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Pyrazoles modulate the inflammatory process through the inhibition of COX-2 activity and leukocytes' oxidative burst

Jorge Miguel Almeida Silva

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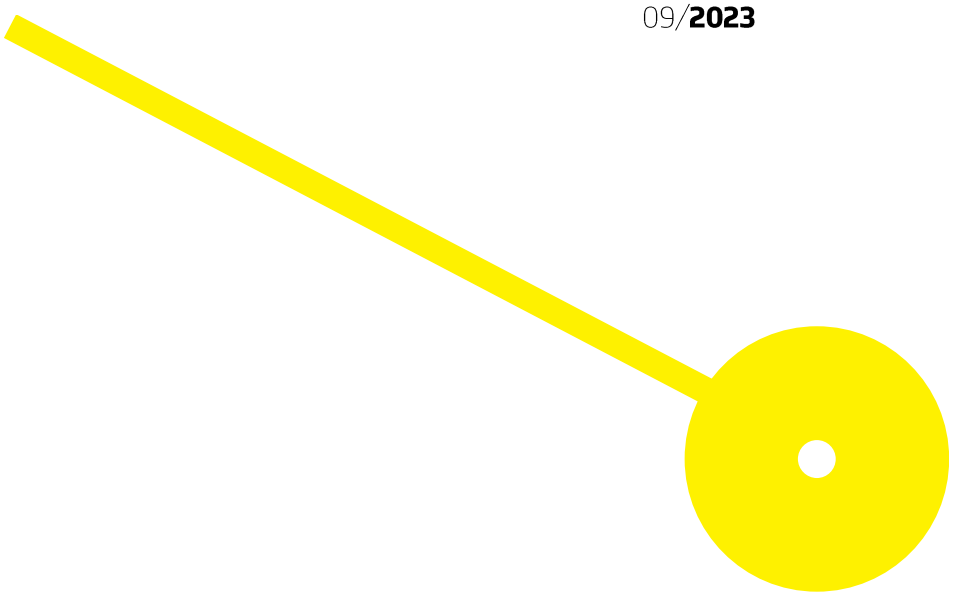

ESCOLA SUPERIOR DE SAÚDE

Pyrazoles modulate the inflammatory process through the inhibition of COX-2 activity and leukocytes' oxidative burst

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Resumo

A inflamação é um processo complexo que inclui várias etapas, destacando-se a atividade da ciclooxigenase 2 (COX-2), bem como, a produção de espécies reativas (RS), durante o *burst* oxidativo dos leucócitos. Atualmente, os fármacos anti-inflamatórios que inibem a COX-2 estão associados a vários efeitos secundários indesejáveis, pelo que será interessante encontrar inibidores seletivos da COX-2 capazes de modular a produção de RS.

Os pirazóis são compostos heterocíclicos aromáticos constituídos por anéis de cinco elementos com três átomos de carbono e dois átomos de azoto. São reconhecidas as atividades biológicas dos pirazóis, nomeadamente a sua ação anti-inflamatória, antibacteriana, antifúngica e anticancerígena.

Neste trabalho, a atividade inibitória de um painel de 28 pirazóis, relacionados estruturalmente foi avaliada nos seguintes mediadores inflamatórios: atividade da COX-2 humana isolada; produção de PGE_2 em sangue; expressão da COX-2 em leucócitos humanos; *burst* oxidativo de leucócitos humanos. Por último, para avaliar a seletividade dos compostos, foi testado o seu efeito inibitório na atividade da COX-1 ovina.

Os resultados revelaram que vários dos pirazóis testados tiveram um efeito inibitório significativo na atividade da COX-2, e os compostos 4 e 11B destacaram-se como os inibidores mais promissores, com $IC_{50} < 25$ µM. No entanto, entre os compostos estudados, apenas o 1A foi capaz de inibir tanto a atividade da COX-2 como a produção de PGE_2. Relativamente à expressão da COX-2, os compostos 14 e 16 destacaram-se por serem capazes de inibir significativamente a sua expressão. O pirazol 11B demonstrou seletividade para a inibição da COX-2, ao contrário do composto 14, que mostrou seletividade para a inibição da COX-1. Muitos dos pirazóis estudados, nomeadamente o composto 4, mostraram um potencial efeito supressor ($IC_{50} < 5$ µM) no *burst* oxidativo dos leucócitos humanos.

Dos pirazóis capazes de inibir ambas as vias (COX-2 e *burst* oxidativo) destacam-se os pirazóis 1B, 4 e 11B.

Este estudo forneceu considerações importantes sobre os pirazóis e o seu promissor efeito modulador do processo inflamatório, o que poderá contribuir para a síntese e desenvolvimento de novas moléculas anti-inflamatórias.

Palavras-chave: Inflamação; Ciclooxigenase 2; *Burst* oxidativo; Pirazóis

Abstract

The inflammation is a complex process which includes several stages, namely the activity of inducible cyclooxygenase 2 (COX-2), as well as the production of reactive species (RS) during the leukocytes' oxidative burst. Currently, the anti-inflammatory drugs that inhibit COX-2 are linked with various undesired side effects, therefore, it would be interesting to find selective inhibitors to COX-2 capable of modulate the RS production.

Pyrazoles are aromatic heterocyclic compounds made up of five-element rings with three carbon atoms and two nitrogen atoms. It is recognised that pyrazoles have a strong anti-inflammatory, antibacterial, antifungal, and anticancer action.

In this work, a panel of 28 structurally related pyrazoles were evaluated through the inhibition of: human COX-2 activity; the production of PGE_2 using a human blood assay; COX-2 expression in human leukocytes; human leukocytes' oxidative burst. Lastly, to assess its selectivity, the compounds were tested *in vitro* against ovine COX-1.

The results revealed that several of the tested pyrazoles had a significant inhibitory effect on COX-2 activity, and compounds 4 and 11B emerged as the most potent inhibitors, with $IC_{50} < 25$ μM. Regardless, amongst the compounds studied only 1A was able to inhibit both the COX-2 activity and the PGE_2 production. Concerning the COX-2 expression, the compounds 14 and 16 stood out since they were able to significantly inhibit its expression. The pyrazole 11B has also demonstrated a selectivity to COX-2, unliked the compound 14, which showed selectivity to COX-1. A multiple of the studied pyrazoles, namely compound 4, showed a potential suppressive effect ($IC_{50} < 5$ μM) against human leukocytes' oxidative burst. At last, various pyrazoles were able to inhibit both pathways (COX-2 and oxidative burst), particularly the pyrazoles 1B, 4 and 11B.

This study provided important considerations about pyrazoles and their promising modulatory effect against inflammation, which might contribute for the design and development of new anti-inflammatory molecules.

Keywords: Inflammation; Cyclooxygenase-2; Oxidative burst; Pyrazoles

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List of abbreviations

AA – Arachidonic acid

AAS – Acetylsalicylic acid

COX – Cyclooxygenase

CYP450 – Cytochrome P450

DAG – Diacylglycerol

DMSO – Dimethyl sulfoxide

EETs – Epoxyeicasatrienoic acid

EPR – Prostaglandin E receptor

HETEs – Hydroxyeicosatetraenoic acid

HHT – 12L-Hydroxy-5,8,10-heptadecatrienoic acid

IC_{50} – Half maximal inhibitory concentration

IL – Interleukin

LOX – Lipoxygenase

LPS – Lipopolysaccharide

MDA – Malonaldehyde

MPO – Myeloperoxidase

MRP4 – Prostaglandin efflux transporter

NADPH – Adenine dinucleotide diphosphate

NK – Natural killer cells

NSAIDs – Non-steroidal anti-inflammatory drugs

PAF – Platelet activating factor

PG – Prostaglandins

PGEs – Prostaglandin E synthase

Phox – Phagocytic oxidases

PI – Propidium iodide

PKC – Protein kinase C

PMA – Phorbol 12-myristate 13 -acetate

Rac 2 – Ras-related C3 botulinum toxic substrate 2

ROS – Reactive oxygen species

RNS – Reactive nitrogen species

RS – Reactive pro-oxidant species

SDS – Sodium dodecyl sulphate

SEM – Standard error of mean

SOD – Superoxide dismutase

TNF – Tumour necrosis factor

Tx – Thromboxanes

TXBSI – Thromboxane synthase inhibitor

General considerations

This work was carried out in the context of the Master's degree in Pharmacy – Technology of Medicines and Health Products from the School of Health of the Polytechnic Institute of Porto. The theme was chosen based on the growing concern around inflammation, and its related diseases. The currently used pharmacotherapy for modulate inflammation through the inhibition of cyclooxigenase-2 is associated with low efficacy and undesirable side effects, so the search for new and safer molecules with capacity to be used to control the inflammatory process is of great importance. Thus, the aim of this study was to evaluate the modulatory effect of a panel of twenty-eight pyrazoles against inflammation, through the inhibition of human cyclooxigenase-2 activity and human leukocytes' oxidative burst.

The experimental component associated with this report was carried out during an internship at LAQV-REQUIMTE, Laboratory of Applied Chemistry, Department of Chemical Sciences, Faculty of Pharmacy of Porto, lasting a total of 1275 hours under the supervision of Doctor Marisa Freitas. The research group where I had the opportunity to do my internship is very diverse, including several master's and doctoral students, as well as post-doctoral researchers.

Structure of the Internship Report

This Internship Report is divided into seven chapters. The first chapter, **Introduction**, refers to a theorical framework with a characterization of inflammation, an approach to its pathophysiology and the associated consequences. In this chapter it is also possible to find information about the pyrazoles. The general and specific objectives of this dissertation are present in the second chapter, **Objectives.** In the third chapter, **Material and Methods**, is described all the experimental aspects related to the *in vitro* activities, namely those related with the evaluation of viability of the cells, inhibition of isolated COX-2 and COX-1, expression of COX-2 and detection of leukocytes' oxidative burst. In the fourth chapter, **Results**, are present all the results obtained for each *in vitro* assay that was performed. In the **Discussion**, there is an integrated discussion of all the obtained results. The chapter six, **Conclusions**, summarizes the main conclusions of the performed work. In the last chapter, **References,** there is the bibliographical references that supported the development of this work.

1. Introdution

1.1. Inflammation

1.1.1. Definition

Inflammation is a physiological response of the body to a local tissue damage or infection (Cavaillon, 2021). Historically, Egyptian papyri dating from around 3000 B.C. already described typical features of inflammation. However, only in the 1st century A.C. a Roman writer, Cornelius Celsus, listed the four cardinal signs of inflammation: redness, swelling, heat and pain. Later, in the 19th century, loss of function was added as the fifth cardinal sign of inflammation (Figure 1) (Freire & Van Dyke, 2013).

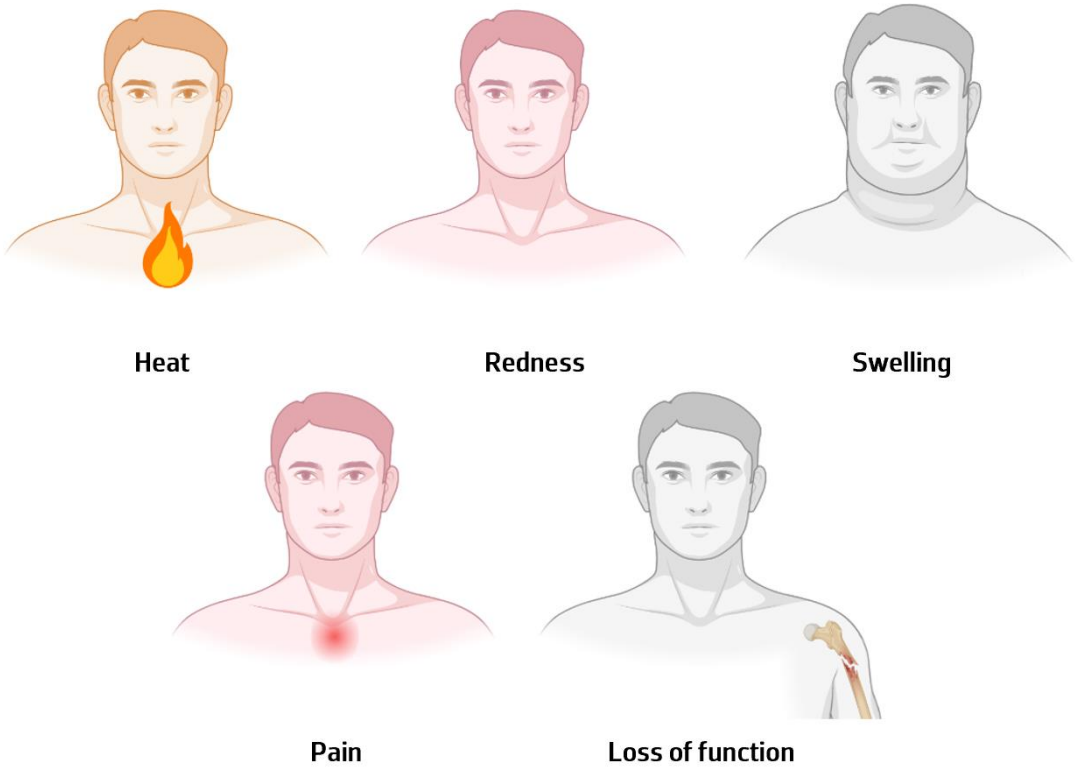


Figure 1: The five cardinal signs of inflammation.

Inflammation represents a carefully orchestrated defence action of the body, involving endothelial cells, epithelial cells, and the recruitment of inflammatory cells, such as neutrophils, monocytes, macrophages, and, in some cases, lymphocytes. During this process, cells are stimulated to release various mediators, namely cytokines, which contribute to the recruitment

of other cells, thus amplifying the inflammatory status (Gusev & Sarapultsev, 2023; Ribeiro, Freitas, Lima, *et al.*, 2015).

The inflammatory process may be classified as acute or chronic, according to the nature and effectiveness of the initial response. Acute inflammation is defined as a transient inflammation, that is an immediate response to damage suffered by the body's cells and tissues. It is rapid and of short duration (hours or few days), characterized by the release of numerous inflammatory mediators (*e.g.* cytokines and arachidonic acid metabolites) leading to the exudation of plasma fluids and proteins (edema), increased vascular permeability and the migration of defence cells (leukocytes, predominantly neutrophils) (Arulselvan *et al.*, 2016).

Acute inflammation can have different causes (Freire & Van Dyke, 2013):

- Bacterial, viral, fungal, or parasitic infections;
- Immune or hypersensitivity reactions;
- Tissue necrosis;
- Hypoxia.

When the acute inflammation is not enough to eliminate the stimulus, it evolves into a chronic inflammatory process (Ribeiro, Freitas, Lima, *et al.*, 2015). Chronic inflammation is defined as a prolonged process (weeks to months) during which tissue damage and reparative efforts coexist. Usually, this type of inflammation manifests itself following an acute inflammation, however, it can begin insidiously, as a low-grade response, without any manifestation of the acute process. This latter form is associated with some diseases that include rheumatoid arthritis, atherosclerosis, tuberculosis and pulmonary fibrosis (Katsicas & Russo, 2015).

The main causes of chronic inflammation are (Medzhitov, 2008):

- Infections by microorganisms that are difficult to eradicate (most common cause);
- Inflammatory diseases, such as autoimmune diseases, which are caused by excessive and inappropriate activation of the immune system;
- Prolonged exposure to endogenous [*e.g.,* lipid mediators like prostaglandins and platelet activating factor (PAF)] or exogenous (*e.g.*, microbial agents or foreign and toxic bodies) agents.

1.1.2. Inflammatory mediators

The inflammatory process involves a variety of mediators, which can be organised into different groups (Figure 2).

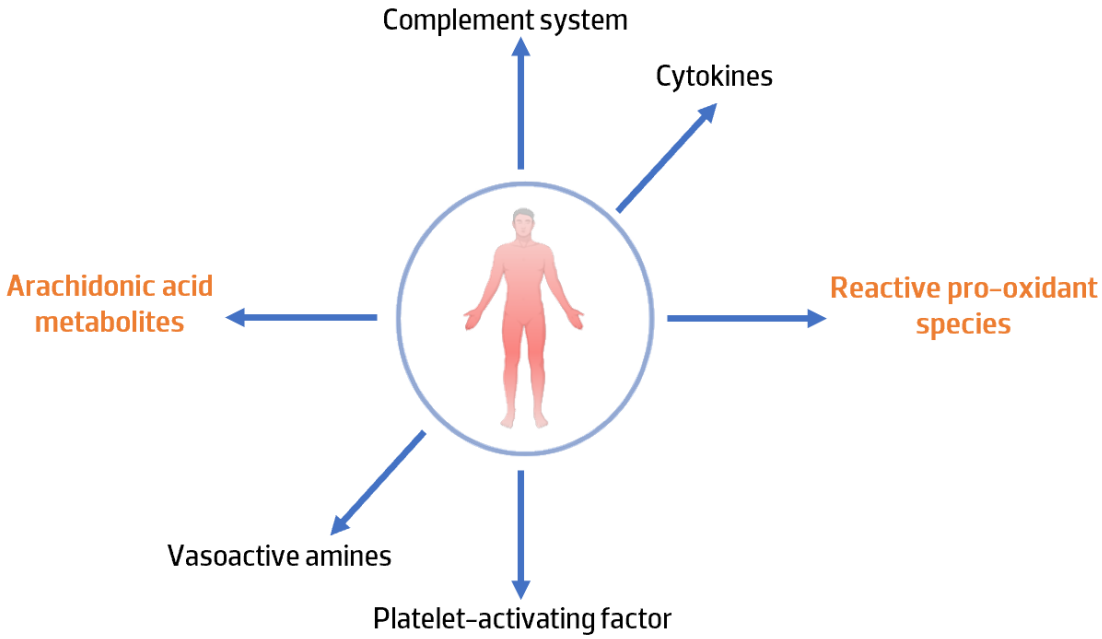


Figure 2: Different routes of the inflammatory process.

