \%^{40 \mu M}$	12.5 – 40
Quercetin		$45.7 \pm 12.0 \%^{40 \mu M}$	3.1 – 40
DPI		$91.5 \pm 3.3 \%^{1.6 \mu M}$	0.016, 1.6

Table 12. Modulation of NETs production, detected by of the inhibition of the binding of SYTOX® green to nucleic acids, ($IC_{50} \pm SEM$, μM or mean percentage of inhibition at the maximum tested concentration, in superscript, $\pm SEM$), with the respective chemical structures and range of tested concentrations.

Similar letters, in superscript, indicate that the difference between the IC_{50} values is not statistically significant and different letters, in superscript, indicate that the difference between the IC_{50} values is statistically significant.

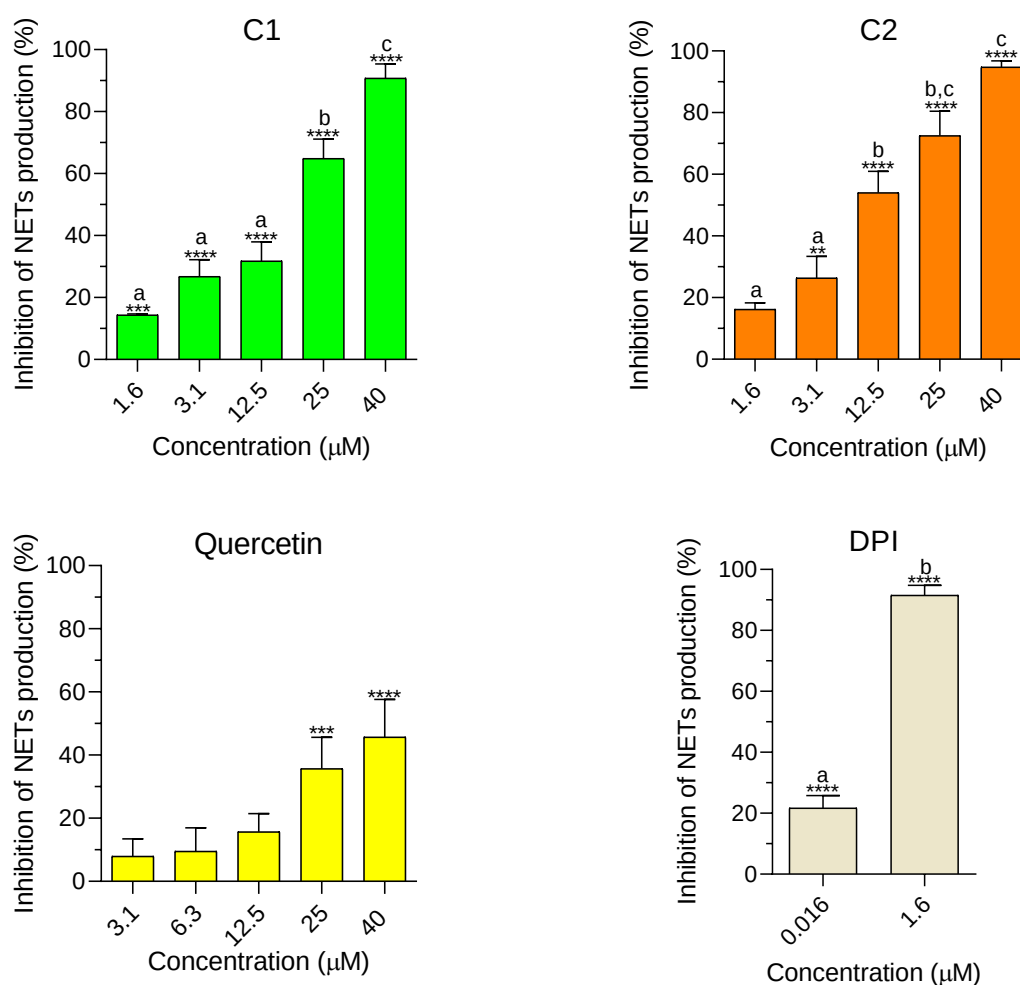


Figure 38. Inhibition of NETs production by the active chalcones C1 ($n=5$) and C2 ($n=4$), quercetin ($n=5$) and the tested positive control, DPI ($n=5$). These results were obtained through the analysis of mean slope values of the reaction kinetics plotted between 3 and 4 hours after PMA (160 nM) stimulation of human neutrophils. $**p \leq 0.01$, $***p \leq 0.001$ and $****p \leq 0.0001$, compared with the control (without compound); similar letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is not statistically significant ($p > 0.05$) and different letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is statistically significant ($p < 0.05$).