Accordingly, the following mediators can be mentioned:

Vasoactive amines: histamine and serotonin stand out as the first mediators to be released during the inflammatory process. They are responsible for vasodilation, as well as, increased vascular permeability. The vasodilation increases the blood flow to the affected area which allows the recruitment of inflammatory cells, the removal of metabolic products and toxins and the increased oxygen and nutrient supply. These mediators can be found in mast cells, basophils, and platelets (Abdulkhaleq *et al.*, 2018).

PAF: is a phospholipid produced by platelets, leukocytes, and endothelial cells. Its secretion is involved in several steps of the inflammatory process. The PAF promotes platelet aggregation, bronchoconstriction, vascular permeability and activation of neutrophils, macrophages, and

eosinophils. The activation of inflammatory cells increases their ability to release pro-inflammatory mediators (Ribeiro, Freitas, Tomé, *et al.*, 2015).

Metabolites of arachidonic acid (AA): are generated through the action of enzymes like cyclooxygenase (COX) and lipoxygenase (LOX), which rapidly convert AA, found in the membrane, into prostaglandins (PG), leukotrienes, and lipoxins. PG are produced by mast cells, leukocytes, and endothelial cells being involved in a variety of physiological functions (healing and vascular tension). Leukotrienes secreted mainly by leukocytes are responsible for chemotaxis and increase of vascular permeability (Bennett & Gilroy, 2016). Lipoxins, on the other hand, produced by lipoxygenase, are inhibitors of inflammation, since they inhibit neutrophil recruitment and instead promote the recruitment of monocytes, which remove dead cells and initiate tissue remodelling (Medzhitov, 2008).

Cytokines: are pleiotropic molecules, produced by various cells, such as lymphocytes, macrophages, endothelial and epithelial cells. They exert their effects both locally and systemically. Cytokines belong to a complex network with synergistic as well as antagonistic interactions and can positively or negatively regulate various target cells (Ribeiro, Freitas, Tomé, *et al.*, 2015). Interleukin (IL) -1β, IL-6, IL-8, and tumour necrosis factor (TNF) play a central role in mediating inflammation. Their secretion can be stimulated by endotoxins, immune complexes, physical injury, and other inflammatory stimuli. Their main action is the regulation of the immune system mainly through the activation of immune cells, as lymphocytes and macrophages (Abdulkhaleq *et al.*, 2018).

Reactive pro-oxidant species (RS): these RS derived from both oxygen (ROS) and nitrogen (RNS) are mainly released by leukocytes after exposure to a foreign agent. Their function is to eliminate invaders, mainly by phagocytosis. A small increase of the levels of these species can also lead to an increase in the expression of cytokines and cell adhesion molecules, which amplifies the inflammatory response (Sies *et al.*, 2022). An example of these species is nitric oxide, a powerful vasodilator, produced by endothelial cells, macrophages, and nerve cells, which has a very relevant role in the inflammatory process (Tenopoulou & Doulias, 2020).

Complement system: is a crucial part of the immune system consisting of over 30 different proteins present in the blood and tissues. Its primary function is to enhance the body's defence against infections and promote the removal of foreign substances. When activated, the complement system can lead to the recruitment and activation of immune cells, the destruction of pathogens through direct lysis, and the generation of inflammatory responses. It also plays a

role in the clearance of immune complexes and the modulation of adaptive immune responses (Ling & Murali, 2019).

Thus, the main purpose of these inflammatory mediators is the resolution of the injury in the organism. However, it inevitably becomes a problem when it goes beyond the physiological balance, resulting in an exaggerated response, thus contributing to the onset and progression of variety of diseases, such as autoimmune diseases, rheumatoid arthritis, and systematic lupus erythematous, and some form of diabetes like type 2 Diabetes *mellitus* (Ribeiro, Freitas, Tomé, *et al.*, 2015). Although all these mediators are essential, this work will delve deeper in the study off AA metabolites, namely the COX and the RS production.

1.1.2.1 Arachidonic Acid: function and metabolism

The AA is an essential fatty acid of the omega 6-family, consisting of a 20-carbon chain with 4 double bonds. This acid cannot be synthesised by the human body and must therefore be obtained from the diet (vegetable oils, meats, eggs and dairy products), or indirectly, by conversion of linoleic acid by desaturases and elongases action (Figure 3) (Martin *et al.*, 2016). AA is the precursor of a large family of bioactive compounds, the eicosanoids, including prostanoids [PG, thromboxanes (Tx)], lipoxins and leukotrienes (Abdulkhaleq *et al.*, 2018). As well as being a precursor of the eicosanoids involved in the inflammation, AA is an essential structural component of cell membrane, providing them with flexibility and fluidity, and regulates both cardiovascular and cerebral function (Tallima & El Ridi, 2018).

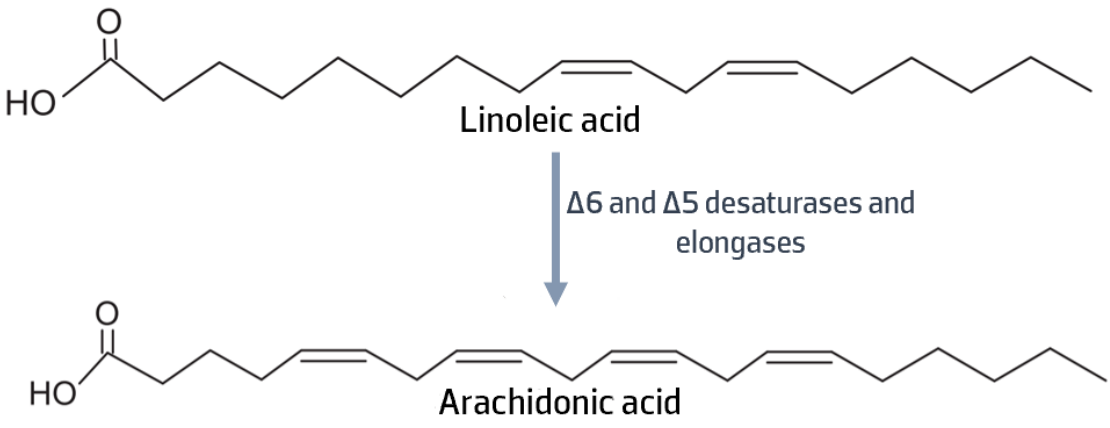


Figure 3: Outline of the conversion of linoleic acid into arachidonic acid, by the combination of desaturases e elongases (Elsherbiny *et al.*, 2013)

The AA can be metabolised in 3 different ways, by the action of enzymes LOX, COX and also by cytochrome P450 (CYP450) (Figure 4). The LOX pathway is a metabolic route in which AA is oxidised, producing lipoxins and leukotrienes. There are several isoforms of LOX, each with different substrate specificities and tissue localisations. The most studied isoforms are 5-LOX, 12-LOX, and 15-LOX. Those enzymes differ in the position at which they introduce oxygen into AA. 5-LOX is widely expressed in cells of the immune system, such as leukocytes, and plays a key role in the synthesis of leukotrienes. The 12-LOX and 15-LOX, on the other hand, have a wider tissue distribution and are involved in various physiological and pathological processes (Zhou *et al.*, 2021).

The AA metabolization pathway by CYP450 results in the formation of AA epoxides namely hydroxyeicosatetraenoic (HETEs) and epoxyeicosatrienoic acids (EETs). These epoxides have multiple biological functions and are involved in the regulation of blood pressure, platelet aggregation and inflammation. The CYP450 pathway adds an additional layer of complexity to the metabolization of AA and the regulation of physiological and pathological processes (Hoxha, 2022).

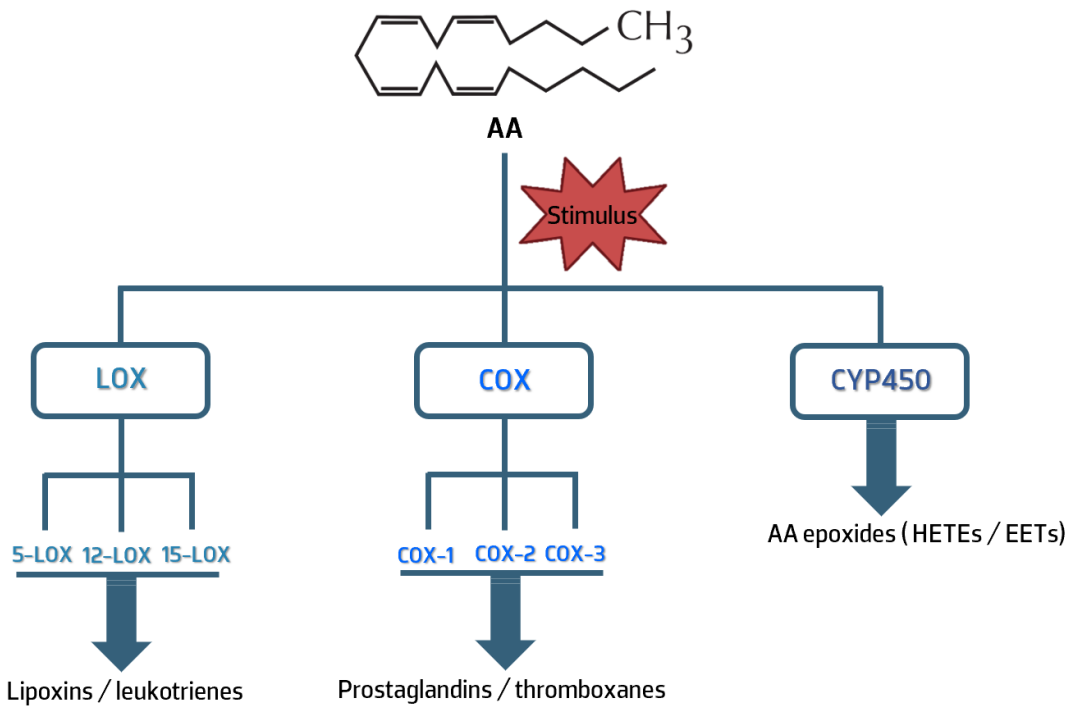


Figure 4: Schematic representation of three AA metabolization pathways (Wang *et al.*, 2019).

Among the various enzymes involved in the metabolism of AA, COX stand out as some of the most extensively studied enzyme. COX enzymes, particularly COX-1 and COX-2 isoforms, are not only central to physiological processes but also prominent targets in the development of anti-inflammatory medications. Given their critical involvement in inflammation, COX enzymes have garnered significant attention, making them a focus of extensive research efforts aimed at uncovering their mechanisms and potential therapeutic applications. In this context, this work aims to delve deeper into the intricate interplay of COX enzymes.

1.2. Cyclooxygenase

COX is a bifunctional enzyme that acts sequentially as a dioxygenase and a peroxidase to perform a complex reaction with RS, which results, in the production of PG and Tx from the metabolization of AA (Bennett & Gilroy, 2016). COX are highly evolutionarily conserved enzymes and there are three distinct isoforms (Figure 6), COX-1, COX-2 and COX-3, which are encoded by two different genes (Ribeiro, Freitas, Tomé, *et al.*, 2015).

COX enzymes are members of the peroxidase family and exhibit several significant structural and functional similarities. When comparing the amino acid sequence of these enzymes, it can be seen that they have a high homology, about 60% of sequences are identical. They also have the same active site, where the AA (substrate) binds and undergoes oxidation. The three-dimensional structure of COX-enzymes is very similar, comprising two main domains, a peroxidase and an epoxide domain (Figure 5) (Kiefer *et al.*, 2000).

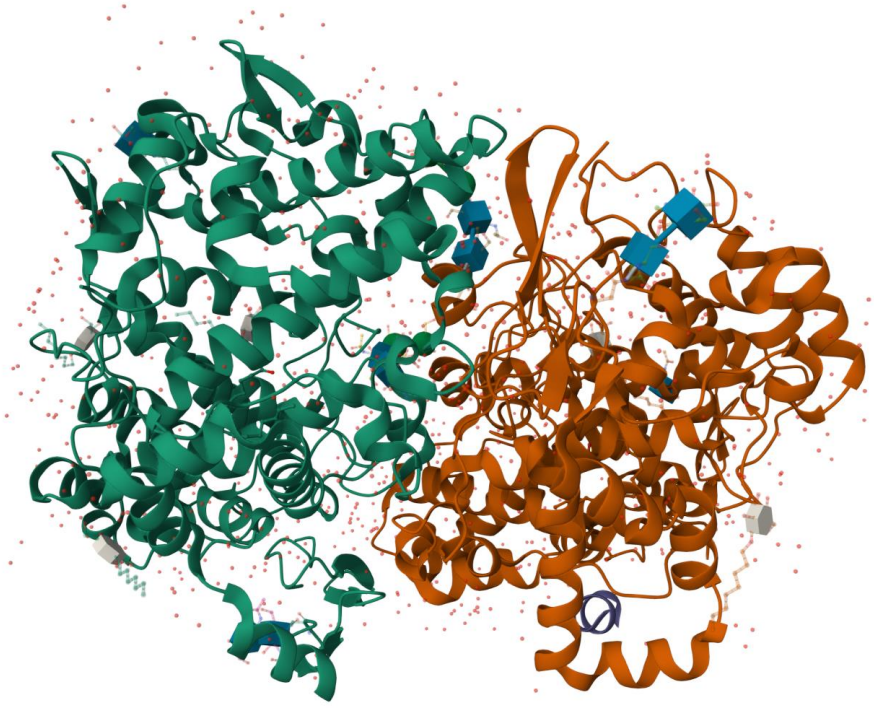


Figure 5: Crystal structure of AA bound to COX-2 active site (Kiefer, 2000)

COX-1, which is constitutively expressed in range of tissues, including endothelium and gastric mucosa, renal collecting tubes and platelets, has been classically classified as the isoform primarily responsible for the homeostatic synthesis of PG (Choi *et al.*, 2009). It plays a key role in several physiological processes, including the adjustment of the acid-base balance in the stomach, protection of the gastric mucosa, maintenance of proper renal function and the regulation of platelet aggregation (Rao & Knaus, 2008).

COX-2, unlike COX-1, is an inducible isoform that can be expressed in cells involved in the inflammatory process, namely leukocytes (*e.g.*, macrophages), fibroblasts, and endothelial cells (Ribeiro, Freitas, Lima, *et al.*, 2015). During the inflammatory process, the expression of the enzyme COX-2, is stimulated by pro-inflammatory cytokines (IL-1 and TNF), to produce PG, important inflammatory mediators (Mohsin & Irfan, 2020). The main functions of COX-2 are then the activation of the inflammatory response, tissue healing (promotion of angiogenesis and cell proliferation), regulation of renal blood flow and also the regulation of normal physiological functions (Botting, 2006).

COX-3 was initially described as an enzyme distinct from COX-1 and COX-2. However, COX-3 is believed to be an analogue of COX-1 expressed in the central nervous system

(hypothalamus), which expresses specific PG in the brain. Nevertheless, its mechanism of action remains unclear (Przybyła *et al.*, 2020). Remarkably, some literature reports that COX-3 may be able to induce remission for chronic inflammation and might be involved in the growth of cervical, ovarian, leukaemia and colon cancer (Kam & So, 2009). In addition, there are promising data that patients with Alzheimer's disease have an increased of COX-3, and it has been reported that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) slightly reduces this illness (Sharma *et al.*, 2019).

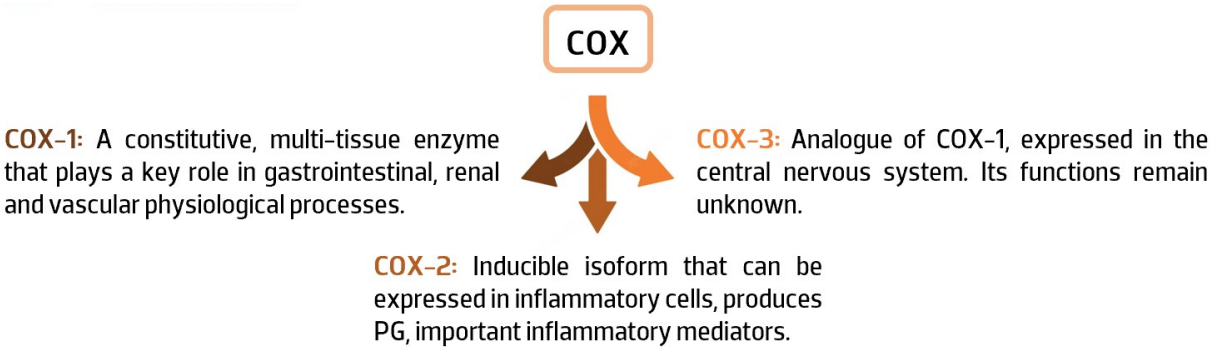


Figure 6: Schematic diagram of the three COX isoforms (Przybyła *et al.*, 2020).

The AA metabolite production cascade (Figure 7) begins with the release of AA, by the enzyme phospholipase A_2, present in phospholipids of cytoplasmic membrane (Wang *et al.*, 2019). COX-1 and COX-2 are responsible for the incorporation of oxygen at carbon-11 of AA, leading to the formation of the five-carbon ring present in PGG_2. Subsequently, PGG_2 is reduced to PGH_2 by COX (Ribeiro, Freitas, Lima, *et al.*, 2015).