In addition to the inhibitory activity of NETs production described above, the mean inhibition percentage values, collected for each tested concentration at 2.5, 3 and 4 hours, after PMA stimulation of human neutrophils, were also analysed and graphically represented. Interestingly, different behaviours on the inhibition of NETs production were found between the positive control tested, DPI, and the chalcones C1, C2 and quercetin.

For the tested positive control, DPI, the inhibition of NETs production was not dependent on the elapsed time after the stimulation of neutrophils with PMA, remaining constant over time (Figure 39).

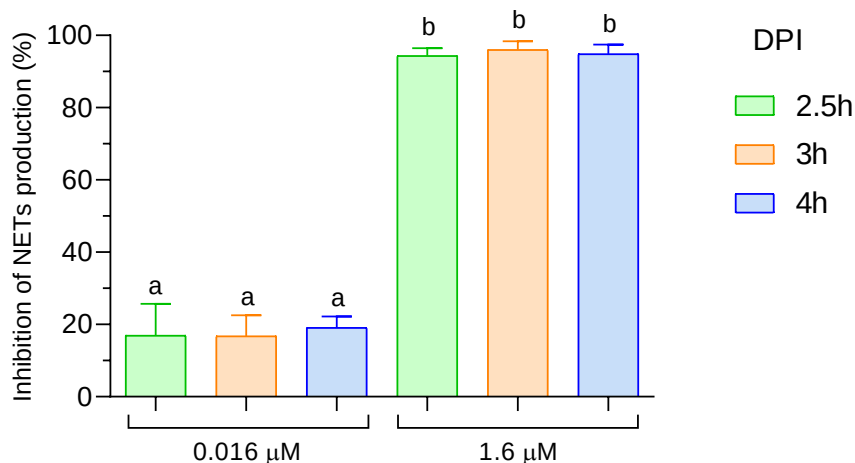


Figure 39. Inhibition of NETs production by the positive control, DPI ($n=5$) at 2.5, 3 and 4 hours after PMA (160 nM) stimulation of human neutrophils. Similar letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is not statistically significant ($p > 0.05$) and different letters above the bars indicate that the difference between the mean inhibition percentage values of each tested concentration is statistically significant ($p < 0.05$).

Interestingly, for chalcones C1 and C2, a distinct inhibitory behaviour was seen. As time elapsed, after stimulation of neutrophils with PMA, the inhibitory activity of the compounds was reduced, suggesting that the active compounds were responsible for either a delay in the production of NETs and not for a drastic inhibition, or due to the amount of produced NETs by 2.5 hours after stimulation being inferior to that released after 3 or 4 hours, resulting in the apparent loss of activity by the chalcones.

Analysing figure 30, it can be seen that for chalcone C1, up to the concentration of 25 μM, there is a statistically significant difference between the mean inhibition percentage values of each concentration at different times, with a tendency for a decrease in said inhibition.

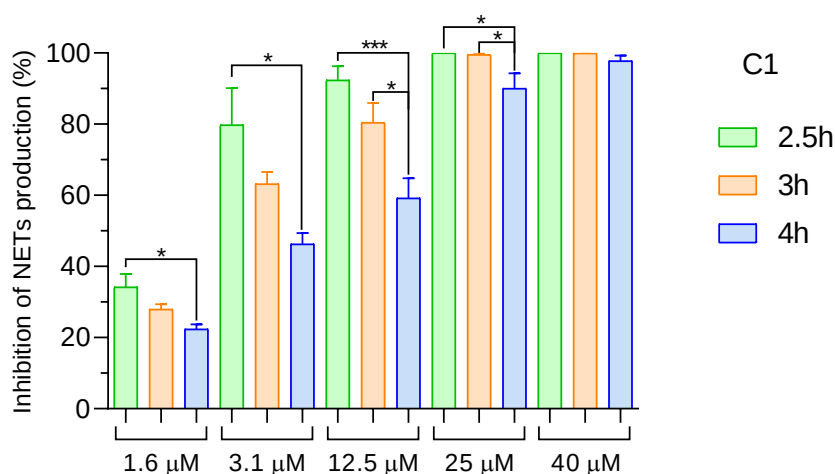


Figure 40. Inhibition of NETs production by the chalcone C1 ($n=5$) at 2.5, 3 and 4 hours after PMA (160 nM) stimulation of human neutrophils. $*p \leq 0.05$ and $***p \leq 0.001$, represent the statistical difference between the mean percentage of inhibition values of the same concentrations at different times.

For chalcone C2, although the differences between the mean inhibition percentage values for the same concentration at different times, apart from at the concentration of 1.6 μM, are not statistically significant, it is possible to visualize the trend of a loss of effect as time elapsed after the stimulation of neutrophils with PMA, up to the concentration of 25 μM (Figure 41).

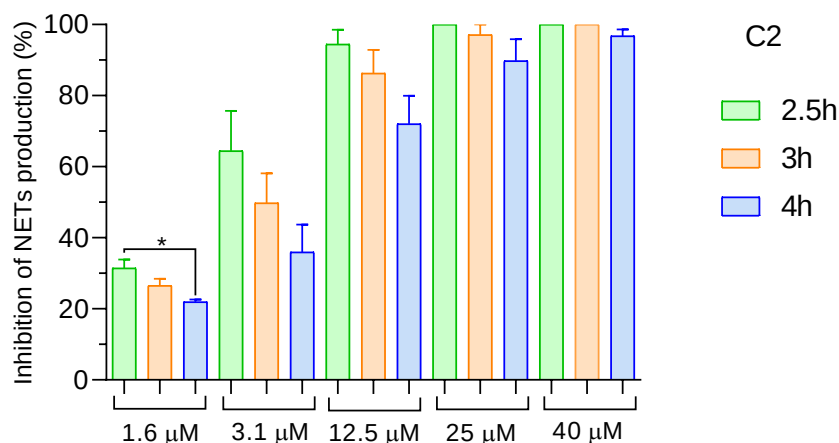


Figure 41. Inhibition of NETs production by the chalcone C2 ($n=4$) at 2.5, 3 and 4 hours after PMA (160 nM) stimulation of human neutrophils. $*p \leq 0.05$, represents the statistical difference between the mean percentage of inhibition values of the same concentration at different times.

6. Discussion

Inflammation is a highly regulated physiological process responsible for the defence of the organism against deleterious stimuli. One of the main cells involved in inflammatory responses are neutrophils, the first responders after the detection of damage and responsible for the orchestration of the innate immune response and for the attempt to eliminate any existing pathogen or damaged cells. These cells can achieve this through different mechanisms, such as the mass production of prooxidant reactive species, phagocytosis and NETosis. Although the importance of these mechanisms is unquestionable, they may get out of control, resulting in further extending the inflicted damage in the affected tissues. Oxidative stress, caused by the excess of generated reactive species, is one of the consequences of an imbalance between the inflammatory response and the body's antioxidant defences and it often leads to tissue damage and the development of diseases. The mechanism of NETosis, if unregulated, is also prone to cause damage to the organism. Therefore, the discovery of compounds capable of treating these conditions, and aiding the organism to recover homeostasis, is of great importance, allowing the development of drugs based on their chemistry.

Natural compounds have been used in medicine for several centuries, as our ancestors discovered and recognized their biological effects and uses to treat ailments and diseases. Chalcones are a family of natural compounds known to be used by Asian cultures in traditional medicine ¹⁰⁷, and, therefore, their biological activities and underlying mechanisms have been studied to better understand what possible applications these compounds could have in the development of new drugs. However, comparing the described results in the literature is difficult, as the studies often vary in the used methodology, the reagents used, the types or lines of cells used, the source of the studied compounds, the concentrations of the tested chalcones (often limited by the used methodology), among other reasons. Therefore, in the present study, the potential antioxidant and anti-inflammatory activities of a panel of seven structurally related chalcones were evaluated through *in vitro* non-cellular ROS and RNS direct scavenging assays and *in vitro* cellular assays, to evaluate the effects of the compounds over the function of human neutrophils. From the selected chalcones, one, in particular, has been extensively studied, albeit not through the used methodologies in the present work, the C1, butein, with several described biological activities, as already enumerated in the introduction section of this thesis. Certain other selected chalcones have also been

studied, using some similar methodologies but, in this present work, their structure-activity relationship will be established for the first time.