PGH_2, being an unstable endoperoxide, readily gives rise to several bioactive PG, including D_2, E_2, $F_{2\alpha}$, I_2, and TxA_2. Furthermore, PGH_2 can be enzymatically converted into malonaldehyde (MDA) and 12L-Hydroxy-5,8,10-heptadecatrienoic acid (HHT) by Tx synthase (Wang *et al.*, 2019). Meanwhile, PGE_2, obtained through the action of prostaglandin E synthase (PGEs) on PGH_2, is one of the most abundant PG produced by the body and has an enormous versatility of biological activities, such as regulation of inflammatory response and modulation of gastrointestinal function. PGE_2 can traverse the cytoplasmic membrane through the activity of a prostaglandin efflux transporter (MRP4) (Li *et al.*, 2021). In turn, exogenous PGE_2 can bind to prostaglandin E receptors 1,2,3 and 4 (EPR_{1-4}), inducing inflammation and tumour growth (Finetti

et al., 2020). This PGE_2 can re-enter the cell by the action of the exogenous prostaglandin E transporter (PGT) (Li *et al.*, 2021).

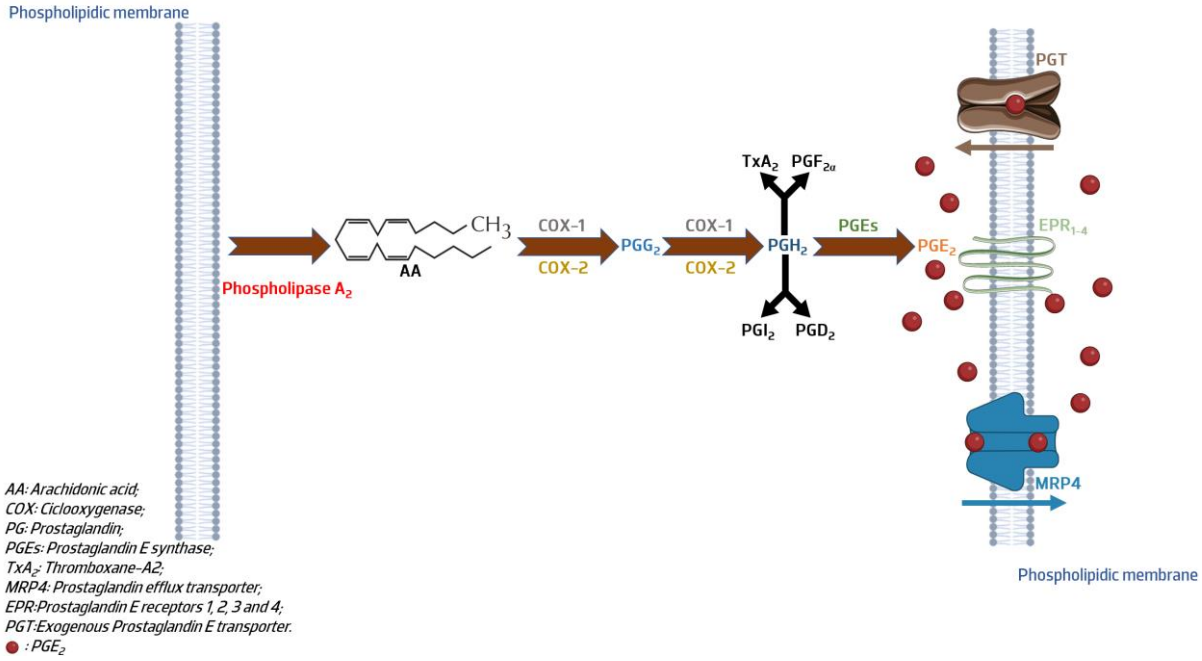


Figure 7: Production cascade of AA metabolites, focusing on PGE2 synthesis (Norberg *et al.*, 2013)

PG are significant inflammatory mediators, especially PGE_2 and PGI_2, since they are potent vasodilators and are involved in the activation of several intracellular signalling pathways, through interaction with G protein-coupled receptors (Li *et al.*, 2021). In addition, PG are involved in blood coagulation, ovulation, initiation of labour, bone metabolism, nerve growth and development, wound healing, kidney function and immune responses (Ricciotti & FitzGerald, 2011). On the other hand, PGD_2 is involved in control of sleep, pain, hypertension, cardiovascular disease, obesity, and diabetes. $PGF_{2\alpha}$ is recognised to regulate the contraction of uterine muscles, the ocular system, and obesity (Fujimori, 2022).

TxA_2 is critically important in controlling haemostasis in the cardiovascular system. It is also a potent vasoconstrictor, which promotes platelet adhesion, and regulates renal haemodynamic, controlling sodium levels in the kidneys (Iñiguez *et al.*, 2008).

1.3. Reactive pro-oxidant species (Oxidative burst)

Leukocytes, also commonly known as white blood cells, are cells of the immune system that play a key role in the defence of our organism. They are produced in the bone marrow and are present in the bloodstream and in other tissues of the body (Carrick & Begg, 2008). There are different types of leukocytes, each with specific functions and distinct characteristics. These are divided into two groups (Figure 8), the granulocytes (eosinophils, basophils, and neutrophils) and the agranulocytes (lymphocytes and monocytes).

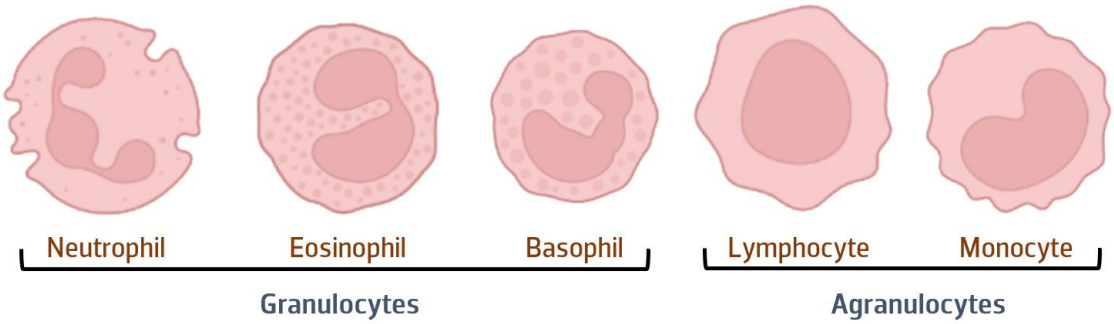


Figure 8: Representation of different leukocyte subtypes (created with BioRender).

Lymphocytes coordinate organism-specific immune responses. There are three main subtypes of lymphocytes: B-lymphocytes, T-lymphocytes, and natural killer (NK) cells. B lymphocytes produce antibodies, proteins that bind to pathogens and label them for elimination. In turn, T lymphocytes, which undergo maturation over time, have the capability to directly target cells that are infected by viruses or are cancerous (Carrick & Begg, 2008). NK cells eliminate virus-infected, cancerous, and aged cells. They also play a role in regulating the inflammatory process since they release cytokines, such as gamma-interferon (INF – γ), which modulates the activity of other cells of the immune system (monocytes and T cells). Therefore, NK cells are an essential part of the immune system, quickly reacting to fight invaders, regardless of whether they have not been exposed to them before, rendering them an important first line of defence for the immune system (Vivier *et al.*, 2008).

Monocytes, the larger sized immune cells, differentiate into macrophages, which are specialised in phagocytosing pathogens, dead cells, and cellular debris. Macrophages also play an

important role in presenting antigens to lymphocytes, thereby activating specific immune responses (Serhan *et al.*, 2010).

Eosinophils are mainly involved in the immune response against multicellular parasites such as worms. They also play a role in allergic reactions, releasing substances that can fight inflammation (Serhan *et al.*, 2010).

Basophils are involved in allergic reactions, releasing substances such as histamine, which promote inflammation and dilation of blood vessels (Carrick & Begg, 2008).

Finally, neutrophils are the predominant leukocytes, comprising ≈70% of the white blood cells, and are specialised in combating infections. They are attracted to the site of infection by chemical signals released by damaged cells or by the microorganisms themselves. Neutrophils are phagocytic cells, which means that they engulf and destroy pathogens by an intracellular digestion processes (phagocytosis, degranulation and formation of neutrophilic extracellular traps) (Liew & Kubes, 2019). Neutrophils are the first line of defence of the organism, as they are the first cells to reach the inflamed areas, where they kill and ingest the invading agents (Jones *et al.*, 2016). Neutrophil functionality is activated upon exposure to a stimulus, which occurs through a sequence of cellular events: margination, rolling, adhesion, diapedesis, chemotaxis and phagocytosis (Figure 9) (Nourshargh & Alon, 2014).

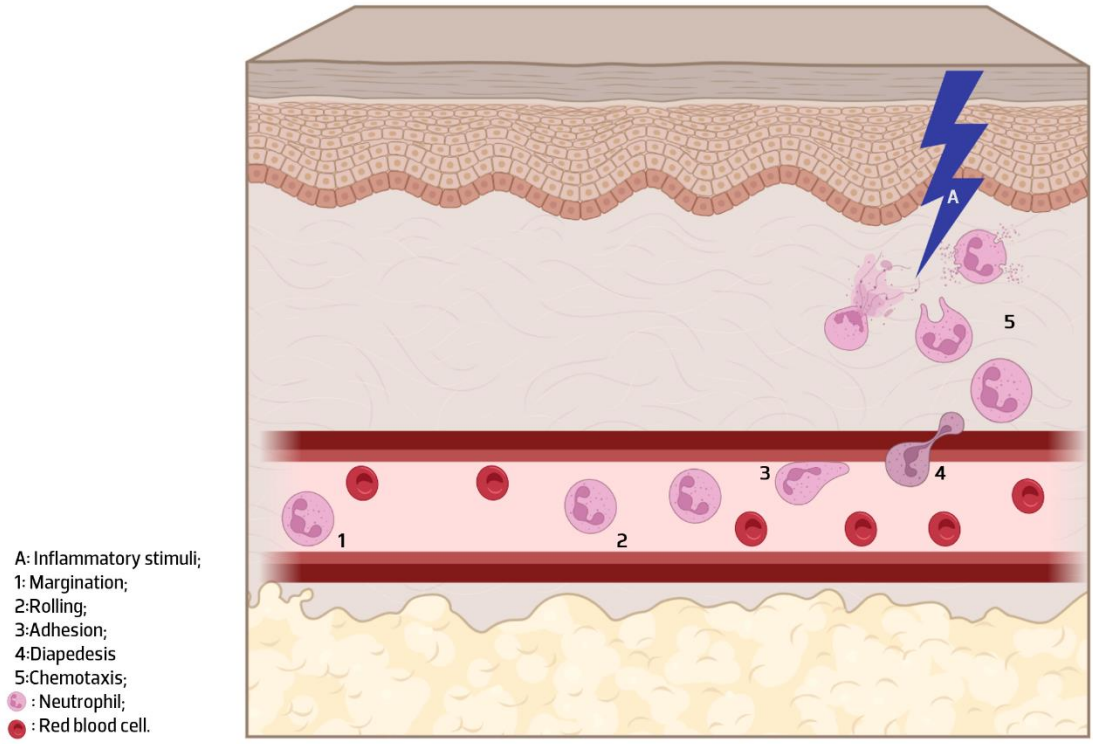


Figure 9: Cellular events sequence for the activation of neutrophils (Nourshargh & Alon, 2014).

Neutrophils move from blood capillaries into connective tissue by margination, rolling, adhesion and diapedesis. Finally, these cells reach to the affected local by chemotaxis, an unidirectional process mediated by chemical stimuli that encourage the movement of neutrophils to a particular site (Figure 9) (Nourshargh & Alon, 2014). They are able to recognise foreign particles or invading microorganism, through the interaction between specific receptors on the neutrophil membrane and ligands present on the surface of the intruder agent. After the recognition, the mechanisms by which neutrophils act in host defence are phagocytosis, degranulation, production of RS, production of cytokines and also the formation of neutrophilic extracellular traps (Amulic *et al.*, 2012). Excessive activation or ineffective clearance of activated neutrophils can lead to tissue damage, cell death and consequently exacerbation of the inflammatory response (Delgado-Rizo *et al.*, 2017).

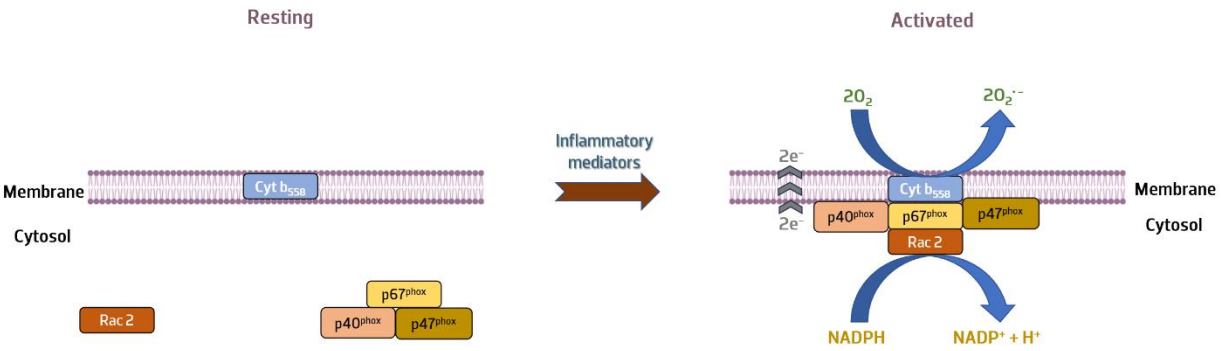
A major aspect associated with the inflammatory and antimicrobial actions of neutrophils is the activation of a potent oxidative burst, during which large amounts of oxygen are consumed and converted into superoxide anion radicals ($O_2^{\cdot -}$), which in turn give rise to other RS. RS are extremely unstable and short-lived compounds. These are made up of ROS and RNS, which can be radical and non-radical reactive chemical species, containing oxygen or nitrogen, respectively (Table 1) (Phaniendra *et al.*, 2015).

Table 1: Examples of radical and non-radical reactive oxygen and nitrogen species (Phaniendra *et al.*, 2015).

	Radical	Non – radical
Reactive species oxygen (ROS)	Superoxide anion radical ($O_2^{\cdot -}$)	Hydrogen peroxide (H_2O_2)
	Hydroxyl radical ($HO^{\cdot}$)	Singlet oxygen ($^1O_2$)
	Peroxyl radical ($ROO^{\cdot}$)	Hypochlorous acid (HOCl)
Reactive species nitrogen (RNS)	Nitric oxide ($^{\cdot}NO$)	Peroxynitrite anion ($ONOO^-$)
	Nitrogen dioxide ($^{\cdot}NO_2$)	Peroxynitrous acid (ONOOH)

When a neutrophil recognises particles for ingestion, the plasma membrane folds, forming first a cup and then a closed phagosome. The nicotinamide adenine dinucleotide diphosphate (NADPH) oxidase complex is activated before the phagosome is fully formed and the intracellular granules fuse with the developing phagosome, releasing various digestive enzymes. The activation of NADPH-oxidase involves increased oxygen consumption, resulting in up to 100-fold increase in basal metabolic activity (Freitas, Lima, *et al.*, 2009).

NADPH-oxidase is a membrane-bound electron transport complex present in different cell types such as fibroblasts, vascular smooth muscle cells, phagocytic cells, endothelial cells (Belambri *et al.*, 2018). This complex is composed by several isoforms, namely, NOX_1 to NOX_5 and $Duox_1$ and $Duox_2$. The biological functional of NADPH-oxidase has a special importance in neutrophils and, therefore, the most studied is NOX_2, which is found in phagocytic cells (neutrophils and macrophages) and B lymphocytes, participating in defence processes through the production of RS (Chocry & Leloup, 2020). This enzymatic complex is composed of multiple subunits, namely, Ras-related C3 botulinum toxic substrate 2 (Rac 2) and 5 phagocytic oxidases (phox), which are glycoprotein $gp91^{phox}$ and polypeptides $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and, $p67^{phox}$ (Figure 10) (Belambri *et al.*, 2018). In its inactive form, the components are found in the cytosol, with the exception of the $gp91^{phox}$ and $p22^{phox}$ subunits. These subunits constitute a membrane-associated complex, called cytochrome b_{558}, which is the catalytic core of NADPH-oxidase, located in the cell membranes of phagosomes, secretory vesicles, specific granules, and the plasma membrane. The biological functions of the 5 subunits are very distinct. The $gp91^{phox}$ is the electron transferase of NADPH-oxidase, while $p40^{phox}$, $p47^{phox}$ and, $p67^{phox}$ make a trimeric regulatory complex in the cytosol of dormant cells. On the other hand, $p22^{phox}$, is the docking site of this trimeric complex. In addition, the $p47^{phox}$ could also exist separately from the regulatory complex. Lastly, Rac 2 boosts ROS production by NADPH-oxidase (Nguyen *et al.*, 2017).



Cyt b_{558}: Cytochrome b_{558}
Phox: Phagocytic oxidases
Rac 2: Ras-related C3 botulinum toxic substrate 2
NADPH: nicotinamide adenine dinucleotide diphosphate

Figure 10: Schematic representation of the resting and activated forms of the phagocytic NADPH oxidase (McCann & Roulston, 2013)

This enzymatic complex may be stimulated by neutrophil adhesion, proinflammatory mediators and toll-like receptors agonists. This first stimulus promotes a state of alert which results in a faster and higher response. The totally activated stage of NADPH-oxidase appears after another stimulus, like microbial products, lipopolysaccharides, and cytokines (IL-8 and TNF) (Mittal *et al.*, 2014).

The activation of this enzymatic complex is regulated by 3 main processes: the NADPH-oxidase subunits' phosphorylation, translocation of cytosolic subunits to membrane, and the Rac 2's activation (Belambri *et al.*, 2018).

NADPH-oxidase subunits' phosphorylation: the activation of the enzymatic complex begins with the $p47^{phox}$ subunit phosphorylation, the first subunit to interact with cytochrome b_{558}, due to the action of protein kinase C (PKC). PKC is triggered by diacylglycerol (DAG) produced by phospholipase, when stimulated by pro-inflammatory mediators (Ribeiro, Freitas, Lima, *et al.*, 2015).

Translocation of cytosolic subunits: the movement of cytosolic subunits to the membrane, which they bind to cytochrome b_{558}, creating a single membrane-associated complex that fuses to the cytoplasm (Chocry & Leloup, 2020).

Rac 2' activation: when Rac2 is activated and subsequently translocate to join the complex, it enables the functionality of NADPH oxidase. The aggregation of all NADPH-oxidase subunits promotes the reduction of oxygen (O_2) into $O_2^{\cdot -}$, using cytosolic NADPH as an electron donor. Indeed, the superoxide dismutase (SOD) catalyses the conversion of $O_2^{\cdot -}$ into compounds with lower reactivity, such as O_2 and hydrogen peroxide (H_2O_2) (Winterbourn *et al.*, 2016).

Subsequently, other reactions may occur. The H_2O_2 in the presence of ferrous ion (Fe^{2+}) or copper ion (Cu^{+}), originating hydroxyl radical ($HO^{\cdot}$) and hydroxide ion (OH^{-}), through the Fenton reaction (Tang *et al.*, 2021). On the other hand, the Haber-Weiss cycle, is where $HO^{\cdot}$ and OH^{-} are formed from the reaction of H_2O_2 and $O_2^{\cdot -}$ catalysed by ferric ion (Fe^{3+}) (Gupta *et al.*, 2016). Moreover, in the presence of chloride ion (Cl^{-}), the H_2O_2 can also form hypochlorous acid (HOCl), through the action of myeloperoxidase (MPO) enzyme. The HOCl is a crucial antimicrobial agent, which plays a major role in the immune response (Figure 11).

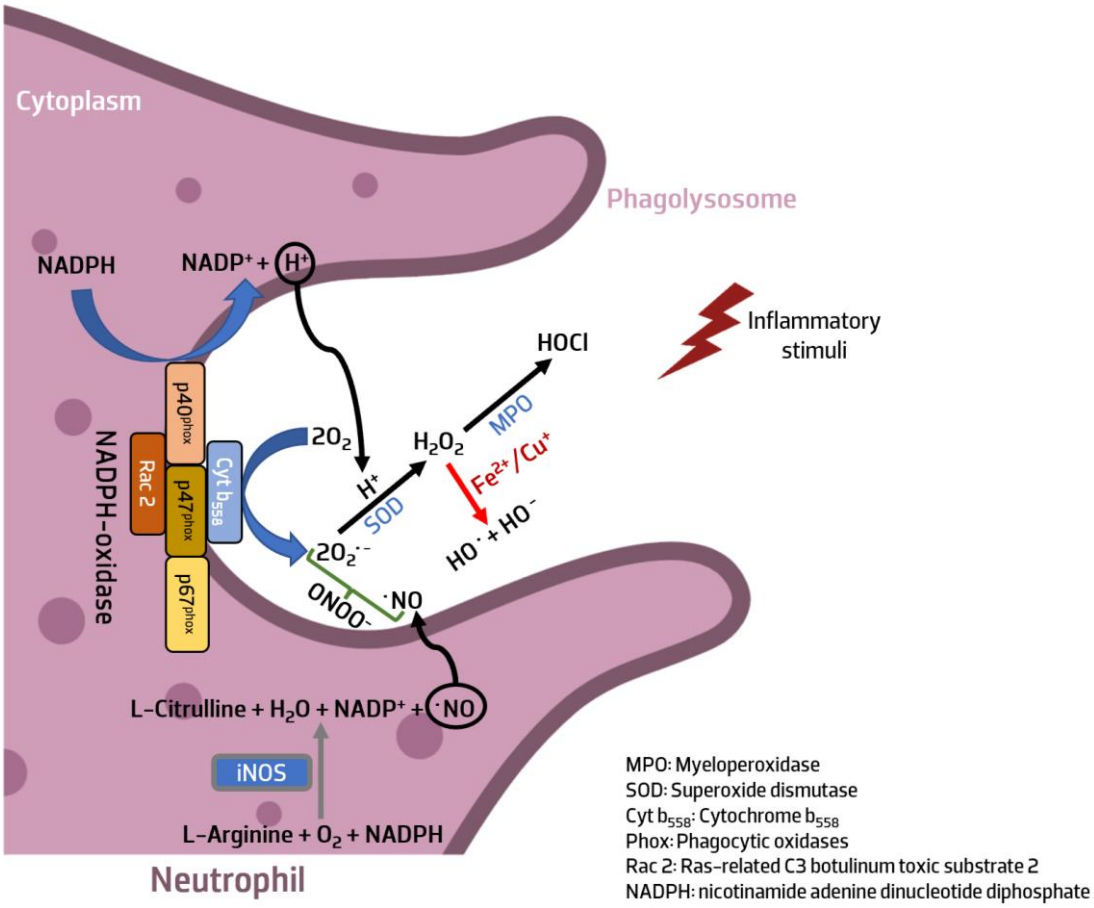


Figure 11: Activation of NADPH-oxidase complex in the cell membrane (Ribeiro, Freitas, Lima, *et al.*, 2015).

The RNS' production begins with the generation of ·NO, by inducible nitric oxide synthase (iNOS) enzyme. iNOS converts the amino acid L-arginine, NADPH and O_2 into L-citrulline, H_2O, oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) and ·NO. ·NO is able to diffuse into phagolysosome and take part in the physiological processes' regulation, host protection, immunity, and inflammation, due to the formation other cytotoxic RNS, as $ONOO^-$, through its fast reaction with $O_2^{\cdot -}$ (Ribeiro, Freitas, Lima, *et al.*, 2015).

The production of ROS and RNS is commonly known as oxidative burst and is essential for our organism.

Though neutrophils are the main providers of RS, other leukocytes also have that ability. Monocytes generate lower quantities of RS, yet their response is characterized by greater sustainability and control. This extended responsiveness proves to be advantageous in combating chronic conditions and more resistant microorganisms. (Manea *et al.*, 2015).

Although RS play a fundamental role in the body's defence, their production must be balanced with the action of antioxidants. Gutteridge and Halliwell (2010) defined the term

antioxidant as "any substance which, when present in concentrations lower than that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate", which means that antioxidants prevent damage to cellular components caused by RS. Antioxidant agents can be endogenous, such as SOD and glutathione peroxidase, or exogenous, namely, vitamin A, ascorbic acid (vitamin C) or polyphenols (Berger *et al.*, 2012).

As described previously, RS are normally produced in the body, however, external factors such as exposure to chemical agents and ultraviolet radiation, smoking and excessive alcohol consumption can dramatically increase their production, in a process called oxidative stress (Halliwell & Gutteridge, 2015).

Therefore, oxidative stress occurs when there is a change in the balance between the antioxidant agents and the pro-oxidant agents, favouring the last ones (Figure 12). The damage caused by oxidative stress can have various negative consequences, such as the oxidation of phospholipids present in cell membrane resulting in lack of membrane integrity (loss of function), the oxidation of proteins which compromises essential cellular processes and also the oxidation of DNA triggering genetic mutations. Furthermore, it is also associated with ageing since the accumulative damage ends up causing cellular degeneration and the functional decline of the organs. In this sense, oxidative stress is implicated in the progression of several illnesses as cancer, diabetes, cardiovascular and inflammatory diseases (Tan *et al.*, 2018).

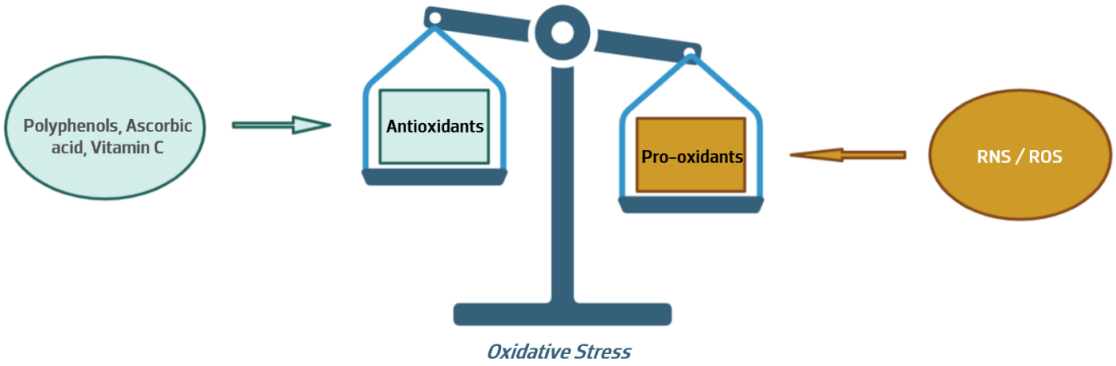
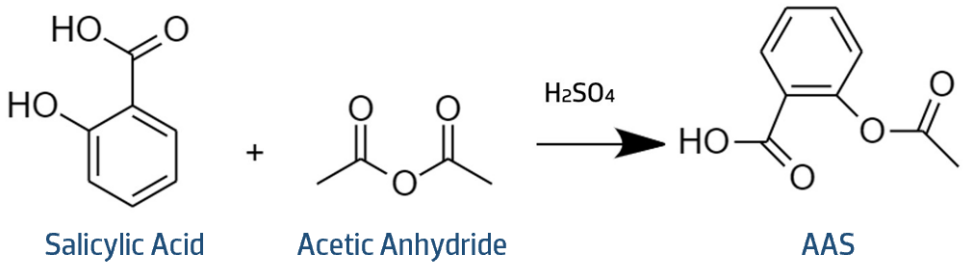


Figure 12: Representation of oxidative stress.

1.4. Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs act by inhibiting COX enzymes, preventing the production of PG and TxA_2, which are the main mediators of the inflammatory process (Patrignani & Patrono, 2015).

The first NSAID appeared, in 1897, by the hand of Felix Hoffman, a Bayer® employee, who decided to acetylate salicylic acid. This strategy of molecular modification aimed to alleviate the bitter taste of this molecule, thus appearing the acetylsalicylic acid (AAS) (Figure 13), which started to be marketed as Aspirin®, by Bayer®, in 1899 (Montinari *et al.*, 2019).



H_2SO_4: Sulfuric acid
AAS: Acetylsalicylic acid

Figure 13: Synthesis reaction of acetylsalicylic acid from salicylic acid and anhydride acetic (Fijałkowski *et al.*, 2022)

The mechanism of action of NSAIDs involves inhibition of both COX-1 and COX-2, although some NSAIDs may have greater selectivity to inhibit one of the enzymes. The NSAIDs mostly elicit inflammatory, analgesic, and antipyretic effects (Brune & Patrignani, 2015):

Anti-inflammatory effect: COX inhibition blocks the conversion of AA into PG, thus reducing the production of these inflammatory mediators. This leads to a decrease in swelling, vasodilatation, pain sensitivity and inflammation (Patrignani & Patrono, 2015).

Analgesic effect: by reducing PG, NSAIDs lower the sensitivity to peripheral and central pain, providing analgesic relief (Brune & Patrignani, 2015).

Antipyretic effect: Since PGE_2 promote an increase in body temperature, with their inhibition, NSAIDs have an antipyretic effect (Brune & Patrignani, 2015).

NSAIDs, according to their selectivity for the 2 COX isoforms, can be divided into 4 distinct groups (Table 2) (Bacchi *et al.*, 2012).

Table 2: Classification of NSAIDs into groups and their examples (Bacchi *et al.*, 2012).

Group 1	Low-selective NSAIDs that fully inhibit both COX-1 and COX-2 (< 5-fold COX-2 selectivity).	Ibuprofen, diclofenac, acetylsalicylic acid, piroxicam
Group 2	Although NSAIDs inhibit both COX, they have a preferential selectivity for COX-2 (5 to 50-fold).	Celecoxib, meloxicam, nimesulide, etodolac
Group 3	NSAIDs that strongly inhibit COX-2 (> 50-fold).	Rofecoxib, NS-398
Group 4	NSAIDs that appear to be weak inhibitors for both enzymes.	Sodium salicylate

Although NSAIDs have anti-inflammatory properties, they are not considered antioxidants. Nevertheless, some studies have suggested that certain NSAIDs such as AAS, ibuprofen, diclofenac, and indomethacin may have antioxidant effects in certain circumstances. It has been proposed that these drugs may have the ability to neutralise free radicals and reduce oxidative stress in some conditions through radical scavenging and metal chelating activities (Kon *et al.*, 2009).

The reality is that non-selective NSAIDs may present several side effects, such as gastric and duodenal ulcer. These undesired effects are explained by the inhibition of COX-1. This is known to be constitutive, and its inhibition results in the blockade of PG synthesis, which regulate several processes involved in gastric protection. In order to avoid these gastrointestinal adverse effects, COX-2 selective inhibitors have been introduced in the market (Botting, 2006).

It is known that although NSAIDs with greater selectivity for COX-2 reduce gastric complications, their prolonged use negatively affects the six main organs of the human body (Figure 14), causing cardiovascular lesions, hepatocellular lesions, kidney damage, gastrointestinal damage and intracerebral haemorrhage (Bindu *et al.*, 2020).

In fact, there is an increase in blood pressure after the start of treatment with COX-2 selective NSAIDs. This variation in individuals with pre-existing vascular disease is recognised to result in an increased risk of cardiovascular morbidity (Salzberg & Weir, 2007).

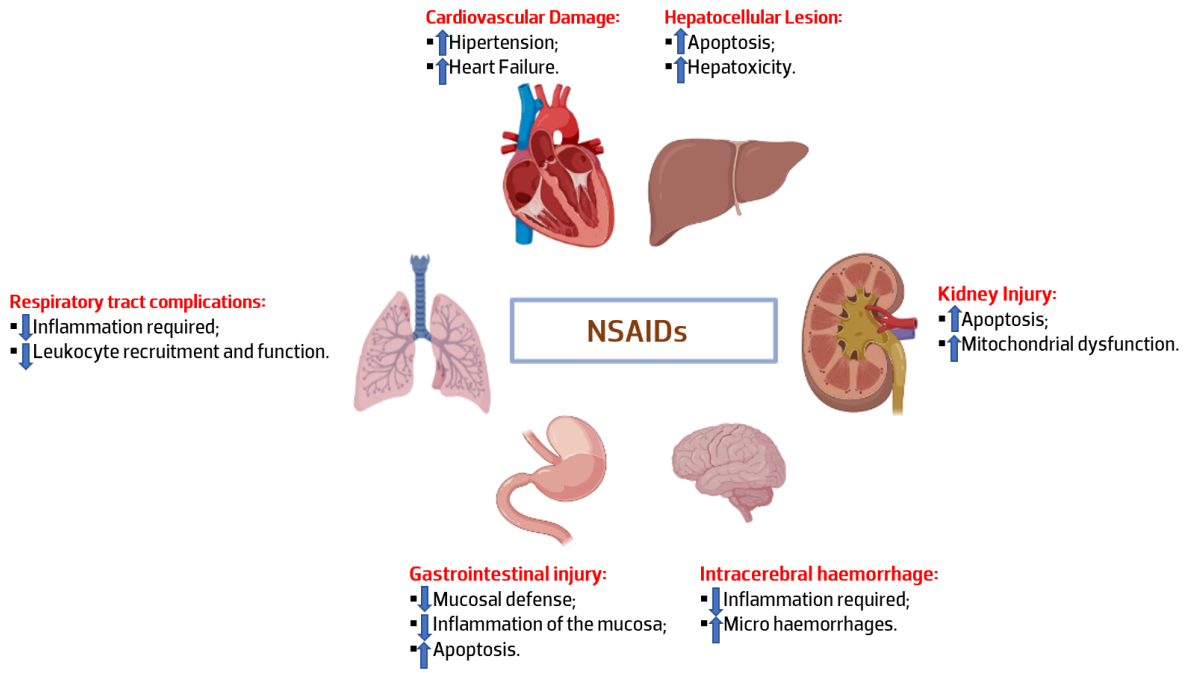


Figure 14: The main side effects of NSAIDs on major organs (Bindu *et al.*, 2020).

Considering the adverse effects of NSAIDs in the market, there is a search by the scientific community for new, safer, and more effective molecules capable of inhibiting COX-2 and also with the ability to inhibit the production of RS. One of the compounds that have aroused interest, are the pyrazoles, which will be described in the following section.

1.5. Pyrazoles

Pyrazoles are aromatic heterocyclic compounds consisting of five-membered rings with three carbon and two nitrogen atoms located in positions 1 and 2 (Figure 15). Possibly due to the complexity in N-N bond formation by living organisms, the fraction of pyrazoles found in natural sources is very small (Rocha *et al.*, 2021).

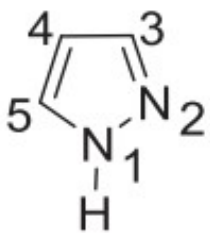


Figure 15: Chemical structure of pyrazole.

In fact, pyrazoles can be substituted by various aromatic and heteroaromatic groups, which makes them particularly interesting as it results in a wide range of biological activities. There are different synthetic routes to access the pyrazole core. However, the main methods for obtaining substituted pyrazoles are (Karrouchi *et al.*, 2018):

- Cyclocondensation of hydrazine and its derivatives with carbonyl systems;
- Dipolar cycloadditions;
- Multicomponent reactions.

Nevertheless, during recent years there has been increasing development of environmentally friendly (green) synthesis pathways such as microwave-mediated solvent-free approaches and the formation of pyrazole derivatives with phthalate fusion (compounds derived by phthalic acid) (Faisal *et al.*, 2019).