In the H_2O_2 scavenging assays, none of the studied chalcones was able to scavenge this ROS. These results seem to indicate that none of the structural characteristics of the studied chalcones benefit the scavenging of H_2O_2 . Several studies highlight the role of the presence of hydroxy groups in the scavenging of H_2O_2 ^{136, 155}. However, by analysing the chemical structure of the studied chalcones, perhaps it is not the number of hydroxy groups that matters, but instead their position in the aromatic rings of chalcones. This claim may be justified by the findings of Sroka et al. ¹⁵⁶, in which the scavenging of H_2O_2 , by phenolic compounds, was seen to be dependent on the presence of three hydroxy groups in ortho position, i.e., in three consecutive carbon atoms of the aromatic ring, a configuration that is seen in neither the A-ring nor in the B-ring of the studied chalcones in this work.

In the $\text{O}_2^{\cdot-}$ scavenging assays, chalcones C1 and C2 were able to scavenge, in a concentration-dependent manner, this ROS, both with similar values of IC_{50} and were approximately six times more potent than the tested positive control, tiron. The chalcones C5 and C6 were able to partially scavenge this ROS. Analysing the structure of the active chalcones, they differ in the number of hydroxy groups present in the A-ring, with C1 having two and C2 having three. In the B-ring, the two chalcones share the presence of a catechol moiety, a structure defined as a benzene ring with two hydroxy substituents in an ortho position to each other. As compounds C1 and C2 are the only ones capable of fully scavenging $\text{O}_2^{\cdot-}$, and simultaneously the only ones with the presence of a catechol group in the B-ring, this feature seems to be the main contributor to the scavenging of $\text{O}_2^{\cdot-}$. It is also important to note that the positive control used, tiron, also contains a catechol group in its chemical structure, reinforcing this conclusion. Analysing the structure of chalcones C5 and C6, both with the presence of three hydroxy groups in the A-ring, the only difference between them is the presence of one hydroxy group in the B-ring of C5, while C6 has an unsubstituted B-ring. The presence of this extra hydroxy group in C5 seems to slightly improve its scavenging activity of $\text{O}_2^{\cdot-}$, as it showed some increase in the inhibition of NBT reduction. Comparing chalcones C2 and C6, which just differ in the presence of a catechol group in the B-ring, the importance of this moiety is once more emphasized, as C2 is more active. Furthermore, comparing the structures of chalcones C1 with C4 and C2 with C3, it seems possible to conclude that the methoxylation of chalcones does not favour the $\text{O}_2^{\cdot-}$ scavenging activity.

In the HOCl scavenging assays, the majority of the tested chalcones were able to scavenge this ROS in a concentration-dependent manner, with chalcones C2, C5 and C6 being the most active (about twice as potent as the tested positive control, ascorbic acid), followed by chalcone C1 (similar activity to the tested positive control), and lastly, the chalcone C4. Analysing the structure of the more active chalcones, C2, C5 and C6, all three have three hydroxy groups in the same positions of the A-ring. In the B-ring, C6 is unsubstituted, C5 has one hydroxy group and C2 has a catechol moiety. Comparing the activity of these three chalcones with the less active ones, C1 and C4, both containing two hydroxy groups in the A-ring, the presence of a larger number of hydroxy groups in this aromatic ring seems to be a contributing structural characteristic for the scavenging of HOCl. These findings are supported by the results of the work of Martins et al.¹⁵⁷, using a similar methodology, in which chalcones with a larger number of hydroxy groups in the A-ring were more active in the scavenging HOCl. When comparing chalcones C1 and C4, it is clear the presence of two hydroxy groups instead of two methoxy groups in the B-ring seems to aid the HOCl neutralising activity, as C1 was roughly three and a half times more potent than C4. Finally, once more, the comparison between chalcones C2 and C3 shows that the methoxylation of these compounds does not favour the scavenging of HOCl. This is also supported by the previously cited work¹⁵⁷, in which methoxylated chalcones also have inferior activity when compared with hydroxylated ones.

In the $\cdot\text{NO}$ scavenging assays, chalcones C1 and C2 were, as seen in the $\text{O}_2^{\cdot-}$ scavenging assays, the only chalcones able to scavenge, in a concentration-dependent manner, this RNS, with C1 being close to four times more potent than the tested positive control, ascorbic acid. Chalcone C2 was slightly less potent than ascorbic acid. None of the remaining tested compounds were able to scavenge this RNS. Analysing the structure of the active chalcones, as previously said, they differ in the number of hydroxy groups present in the A-ring, with C2 having one more hydroxy group than C1. As seen in the $\text{O}_2^{\cdot-}$ scavenging assays, the increased number of hydroxy groups in this aromatic ring does not also seem to influence the scavenging of $\cdot\text{NO}$, as the chalcone C2 is less potent than C1. The presence of a catechol moiety in the B-ring seems, once again, to be the determining factor for the scavenging of the reactive species, as C1 and C2 are the only studied compounds with this structural characteristic and, simultaneously, the only ones capable of actively scavenging $\cdot\text{NO}$. The structure-activity relationship analysis of chalcones C1, C2, C3 and C4 seems to indicate that methoxylation of chalcones once more does not favour the scavenging activity.

The results obtained in the *in vitro* non-cellular reactive species scavenging assays, indicate chalcones C1 and C2 as consistently more active than the remaining studied chalcones, and also generally more potent than the tested positive controls. This means these two chalcones have a broader antioxidant activity spectrum and are also very interesting because of the higher activity compared to reference compounds, like the ones used as positive controls. Chalcones C4, C5 and C6 were only able to scavenge HOCl, in a concentration-dependent manner, meaning these compounds are more selective. Although it may be interesting to find the activity of a compound to be less selective, i.e., capable of scavenging several reactive species, as is the case of C1 and C2, it may also be important to find selective compounds. Selectivity is important in reducing off-target and side-effects, as well as assuring that the scavenging of reactive species will occur with precision, being a factor often taken into account in drug development ¹⁵⁸. It was also observed the importance of the presence of a catechol group in the scavenging of $O_2^{\cdot-}$ and $\cdot NO$. This is due to the readiness of this structure to donate one of the hydrogen atoms to these free radicals, easily neutralizing them. The high ability of compounds containing catechol moieties to scavenge these radical reactive species is also due to a single catechol group being able to neutralize two free radical molecules, as described by Zafar et al. ¹⁵⁹. It should also be highlighted the superior activity of hydroxylated chalcones when compared to methoxylated ones. This is also supported by the review of Zafar et al ¹⁵⁹, as it describes the role of hydroxy substituents in the aromatic rings of polyphenols, where, as previously mentioned, the neutralization of reactive species occurs through the donation of the hydrogen atom of the hydroxy group followed by the stabilization of the molecules through electronic resonance, as the free electron is delocalized in the aromatic structure ¹⁵⁹.

Following the evaluation of the ability of the studied chalcones to directly scavenge physiologically relevant ROS, *ex vivo* cellular studies followed, aiming to evaluate the ability of the studied chalcones to modulate the function of human neutrophils during an inflammatory response. Firstly, as cells may be sensitive to the interaction with chalcones, the cytotoxicity of the studied compounds had to be assessed. Also, there are some indications that the organic solvent DMSO is capable of interfering with the viability and activity of living cells ¹⁶⁰. Thus, a primary study was carried out to assess whether DMSO could harm neutrophil viability. The results show that only above the maximum percentage of DMSO used, 4 %, there was a loss in cell viability. The following cytotoxicity tests carried out showed that, in general, the active chalcones did not present any cytotoxicity at the range of tested concentrations.