Molecules with pyrazole core in their structure have interest in multiple areas, including medicine, agriculture, and biotechnology (Karrouchi *et al.*, 2018). Regarding the biological activities pyrazoles (Figure 16) the most common are (Ramadan *et al.*, 2021):

- anti-inflammatory (inhibition of the production of inflammatory mediators or modulation of the immune response involved in inflammatory processes);
- antibacterial (inhibition of the bacterial growth or interference with crucial metabolic processes in bacteria);
- antioxidant (neutralisation and reduction of RS);
- antifungal (inhibition of fungal growth, hyphae formation and the synthesis of essential cellular components of the fungi);
- anticancer (promotion of cancer cell apoptosis);
- analgesic (modulation of pain transmission pathways and interaction with the receptors involved in pain sensation).

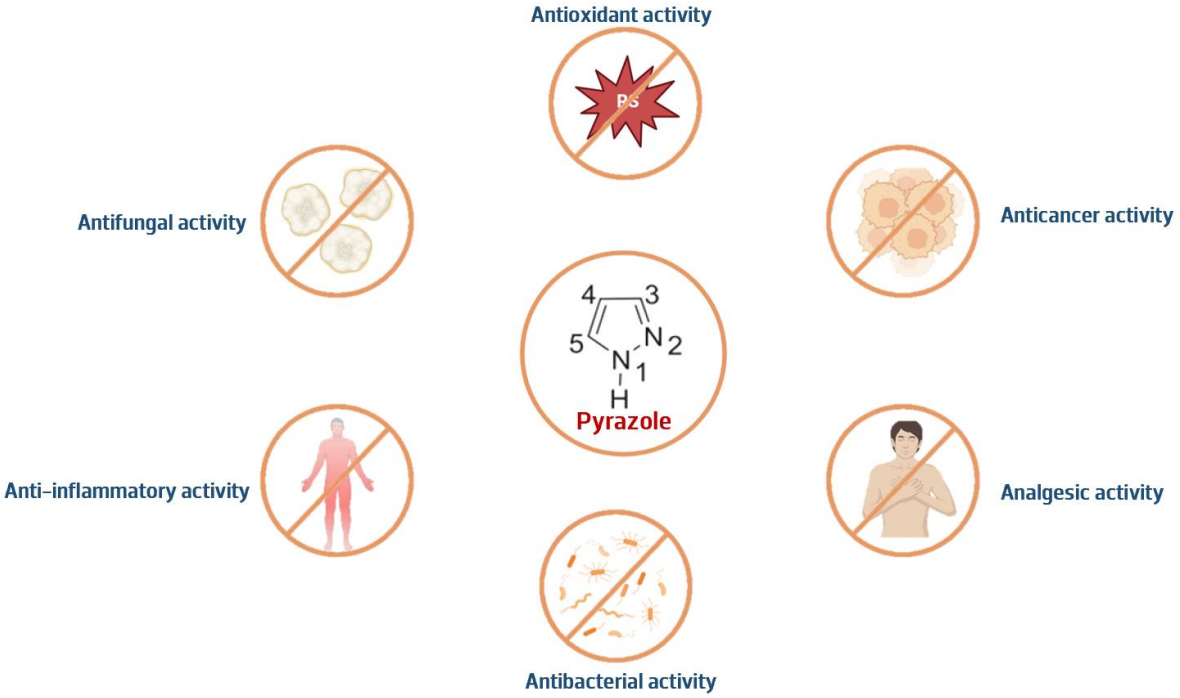


Figure 16: Biological activities of pyrazoles (Ramadan *et al.*, 2021).

In fact, there are numerous pharmaceutical drugs on the market that are pyrazole derivatives, as shown in Figure 17.

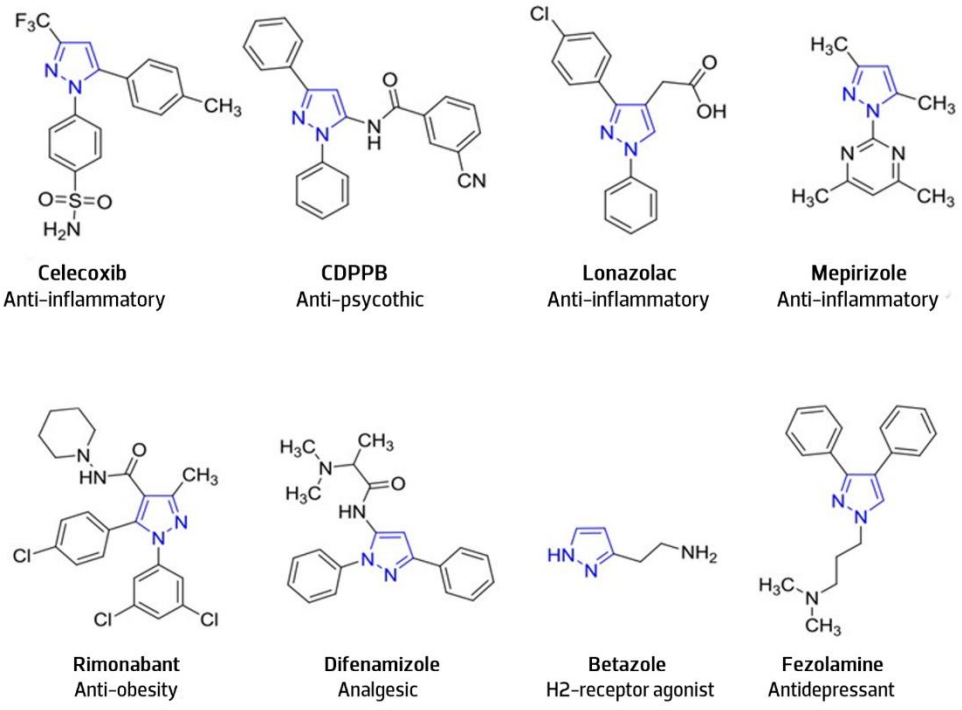


Figure 17: Drugs based on pyrazoles moiety and their therapeutic activities (CDPPB: 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl) benzamide) (Karrouchi *et al.*, 2018).

Considering the anti-inflammatory activity, NSAIDs as antipyrine, aminopyrine, metamizole and celecoxib have in their structures the pyrazole ring. This core has been used in the preparation of various molecules in an attempt to minimise the undesired effects of COX-2 inhibitors (Ramadan *et al.*, 2021). Noteworthy, celecoxib was the first COX-2 selective inhibitor to be introduced into clinical practice. It was developed to provide similar anti-inflammatory and analgesic activity to non-selective NSAIDs, without the gastrointestinal toxicity that is thought to result primarily from COX-1 inhibition. Celecoxib is authorised in the European Union for the treatment of osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis. However, it must be used in lower concentrations as it use increases the cardiovascular risk (McCormack, 2011).

Finally, a search in the PubMed database, with the keywords "Pyrazole and COX" resulted in a total of 3486 articles which demonstrate the interest of the scientific community in this issue. Meanwhile, in relation to keywords "Pyrazole and Oxidative burst", it was possible to conclude that there are just 38 reports on this subject.

2. Objectives

The main objective of this work was to evaluate the modulatory effect of a panel of twenty-eight pyrazoles against inflammation, through the inhibition of human COX-2 activity and human leukocytes' oxidative burst.

The following specific aims were established based in the activity of pyrazoles on:

- the cell viability of blood cells (leukocytes and erythrocytes);
- isolated human COX-2;
- PGE_2 production;
- COX-2 expression;
- Isolated ovine COX-1;
- human leukocytes' oxidative burst.

3. Methods

3.1. Chemicals

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA): Histopaque 1077, Histopaque 1119, trypan blue 0.4%, Dulbecco's phosphate buffer saline without calcium and magnesium ions (PBS), luminol, Vas-2870, phorbol 12- myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), AAS, glycine, tween® 20, cremophor®, glutamine, lipopolysaccharide (LPS), sodium dodecyl sulphate (SDS), 2-mercaptoethanol, N,N,N',N'-tetramethyl ethylenediamine (TEMED), penicillin/streptomycin, gentamicin sulphate, trizma, D-glucose and calcium chloride ($CaCl_2$). The ethylenediamine tetra acetic acid K_3 (EDTA) and heparin tubes to collect the blood were purchased from Vacuette S.A. (Porto, Portugal). The potassium chloride (KCl) was purchased from Pronolab (Lisbon, Portugal), the sodium chloride (NaCl) was purchased from VWR Chemical (Pennsylvania, USA) and the magnesium sulphate ($MgSO_4$) and methanol were purchased from Merck (Darmstadt, Germany). Absolute ethanol was obtained in Fischer Chemical (New Hampshire, USA). The RPMI medium and fetal bovine serum (FBS) were purchased from Thermo Fischer Scientific (Massachusetts, EUA). The primary antibodies, rabbit monoclonal anti-COX-2 and mouse anti-β-actin, and the secondary antibodies (anti-mouse and anti-rabbit HRP-conjugated) were purchased from Santa Cruz Biotechnology (Dallas, USA). The reagents below were obtained at Bio-Rad (Hercules, USA): polyvinylidene fluoride (PVDF) membranes, filter paper, blocking buffer, denaturant solution, acrylamide, and transfer buffer 10×. The Elisa kit for the determination of PGE_2 was purchased from Enzo Life Sciences (Lausen, Switzerland) and the fluorometric kits to assess inhibition of COX enzymes (1 and 2) were purchased from Abcam (Cambridge, United Kingdom).

The group of pyrazoles under study (Table 3) and thromboxane synthase inhibitor (TXBSI) were synthesized by the Chemistry Department and QOPNA of Aveiro University, as previously described (Cardoso *et al.,* 2015; Ferreira *et al.,* 2013; Silva *et al.,* 2004; Silva *et al.,* 2009; Silva *et al.*, 2007; Silva *et al.*, 2010).

Table 3: Panel of the studied pyrazoles.

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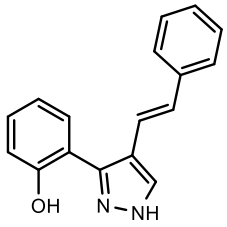
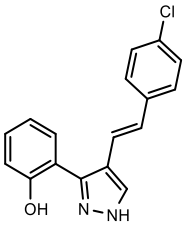
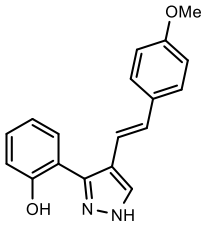
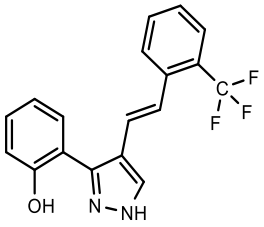
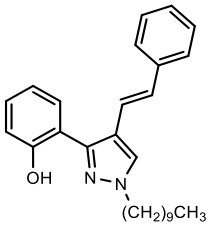
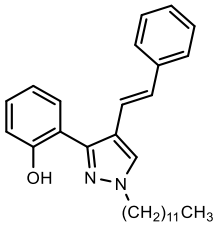
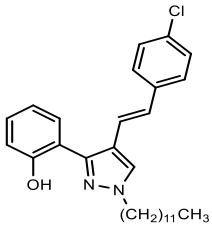
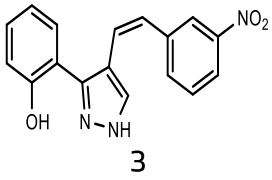
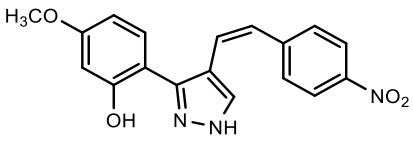
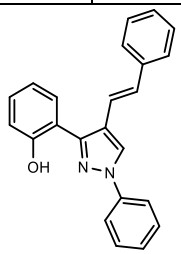
 1A	 1B	 1C	 1D
 2A	 2B	 2C	 3
 4	 16		
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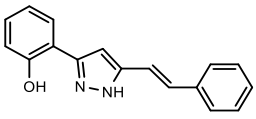
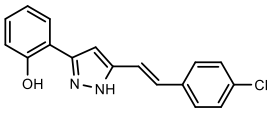
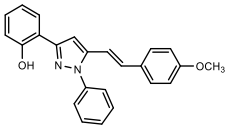
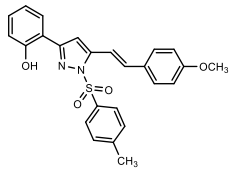
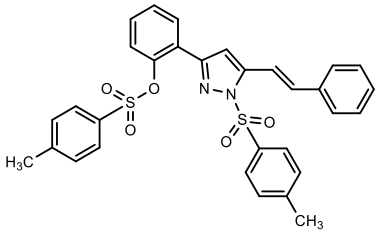
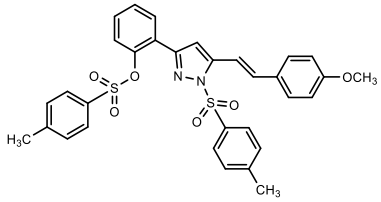
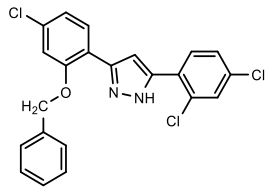
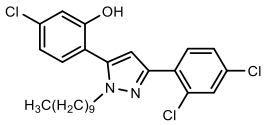
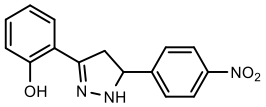
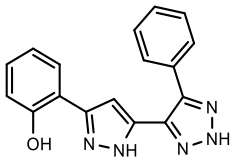
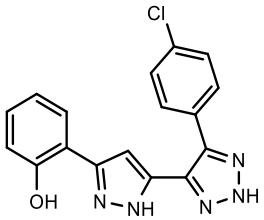
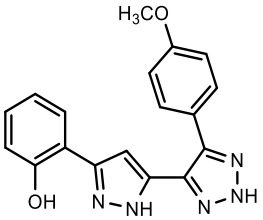
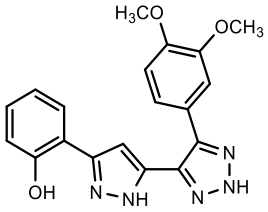
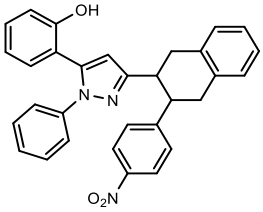
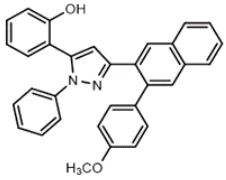
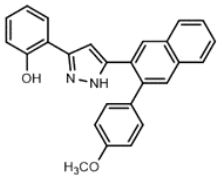
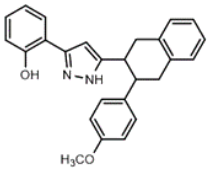
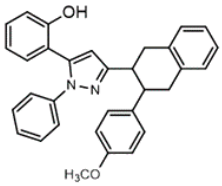
 5A	 5B	 6A	 6B
 7A	 7B		

Table 3: Panel of the studied pyrazoles

Miscellaneous			
 8	 9	 10	 11A
 11B	 11C	 11D	 12
 14	 15	 17	 18

3.2. Equipment

Fluorescence and chemiluminescence readings were performed in a microplate reader Synergy HT (BIO-TEK). Centrifugations were performed in an Allegra X-30R centrifuge (Beckman Coulter). Microscopy analyses were carried on an Eclipse E 400 optical microscope (Nikon). Flow cytometry was performed using a flow cytometer (BD Accuri C6). Lastly, the bands of western blot were detected by Chemidoc Imaging System (Biorad, Hercules, CA, USA).

3.3. Blood collection

Blood was collected from healthy human volunteers after their informed consent at the *Santo António* University Hospital Center, by peripheral venipuncture into lithium heparin vacuum tubes or K_3EDTA vacuum tubes, depending on the assay.

3.4. Viability of erythrocytes

The human whole blood (800 µL), collected in heparin vacuum tubes, was placed in a microtube with 110 µL of DPBS-gentamicin and 100 µL of the tested compounds (0-50 µM), dissolved in a mixture of DMSO/chremophor/ethanol 1% (1:10). Following this step, 600 µL of the previously prepared mixture was placed in each well of a 12-well plate, and 200 µL of PBS was added. Gently, the mixture was homogenized and incubated for 5 hours at 37°C.

Thereafter, the content of each well was resuspended and transferred to a conical microtube, which was centrifuged at 1500 *g*, for 5 minutes, at 4 °C, as illustrated in Figure 18. 100 µL of the supernatant obtained was placed in a transparent 96-well plate. The absorbance was read at 540 nm (maximum absorbance of haemoglobin) and 630 nm (maximum absorbance of methaemoglobin) in a microplate reader (single readings). The results are expressed as the percentage of haemolysis and represent at least 3 independent experiments. The positive control was triton X-100 (1.25 % and 0.625 %).