The evaluation of the modulation, by the studied chalcones, of the human neutrophils' oxidative burst was carried out, firstly, using a non-specific luminometric probe, luminol, to analyse their ability to interfere with this mechanism globally, as the used probe detects several ROS generated during an inflammatory response. Luminol is a small lipophilic molecule that detects both intra and extracellular reactive species. Then, since in the *in vitro* non-cellular assays of HOCl scavenging, several of the studied chalcones showed to be able to scavenge this ROS, it was studied the modulation of neutrophils' oxidative burst through the detection of HOCl by a specific fluorescence probe, APF, to determine if the previously observed activity upon HOCl would occur in a more complex and physiological-like cellular system.

In the assays using luminol to evaluate the modulation of the human neutrophils' oxidative burst, the most active compounds were C1 and C2, with a very similar potency when compared to the positive control tested, quercetin. The chalcone C0 was also able to modulate oxidative burst in a concentration-dependent manner, although with a higher IC₅₀ value. The remaining chalcones presented some inhibition of luminol oxidation, at the maximum tested concentration. Analysing the chemical structure of the most active chalcones, C1 and C2, the presence of a catechol group still seems to be an essential structural characteristic, as they are the ones that possess this moiety. As previously observed in the reactive species scavenging assays, the substitution of hydroxy groups for methoxy groups results in a significant loss of activity, as seen for compounds C3 and C4. These results are consistent with the observations of Martins et al.¹⁵⁷, where the ability of a panel of chalcones to modulate human neutrophils' oxidative burst was evaluated using the same methodology as in the present work. From the panel of compounds, some chalcones were common to those studied in the present work, namely C0, C1, C2, C3 and C4. The apparent importance of catechol groups in the modulation of neutrophils' oxidative burst was described in the work of Lucas et al.¹⁶¹, where a group of chromone derivatives, 2-styrylchromones, containing a catechol group, were seen to modulate human neutrophils' oxidative burst more potently than structurally related compounds lacking this moiety, using the same methodology as in the present work.

One factor that must be considered when studying the activity of compounds in cellular systems, is the ability of these molecules to penetrate the cellular membrane, something which usually depends on their lipophilicity, size, and charge. Chalcones are small and uncharged molecules, meaning that the permeability of membranes to these compounds depends, mainly on their polarity. Therefore, as it is the most apolar chalcone in the study, C0 showed, for the first time in this study, some activity. This may be linked to it

having the best lipophilicity to penetrate the cells and, once within the neutrophils, modulate their activity. Chalcones C1 and C2 are not as lipophilic, as they possess polar hydroxy groups, however, they showed very interesting activity, by inhibiting the luminol oxidation in a similar potency as the well-known flavonol, quercetin. Also, it may not be necessary for chalcones to enter cells, as their reactive species scavenging may happen, mainly, outside the cytoplasmic membrane. Finally, as all chalcones showed at least some ability to modulate the neutrophils' oxidative burst, it may mean that the chalconoid structure is relevant to this kind of activity.

In the assays using APF to evaluate the modulation of the human neutrophils' oxidative burst, the most active chalcone was C1 (with similar potency to the positive control used, quercetin), followed by C2 and C6, which showed similar activities to each other. It seems that the presence of a catechol group in the B-ring of C1 and C2 aids in their activity, with these compounds showing a potent activity. This finding is supported by the work of Ribeiro et al.¹⁴¹, as the studied flavonoids containing a catechol group presented low IC₅₀ values, in a similar range to the results hereby presented. However, this feature is not determinant as chalcone C6 does not have a catechol and it is almost as potent as chalcone C2. Interestingly, chalcones C1, C2, and C6 were also the most active in the assays where the direct scavenging of HOCl was studied. This may mean that they may modulate neutrophils' oxidative burst by the scavenging of HOCl. In the non-cellular HOCl scavenging assays, the ability of quercetin to neutralize this ROS was also evaluated and it proved to be one of the most active compounds. This showed that the HOCl scavenging activity of quercetin was replicable in a more complex cellular system.

As these chalcones are shown to be active in the modulation of the human neutrophils' oxidative burst, it can be said that one of the mechanisms by which this anti-inflammatory activity is achieved is through the direct scavenging of reactive species generated during the oxidative burst phenomenon.