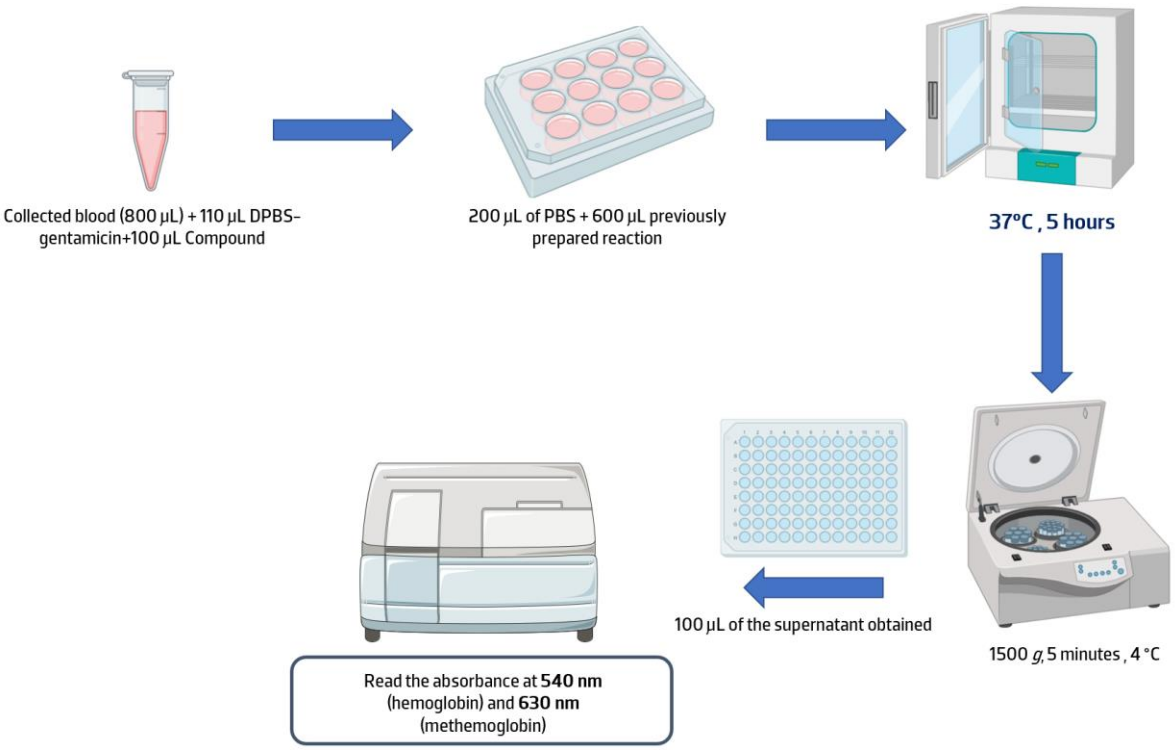


Figure 18: Schematic representation of the erythrocytes' viability assay.

3.5. Viability of leukocytes

The human whole blood (800 µL), collected in heparin vacuum tubes, was placed in 12-well plate, with 110 µL of DPBS-gentamicin and 100 µL of the compound under study (0-50 µM), dissolved in mixture of DMSO/chremophor/ethanol 1% (1:10). The plate was softly homogenized to ensure proper mixing and incubated for 5 hours at 37°C (Figure 19).

After the incubation period, the content of each well was resuspended and 100 µL of the solution was transferred to 15 mL centrifuge tubes. Hereafter was added 6 mL of lysis buffer at a ratio of 1:10, followed by an incubation of 10 minutes in the dark, at room temperature. After the incubation time, the tubes were centrifuged at 400 *g*, for 5 minutes, at 20°C and the supernatant was discarded.

Following this step, the pellet was resuspended in 2 mL of PBS and centrifuged at 400 *g*, for 5 minutes, at 20°C. The supernatant was discarded and 100 µL of propidium iodide (PI) (1 µg/mL) was added. The tubes were incubated for 15 minutes, in the dark, at room temperature, then 500 µL of PBS was added followed by the readings in the flow cytometer. The data are expressed as the percentage of PI positive cells and represent at least 3 independent experiments.

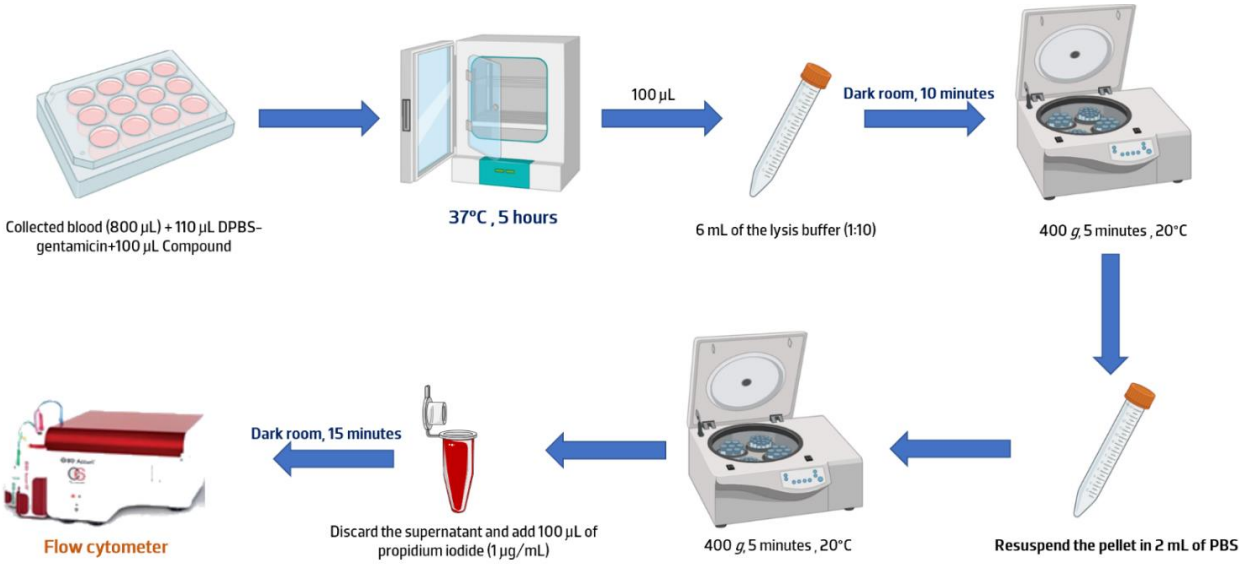


Figure 19: Schematic representation of the leukocytes' viability assay.

3.6. Isolation of human leukocytes by the density gradient centrifugation method

Approximately 4 mL of blood was collected, by antecubital venipuncture, into K_3EDTA vacuum tube from each healthy volunteer. The leukocytes were separated using two solutions of different densities, Histopaque 1119 (density 1.119) and Histopaque 1077 (density of 1.077). 3 mL of each solution was pipetted into a 15 mL polypropylene tube at room temperature, with the denser placed, at the bottom and the less dense solution on top. Polypropylene was chosen as the material for the tube to prevent the cells from adhering to the walls. It was important to pipette the less dense solution (Histopaque 1077) slowly and carefully to prevent the mixing of the solutions. Finally, blood was gently added on top of this discontinuous density gradient. After centrifugation at 900 *g,* for 30 minutes, at 20°C, the cells were distributed over the tube according to their density (Figure 20). Erythrocytes were deposited at the bottom of the tube, granulocytes (neutrophils, eosinophils, and basophils) at the Histopaque 1077/Histopaque 1119 interface and agranulocytes (lymphocytes and monocytes) as well as platelets at plasma/Histopaque 1077 interface. The two leukocyte layers were collected, using a *Pasteur* pipette, into a centrifuge tube in which PBS buffer without Ca^{2+} and Mg^{2+} was added (≈ 12 mL), to promote cells washing. The tube was then centrifuged at 850 *g,* for 5 minutes, at 4°C to preserve cell integrity. The resulting supernatant was discarded, and the pellet was resuspended in 1.25 mL of PBS without Ca^{2+} and Mg^{2+}. To avoid leukocytes being contaminated with erythrocytes, the remaining red blood cells were lysed by adding 4.00 mL of distilled water. The polypropylene tubes were carefully inverted and after approximately 3 minutes, isotonicity was re-established by the addition of 2.2 mL of a NaCl 3% solution (m/v). The suspension was submitted to a centrifugation at 850 *g,* for 5 minutes, at 4°C. Finally, the supernatant was discarded, and the cells were resuspended in Tris-glucose buffer (1.26 mM $CaCl_2$, 5.37 mM KCl, 0.81 mM $MgSO_4$, 140 mM NaCl, 25 mM Trizma, pH=7.4) enriched with 5.5 mM glucose, to perform the oxidative burst assay as described in section 3.12. For the COX-2 expression assay, as outlined in 3.11, the cells were resuspended in RPMI medium supplemented with 10% FBS, 1% of penicillin/streptomycin, and 1% of glutamine. To maintain viability, the isolated leukocytes were kept in ice and under stirring in an orbital shaker, until use.

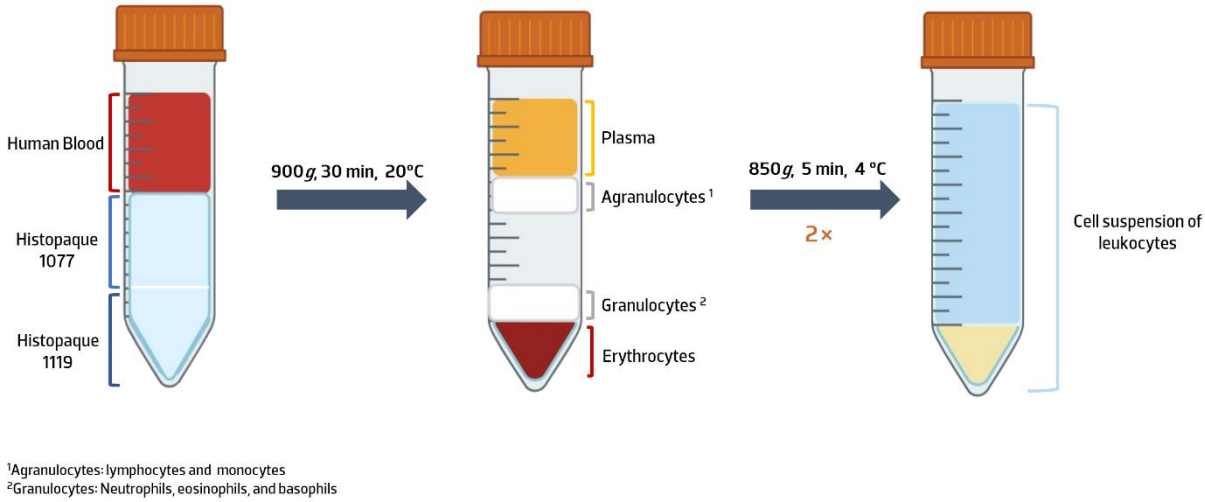


Figure 20: Schematic representation of the isolation of human leukocytes by the density gradient centrifugation method.

3.7. Evaluation of viability and human leukocyte count

At the end of the isolation of the cells, the viability of the human leukocytes was assessed by the trypan blue assay. Trypan blue is a negatively charged dye that only interacts with cells that have damaged membranes. As such, when the leukocyte is viable, it is shiny and colourless (without staining). The non-viable cell, on the other hand, is stained blue (Figure 21) (Tran *et al.*, 2011).

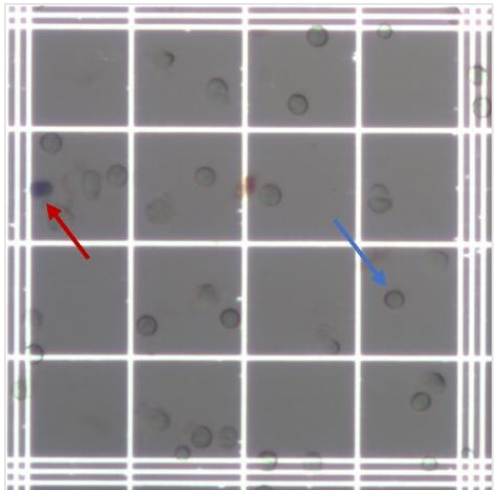


Figure 21: Photograph taken in optical microscope, immediately after the isolation of human leukocytes and incubation of the cells with trypan blue. Amplification 40 × (blue arrow – viable cell; red arrow - non-viable cell).

To determine the leukocyte viability, 20 µL of cell suspension and 10 µL of trypan blue solution were placed in a microtube. After homogenization, the content was placed in a Neubauer chamber, and the leukocytes were counted in the quadrant outlined in blue (Figure 22).

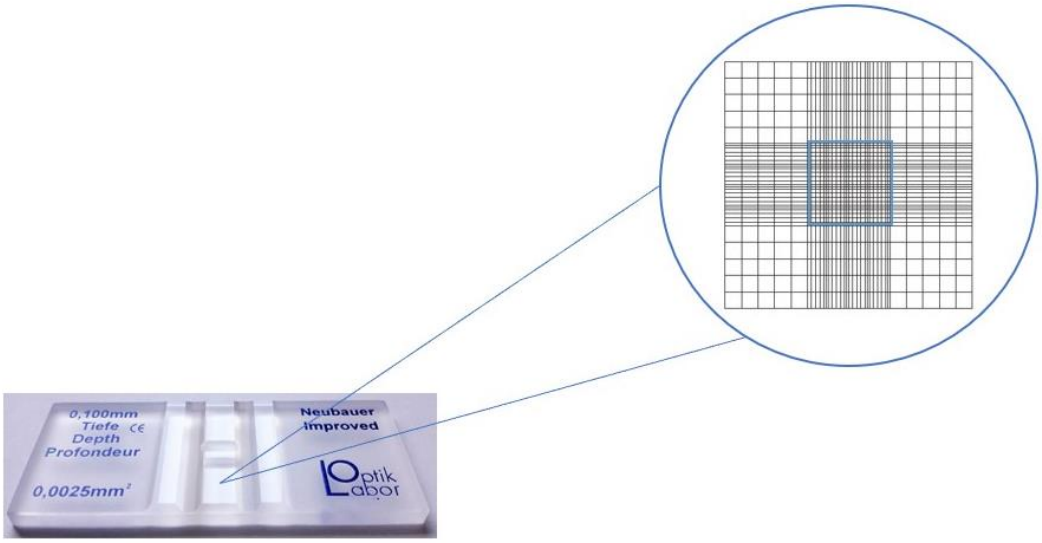


Figure 22: Illustration of the quadrants of the Neubauer chamber.

The cell viability was obtained from equation 1.

Equation 1: $$Cell\ viability = \frac{Nº\ of\ viable\ cells}{Total\ number\ of\ cells\ (viable+non-viable)}$$

3.8. COX-2 inhibition

The COX-2 activity was measured using a fluorometric inhibitor screening kit following the manufacturer's instructions. According to the kit datasheet, COX-2 was reconstituted with 110 µL of sterile H_2O and the AA reconstituted in the vial in 55 µL of 100% ethanol. Before the assay, reaction mix was prepared (76 µL COX assay buffer, 1 µL probe, 2 µL diluted COX cofactor and 1 µL COX-2). As shown in Figure 23, in a black 96-well plate, 5 µL of the compounds under study (0-50 µM), 40 µL of the reaction mix and 5 µL of AA were added on each well. The fluorescent signal was measured ($\lambda_{Ex/Em}$ = 535/587 nm) at 25°C, for 15 minutes. The results of the *in vitro* inhibitory activity of COX-2 corresponds to the slope of the kinetic reaction between 4 and 10 minutes. The positive control was celecoxib (0.03–1 µM), a selective COX-2 inhibitor.

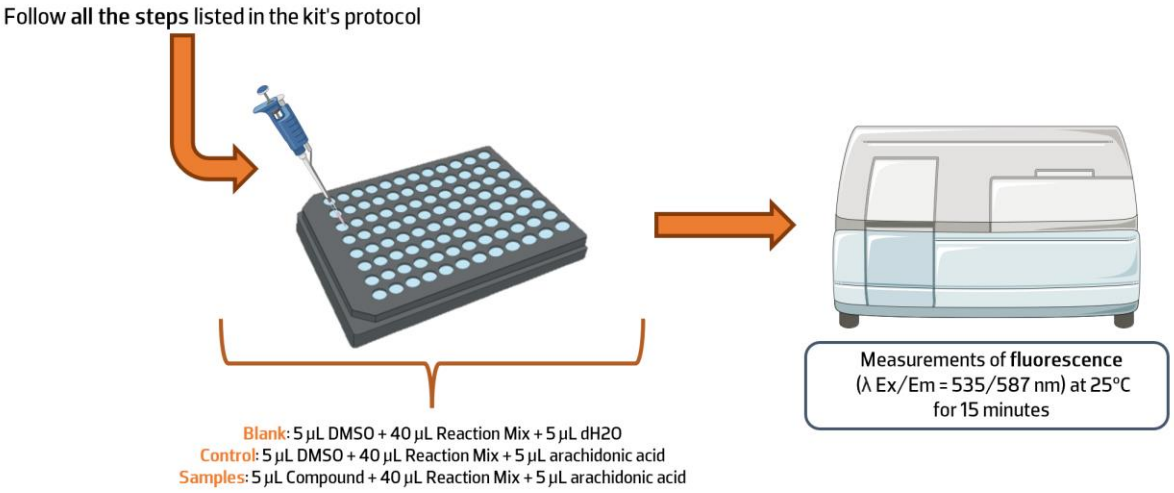


Figure 23: Schematic representation of the COX-2 inhibitor screening assay.

3.9. Determination of PGE_2 production

The collected human blood (800 µL) was placed in a 12-well plate and incubated at 37°C for 15 minutes, with 10 µL TXBSI (1 µM), 50 µL AAS (10 µg/mL) and 100 µL of compounds under study (0-50 µM), dissolved in a mixture of DMSO/chremophor/ethanol 1% (1:10). Then, 50 µL LPS (10 µg/mL) was added, and the mixture was incubated for 5 hours (Figure 24). The reaction was stopped by adding cold DPBS-gentamicin buffer (1 mL) to the samples and placing them on ice for 10 minutes. The samples were subsequently centrifuged at 1000 *g*, for 15 minutes, at 4°C to separate the blood cells from the supernatant. The supernatant was then collected and stored at -20°C until use. The solvents used did not have inhibitory effects and none affected cell viability. The amount of PGE_2 in the samples was measured using the Elisa kit, according to the manufacturer's instructions, as an indirect indicator of COX-2 activity. A COX-2 inhibitor, celecoxib (0.5-5 µM), was used as positive control.

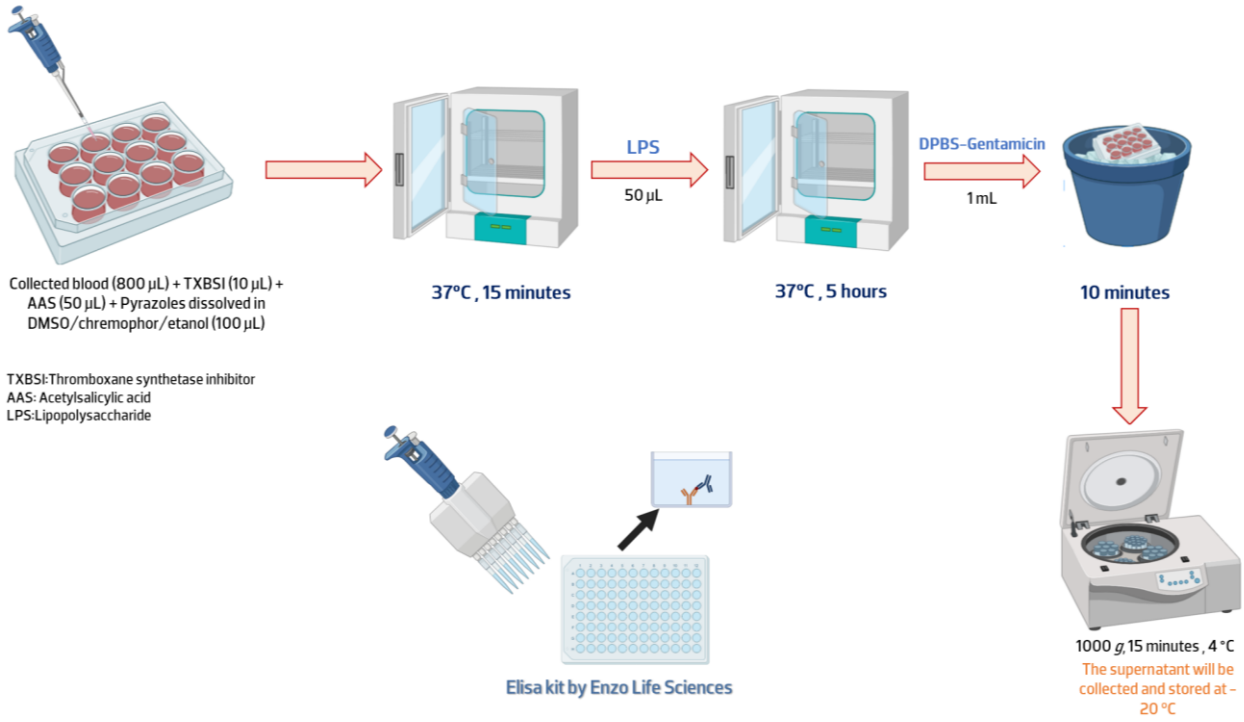


Figure 24: Schematic representation of the determination of PGE_2 production.

3.10. COX-1 inhibition

The COX-1 activity was measured using a fluorometric inhibitor screening kit following the manufacturer's instructions. According to the kit datasheet, COX-1 was reconstituted with 110 µL of sterile H_2O and the AA reconstituted in the vial in 55 µL of 100% ethanol. Before the assay, reaction mix was prepared (76 µL COX assay buffer, 1 µL probe, 2 µL diluted COX cofactor and 1 µL COX-1). As shown in Figure 25, in a black 96-well plate, 5 µL of the compounds under study (0-50µM), 40 µL of the reaction mix and 5 µL of AA were added on each well. The fluorescent signal was measured (λ Ex/Em = 535/587 nm) at 25°C for 15 minutes. The results of the *in vitro* inhibitory activity of COX-1 corresponds to the slope of the kinetic reaction between 4 and 10 minutes. The positive control was SC-560, a selective COX-1 inhibitor (0-25 nM).

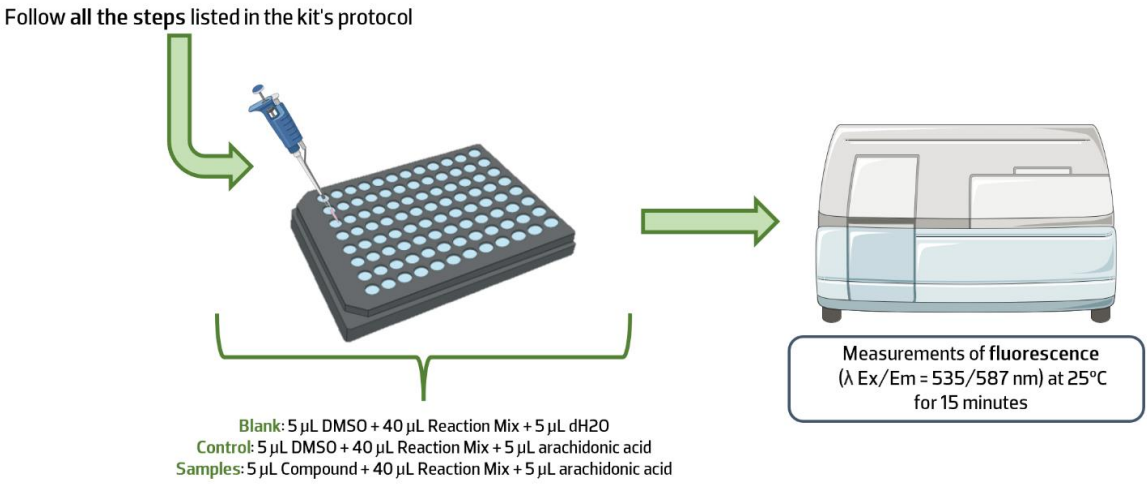


Figure 25: Schematic representation of the COX-1 inhibitor screening assay.

3.11. COX-2 expression on human leukocytes

The pellet resulting from the isolation of human leukocytes was resuspended in RPMI culture medium with 10% FBS, 1% of penicillin/streptomycin, and 1% of glutamine. In each well of a 12-well plate, 2000 µL of isolated human leukocytes (1×10^6 cells/mL) and 20 µL of the compounds under study (0 – 12.5 µM), dissolved in a mixture of DMSO/chremophor/ethanol 1% (1:10), were added and incubated for 15 minutes at 37°C. Following that incubation time, 50 µL of LPS was added to the control and samples, and the plate was incubated for 3 hours. The content was collected in a centrifuge tube and centrifuged at 380 *g*, at 4°C, for 10 minutes. The supernatant was collected and washed with 500 µL of PBS followed by another centrifugation in the same conditions. Then, RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 1.0 mM EDTA, 0.1 % SDS, pH=7.4) with the protease and phosphatase inhibitors was added and the samples were placed on ice for 30 minutes. Finally, a last centrifugation at 18000 *g*, at 4°C, for 10 minutes was performed, and the supernatant was collected and stored at -20°C (Figure 26).

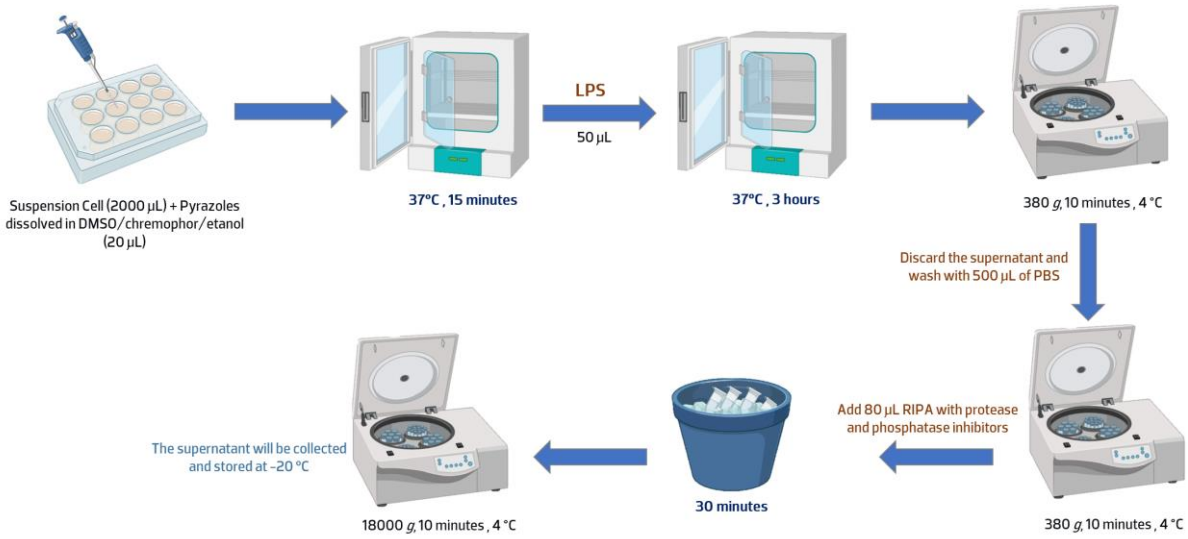


Figure 26: Schematic representation from the initial part of COX-2 expression on human leukocytes.

After these steps, the protein concentration of samples was determined by the Lowry method and cell lysates were denatured (denaturant solution with 2-mercaptoethanol). Proteins (30 µg/well) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in blocking buffer approximately 10 minutes, at room temperature. Subsequently, the membrane was incubated overnight, with the primary antibody, rabbit monoclonal anti-COX-2. Mouse anti-β-actin monoclonal was used to detect β-actin as the loading control. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were used as secondary antibodies. Immune complexes were detected with the Clarity™ western ECL substrate using Chemidoc Imaging System (Biorad, Hercules, CA, USA), and the bands were analysed using QLICS software (version 7.0, Total Lab), as illustrated in Figure 27. The results were normalized by calculating the ratio between the intensities of the bands corresponding to the protein of interest and the actin used as the loading control. The positive controls were apigenin and dexamethasone (6.25 and 25 µM).

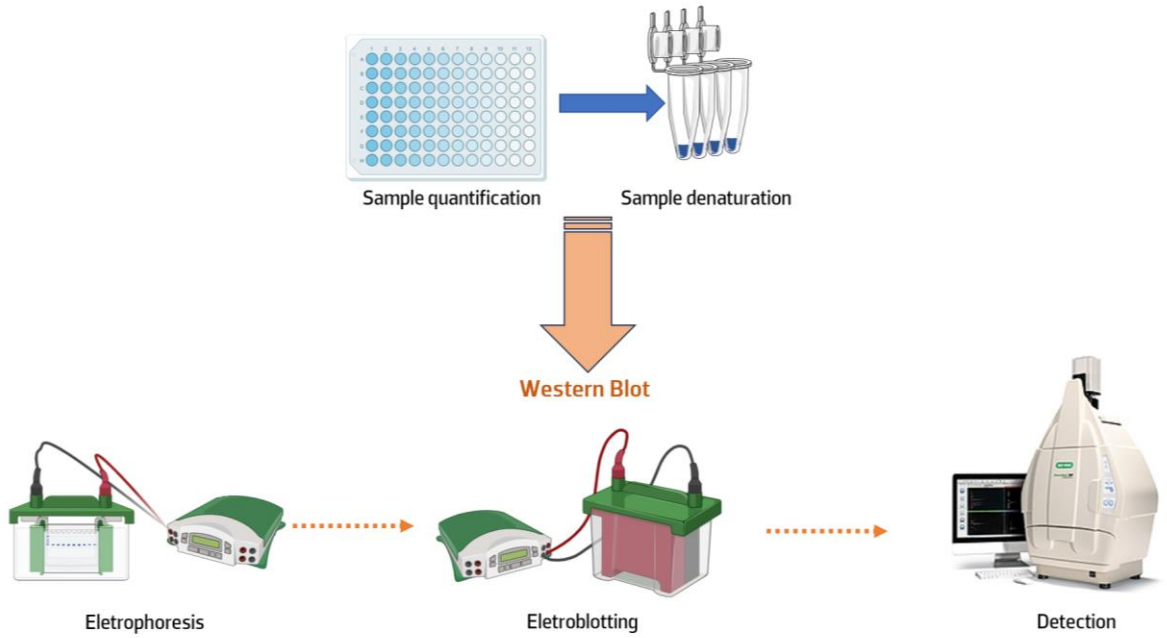


Figure 27: Schematic representation of endpoint of COX-2 expression on human leukocytes.

3.12. Detection of human leukocytes' oxidative burst

In a 96-well plate, 140 µL of isolated human leukocytes (1×10^6 cells/mL), 50 µL luminol (500 µM) and 10 µL of the compounds under study (0–50 µM) were added in each well. After a 5-minute incubation at 37°C, 50 µL of PMA (160 nM), an inducer of NADPH-oxidase activity, was added. The chemiluminescence readings were performed for 30 minutes, at 37°C (Figure 28). The Vas-2870 (0-5 µM), a known inhibitor of NADPH-oxidase was used as positive control. The results of the *in vitro* inhibitory activity of human leukocytes' oxidative burst corresponds to the highest value obtained in chemiluminescence reading.

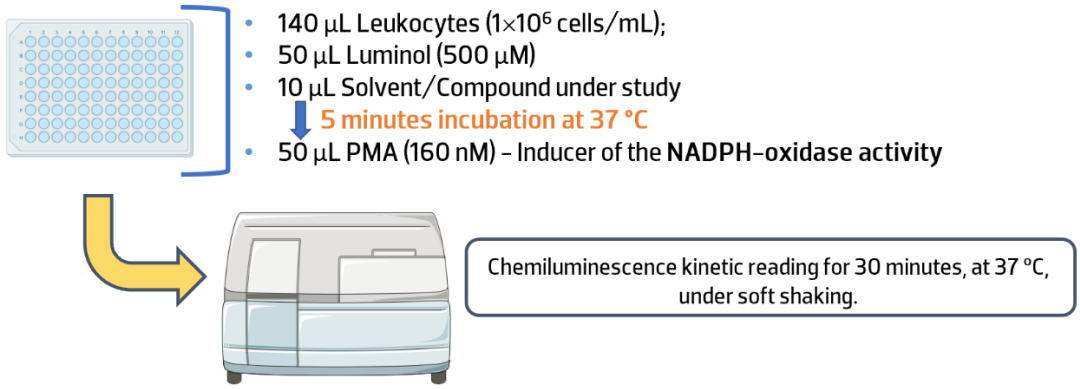


Figure 28: Schematic representation of pyrazoles effect on human leukocytes' oxidative burst.

3.13. Statistical analysis

The results of the *in vitro* tested inhibitory activities of the pyrazoles were expressed as mean percentage inhibition ± standard error of mean (SEM) or half maximal inhibitory concentration (IC_{50}) ± SEM ($n \geq 3$). A statistical comparison among the most active pyrazoles will be estimated using one-way analysis of variance (ANOVA), considering a p value < 0.05 statistically significant. The IC_{50} and all statistical analyses were performed using GraphPad Prism™ (version 9.0.0; GraphPad Software).

4. Results

4.1. Viability of erythrocytes

The evaluation of the effect of pyrazoles in the erythrocytes' viability is depicted in Figure 29. The tested concentration for the compounds under study was 50 µM, except for pyrazoles 2A, 2B and 2C, due to solubility limitations. Therefore, in those cases the concentration that was tested was 25 µM.

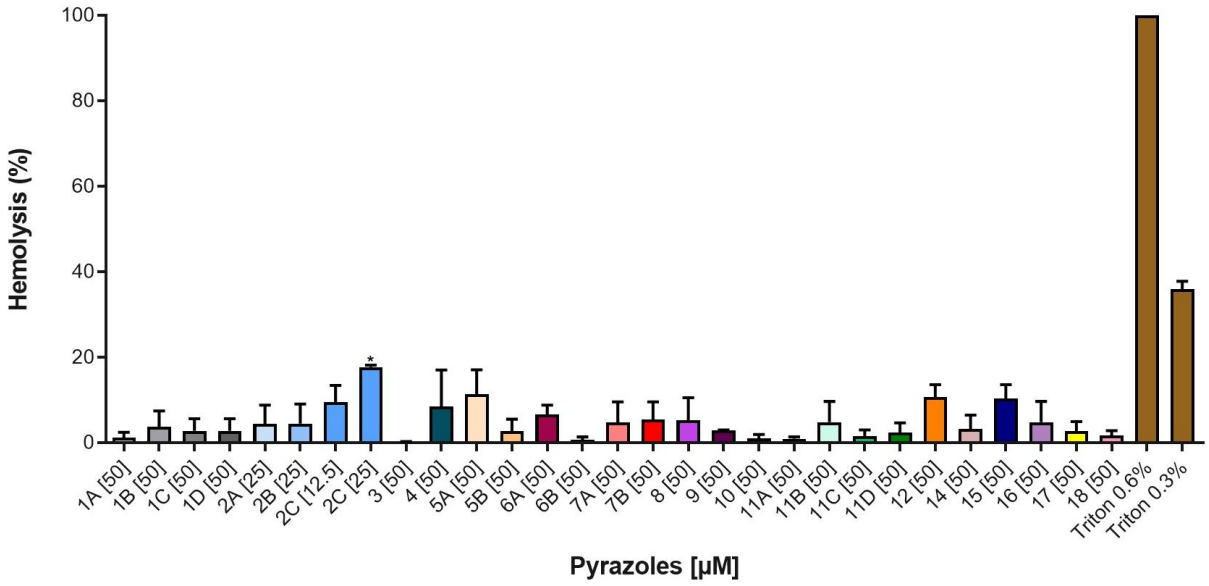


Figure 29: Erythrocyte viability based on the hemolysis values (%). *p < 0.05 when compared with the control (without pyrazoles). The results are represented as mean ± SEM (n ≥ 3).

As it can be seen in Figure 29 none of the compounds affect the viabilty of the erytrocytes, except the compound 2C, that presented a significant decrease at concentration of 25 µM. In this sense, all compounds were used at their maximum tested concentration with the exception of pyrazole 2C (maximum concentration tested was 12.5 µM).

4.2. Viability of leukocytes

The maximum concentration used to study leukocyte viability was 50 µM, with the exception of the compounds 2A, 2B, and 2C, due the above-mentioned solubility limitations. As shown in Figure 30 none of the concentrations tested affect the percentage of PI- positive cells, indicating

that the pyrazoles did not affect the viability of the leukocytes. Therefore, those concentrations were used in the following assays.