Following the evaluation of the human neutrophils' oxidative burst by the studied chalcones, the most promising ones were chosen to perform the studies to evaluate their potential to modulate the release of NETs, detected by NET-bound SYTOX™ green, a fluorescent molecular probe that binds to nucleic acid molecules. The study of NETosis is recent and the mechanisms underlying this process are still not fully understood, therefore, the findings in our study may help its understanding. The chosen chalcones were C1 and C2, as they were consistently active in the direct scavenging of physiologically relevant prooxidant reactive species and in the modulation of human neutrophils' oxidative burst, as well as generally presenting stronger activities than

reference compounds used as positive controls. The chalcone C6 was also chosen, as it was able to scavenge HOCl, which may be of interest, as, during the mechanism of NETosis, the enzyme MPO, responsible for the production of this ROS, is involved and there is evidence that HOCl is actually a promoter of NETosis, as it was described by Hallberg et al.¹⁶², even being a more potent NETosis inducer than PMA.

In these assays, C1 and C2 were able to inhibit the release of NETs in a concentration-dependent manner, although with lower activity than the positive control used, DPI. DPI indirectly inhibits NETosis as it is a NADPH oxidase inhibitor, inhibiting the generation of ROS and, therefore, the pathway that leads to nucleic acid release^{163, 164}. Quercetin was also studied to better understand if the activity of the active chalcones was dependent on the neutralization of reactive species, however, this flavonoid was not able to fully inhibit NETs release. As the most active chalcones, C1 and C2, were able to inhibit the production of NETs more potently than quercetin and these three compounds showed similar activities in the scavenging of prooxidant reactive species, this could mean that chalcones C1 and C2 modulate the production of NETs through a mechanism other than the neutralisation of reactive species. Chalcone C6 was also not capable of fully inhibiting NETosis. It only partially modulated it, at the maximum tested concentration. This was perhaps not expected, as this compound was capable of scavenging HOCl and inhibiting neutrophils' oxidative burst with low IC₅₀ values.

The active chalcones, C1 and C2, showed a distinct behaviour in the inhibition of NETosis when compared with the positive control. The activity of these chalcones was variable as time passed since neutrophils' stimulation, decreasing with time, while DPI activity remained unchanged. This may indicate, either, that the increase in produced and released NETs over time results in a reduced activity of chalcones due to insufficient quantity of the compound in contact with NETs, or that the chalcones modulate this mechanism by delaying its onset. This can be interesting as it may regulate the NETosis phenomenon in the first hours of activation of neutrophils during an inflammatory response, aiding the immune response by reducing the scale of the cellular events happening, the possible damage inflicted to the affected tissue, and, also, avoiding thrombotic events as the recruitment of inflammatory cells.

In the work of Sousa et al.¹⁴⁹, the modulation of NETs release by a panel of chalcones was evaluated, with some of these compounds being common to our work, namely C0, C2 and C4. The methodology used was similar to the one used in the present study; however, this work was focused on the chalcones effects in a glucose-enriched

environment. From the studied compounds only the chalcone C2 presented some activity in neutrophils treated with the higher glucose levels.

Although we observed, in this study, that the active chalcones are capable of directly scavenge and neutralize ROS and RNS, there are other possible mechanisms through which these compounds could modulate neutrophils' oxidative burst and NETs release, such as through the interaction with important enzymes involved in the process, especially NADPH oxidase, and through the interference with transcriptional pathways, responsible for the synthesis of signalling molecules and enzymatic systems. These compounds may be exerting their modulatory activity by binding to specific residues in regulatory centres of enzymes' subunits, affecting their function. A way in which inhibitors bind to the specific site in enzymes is through hydrogen bonds. These bonds are possible by sharing a hydrogen atom, donated by a donor group, usually an amine or a hydroxy group, and accepted by an acceptor group. There are compounds capable of inhibiting NADPH oxidase through this mechanism, such as some derivatives of apocynin, a natural methoxy-substituted natural catechol. In the work of Jiang et al.¹⁶⁵, a model for interaction between NADPH oxidase and the analogues of apocynin was used and it was shown that the stronger inhibitors of this enzyme can interact with a lysine residue of the regulatory complex through hydrogen bonds. This interaction results in the inhibition of the translocation of the regulatory subunits to the cell membrane and in the binding of these subunits to the membrane-bound catalytic centre of the enzyme. Indeed, the active compounds possessed free hydroxy groups and it is interesting to see the stronger inhibitor studied, ferulic acid, is structurally similar to chalcones. Enzyme inhibition by certain chalcones has already been described, as is the case of butein (C1), known to be able to inhibit tyrosine kinases¹⁶⁶, α -amylase and α -glucosidase¹⁶⁷.

Several studies also describe the inhibition of several inflammatory transcriptional pathways, such as the NF- κ B pathway, by polyphenols closely related to chalcones. Also, in the work of Pinho-Ribeiro et al.¹⁶⁸, hesperidin methyl chalcone was shown to inhibit the NF- κ B pathway.

The characteristics of the biological activities described for the active chalcones in this work are of major interest for potential application in the treatment of inflammation-related pathologies. It must be highlighted the modulatory effects of chalcones C1 and C2 over NETs production and release, showing a potent inhibitory activity during the first hours of the inflammatory response, but showing a decrease in this activity over time. This mode of action may be extremely important in the regulation of the inflammatory response, controlling the exacerbated inflammatory response in a first stage, where the

massive production of prooxidant reactive species and NETs is undergoing, but then allowing it to occur, as it is essential for the elimination of any pathogen or damaged cells and to allow the organism to recover its homeostasis^{35, 52}. An example in which these chalcones may be of interest for therapeutical use is during sepsis. Sepsis is defined as a consequence of several unregulated defence mechanisms against infection, with hyper-inflammation and immune suppression processes happening simultaneously. This pathologic condition is life-threatening, as the organism is overwhelmed, leading to organ dysfunction¹⁶⁹. During sepsis, the hyper-inflammatory environment, characterized by massive release of proinflammatory mediators, induces the production of prooxidant reactive species and NETs in neutrophils. In the works of Gardiner et al^{170, 171}, NETs are described as having undesirable effects over optimal blood circulation, promoting clotting phenomena and endothelial disruption during sepsis, conditions that may cause death¹⁷². Thus, C1 and C2 anti-inflammatory activities may aid the therapeutical treatment of sepsis, as these chalcones can modulate the action of neutrophils during this condition, reducing the hyper-inflammatory process, allowing the organism to defend itself against the existing infection, but also allowing the regaining of homeostasis and reducing undesirable tissue damage caused by prooxidant reactive species and NETs.

As the discovery of NETs is relatively recent, the pharmacological compounds that could be used to inhibit their production and release, such as hydroxychloroquine or methotrexate, are still undergoing pre-clinical or clinical trials, making it difficult to compare the activity of these compounds with the active chalcones in the present study

Conclusion

In this work, the ability of seven structurally related chalcones to interfere with human neutrophils' function was evaluated. To assess the mechanisms through which chalcones exerted this effect, their antioxidant and anti-inflammatory activities were evaluated. Among the panel of studied chalcones, C1 and C2 presented the most interesting activities, being able to directly scavenge three of the four physiologically relevant prooxidant reactive species studied ($O_2^{\cdot-}$, HOCl and $\cdot NO$), to modulate human neutrophils' oxidative burst, and to inhibit the release of NETs, generally, with superior potency than the positive controls tested.

Concerning the mechanisms by which chalcones act, it is possible to state, based on the results obtained in the non-cellular reactive species scavenging assays, that the compounds may exert their anti-inflammatory activity by directly scavenging ROS and RNS.

Following the analysis of the structure-activity relationships elaborated from the results of this research, certain conclusions can be drawn about the importance of the presence of specific chemical features for the antioxidant and anti-inflammatory activities of chalcones. Firstly, an advantageous feature is the presence of hydroxy groups as substituents in the aromatic rings. Subsequently, it was observed that the replacement of these groups by methoxy substituents is not beneficial for these activities. Secondly, the presence of catechol groups in the B-ring of chalcones also generally favours their activity.

Although chalcones C1 and C2 were largely active in all the performed studies and consequently very promising compounds, it is needed to further investigate their full mechanisms of action, such as evaluating the modulation of the production and release of other inflammatory mediators, the inhibition of inflammation-related enzymes and enzyme complexes, among other functional studies.

This study, therefore, described important information about the structure of biologically active chalcones that can be used in the manipulation of the chalcone scaffold for the design of antioxidant and anti-inflammatory drugs, capable of both inhibiting and modulating neutrophils' inflammatory mechanisms.

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