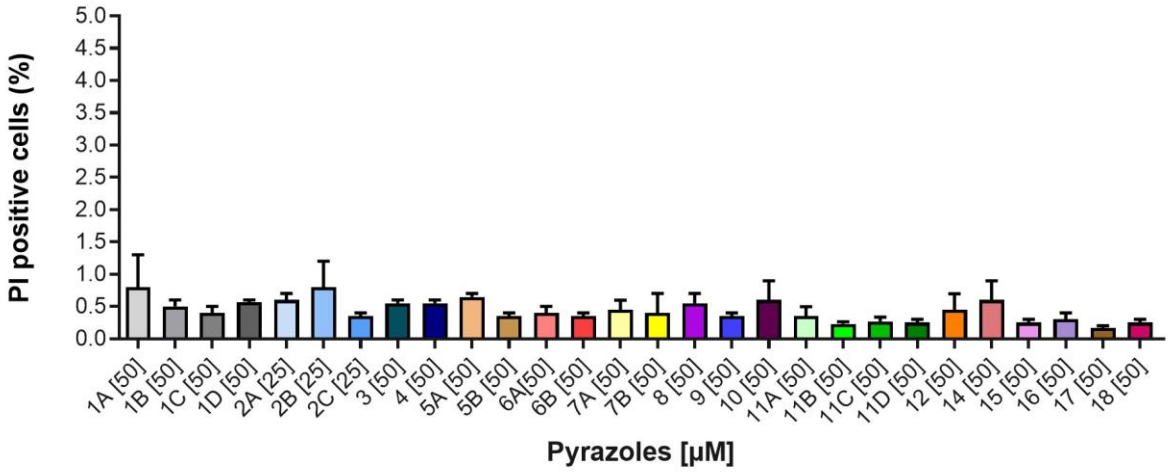


Figure 30: Viability of leukocytes based on PI positive cells (%). The results are represented as mean ± SEM (n ≥ 3).

4.3. COX-2 inhibition

The COX-2 inhibitory activity by pyrazoles was measured using a fluorometric inhibitor screening kit according to the manufacturer's instructions. The positive control used was celecoxib and presented an IC_{50} of 0.3 ± 0.1 µM (Figure 31).

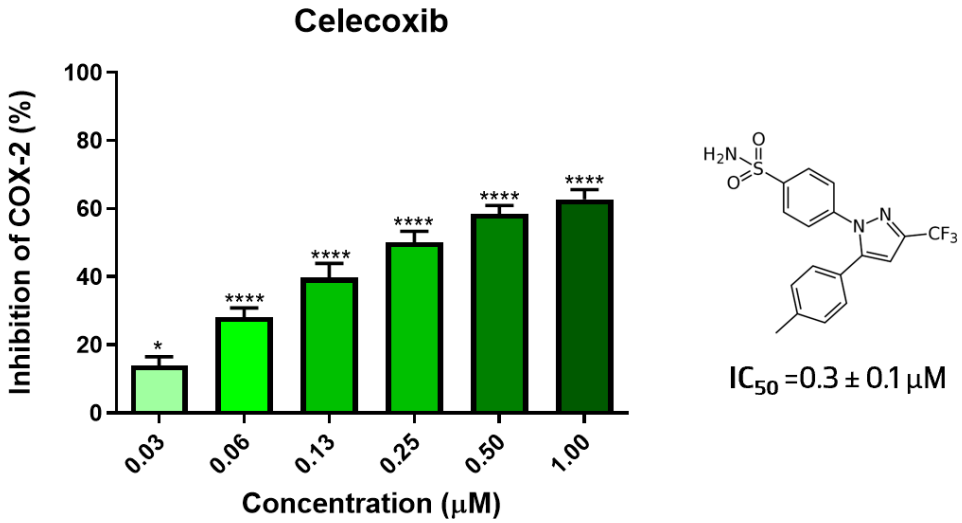


Figure 31: Inhibitory effect of celecoxib against the COX-2 activity. *p < 0.05, ****p < 0.0001 when compared with control (without pyrazoles). The results are represented as mean ± SEM (n ≥ 3).

The inhibitory effect against COX-2 activity of the pyrazoles is shown in Table 4. The compounds were divided into 3 groups based on their chemical structure. The 4-styrylpyrazoles from the compound 1 to the 4 and 16 which have a styryl substituent at position 4 of the pyrazole structure. The 5-styrylpyrazoles (5 to 7B) that have a styryl group in position 5 of the pyrazole. Lastly, the miscellaneous compounds from 8 to 18, except the pyrazole 16, which have chemical structures that are very diverse of each other and have been grouped in a single cluster.

Table 4: Chemical structures and *in-vitro* COX-2 inhibition activity of pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

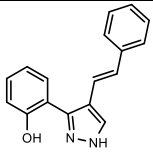
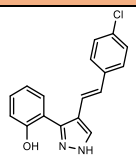
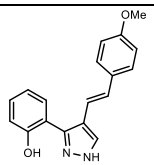
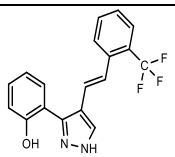
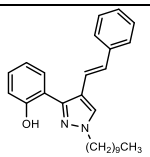
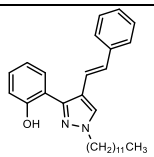
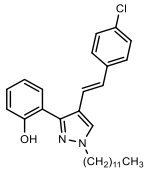
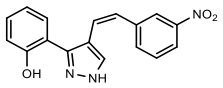
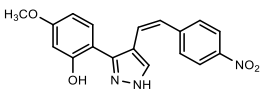
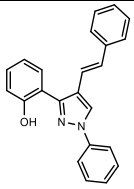
4-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 1A	58 ± 2 µM	 1B	43 ± 4 µM
 1C	62 ± 4 µM	 1D	62 ± 3 µM
 2A	<30% $^{25\,\mu M}$	 2B	<30% $^{25\,\mu M}$
 2C	<30% $^{12.5\,\mu M}$	 3	41 ± 1% $^{50\,\mu M}$
 4	23 ± 3 µM	 16	<30% $^{50\,\mu M}$

Table 4: Chemical structures and *in-vitro* COX-2 inhibition activity of pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

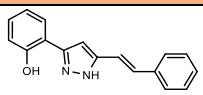
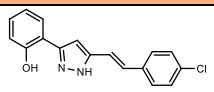
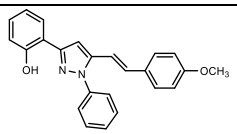
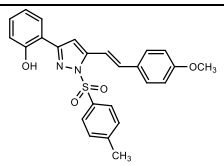
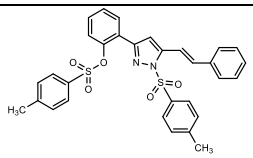
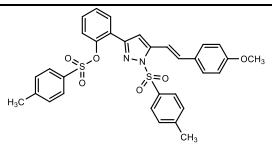
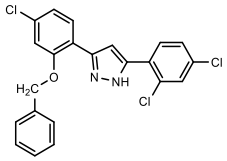
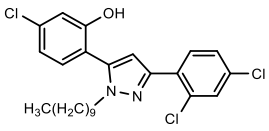
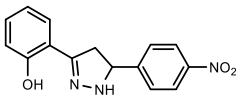
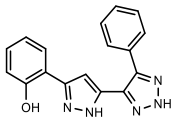
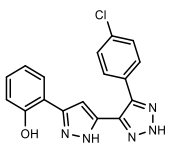
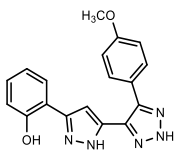
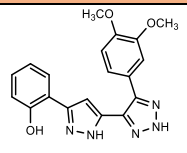
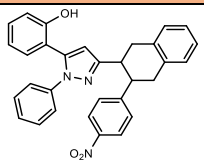
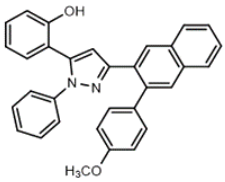
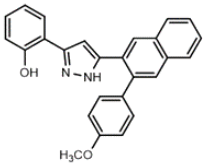
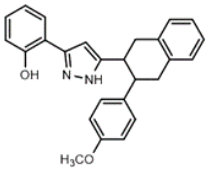
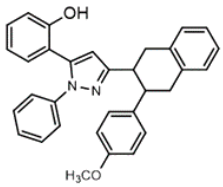
5-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 5A	<30% $^{50\,\mu M}$	 5B	<30% $^{50\,\mu M}$
 6A	<30% $^{50\,\mu M}$	 6B	<30% $^{50\,\mu M}$
 7A	<30% $^{50\,\mu M}$	 7B	<30% $^{50\,\mu M}$
Miscellaneous			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 8	<30% $^{50\,\mu M}$	 9	<30% $^{50\,\mu M}$
 10	<30% $^{50\,\mu M}$	 11A	34 ± 3% $^{50\,\mu M}$
 11B	15 ± 2 µM	 11C	41 ± 2 µM

Table 4: Chemical structures and *in-vitro* COX-2 inhibition activity of pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

Miscellaneous			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 11D	37 ± 3%$^{50\,\mu M}$	 12	<30%$^{50\,\mu M}$
 14	<30%$^{50\,\mu M}$	 15	<30%$^{50\,\mu M}$
 17	<30%$^{50\,\mu M}$	 18	<30%$^{50\,\mu M}$

Considering the 4-styrylpyrazoles, it was observed that all the compounds belonging to subgroup 1 (1A-1D), which have an identical chemical structure differing only the substituents attached to benzene group, showed inhibitory activity. The most active compound was the pyrazole 1B, with an IC_{50} of 43 ± 4 µM. This result showed that the chlorine substitution at position 4 of the benzene of the styryl group is favourable for the inhibitory effect. On the other hand, in subgroup 2 (2A-2C), none of the pyrazoles showed inhibitory activity at the maximum tested concentration. Regarding the remaining compounds of this group, the pyrazole 4 has a high activity since presents a IC_{50} lower to 25 µM (IC_{50} = 23 ± 3 µM), the compound 3 showed some activity, although it was not possible to determine its IC_{50} (42 ± 1%$^{50\,\mu M}$) and the 16 did not revealed an inhibitory activity.

In the 5-styrylpyrazoles group, none of the compounds showed inhibitory activity towards COX-2, which seems to indicate that the presence of the styryl group in position 5 of the pyrazole does not promote the inhibition of this enzyme.

Regarding the miscellaneous group, the most active pyrazoles were 11B and 11C presenting an IC_{50} of 15 ± 2 μM and 41 ± 2 μM, respectively. This result suggests that the substitution with chloro in position 4 of the phenyl attached to the triazole group is more favorable than the presence of methoxyl for COX-2 inhibitory activity.

4.4. Determination of PGE_2 production

The determination of PGE_2 production, which is an indirect method of assessing COX-2 inhibition, was performed in a more complex way using a whole blood assay. The positive control of the method was the celecoxib that presented an IC_{50} of 0.4 ± 0.1 μM, as it can be observed in Figure 32.

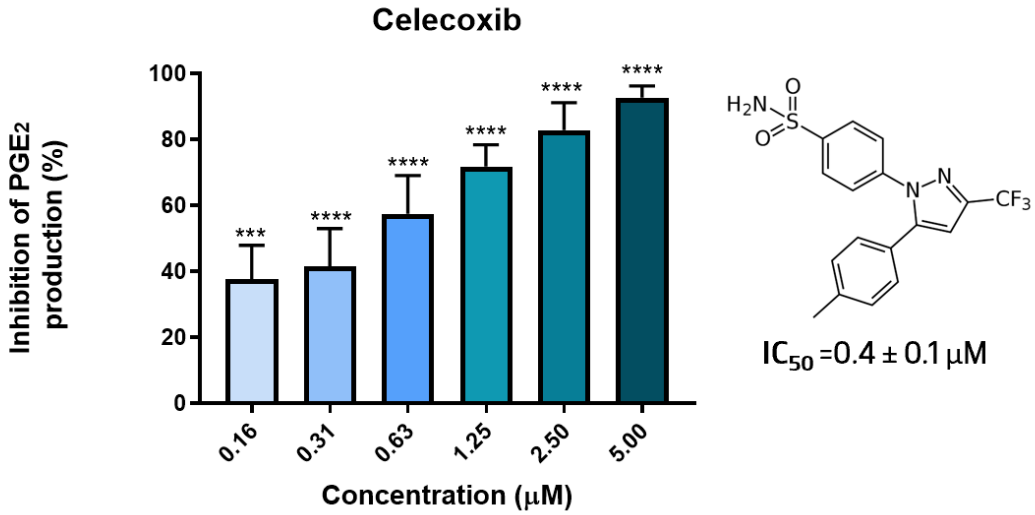


Figure 32: Inhibitory effect of celecoxib against the PGE_2 production. ***p < 0.001, ****p < 0.0001 when compared with control (without pyrazoles). The results are represented as mean ± SEM (n ≥ 3).

The inhibitory effect against the PGE_2 production by pyrazoles is shown in Table 5.

Table 5: Chemical structures and inhibition of PGE_2 production by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

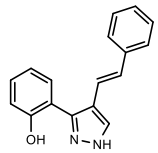
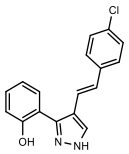
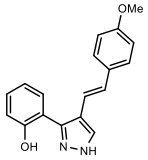
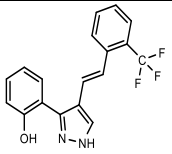
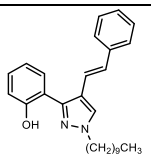
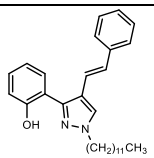
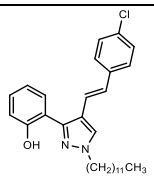
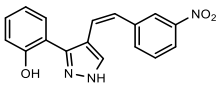
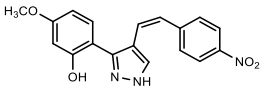
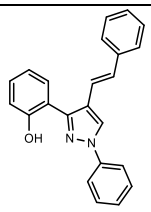
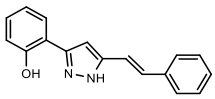
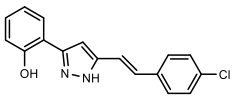
4-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 1A	10 ± 1 µM	 1B	<30% $^{50\ \mu M}$
 1C	<30% $^{50\ \mu M}$	 1D	<30% $^{50\ \mu M}$
 2A	<30% $^{25\ \mu M}$	 2B	<30% $^{25\ \mu M}$
 2C	<30% $^{12.5\ \mu M}$	 3	<30% $^{50\ \mu M}$
 4	<30% $^{50\ \mu M}$	 16	4.9 ± 0.4 µM
5-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 5A	<30% $^{50\ \mu M}$	 5B	<30% $^{50\ \mu M}$

Table 5: Chemical structures and inhibition of PGE_2 production by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

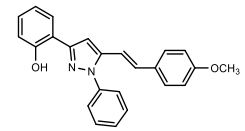
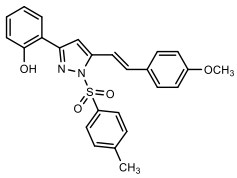
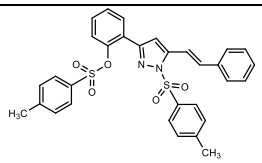
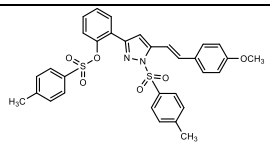
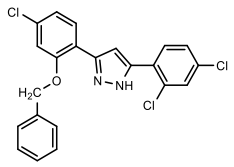
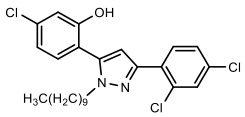
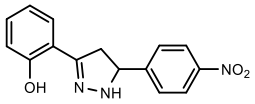
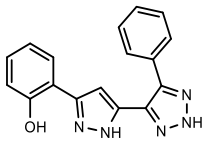
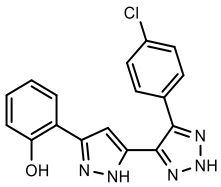
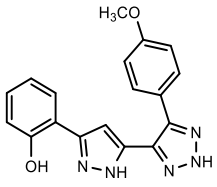
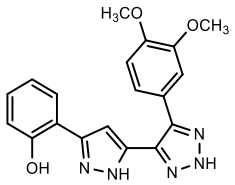
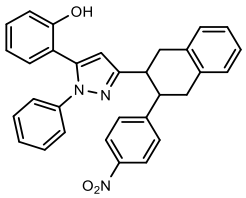
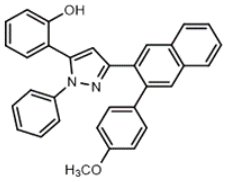
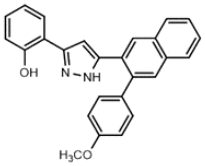
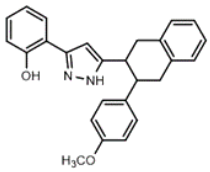
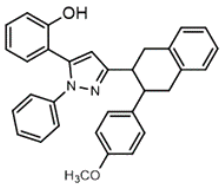
5-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 6A	9 ± 1 µM	 6B	<30% $^{50\ \mu M}$
 7A	<30% $^{50\ \mu M}$	 7B	<30% $^{50\ \mu M}$
Miscellaneous			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 8	<30% $^{50\ \mu M}$	 9	<30% $^{50\ \mu M}$
 10	<30% $^{50\ \mu M}$	 11A	<30% $^{50\ \mu M}$
 11B	<30% $^{50\ \mu M}$	 11C	<30% $^{50\ \mu M}$

Table 5: Chemical structures and inhibition of PGE_2 production by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

Miscellaneous			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 11D	<30% $^{50\,\mu M}$	 12	<30% $^{50\,\mu M}$
 14	22 ± 2 µM	 15	<30% $^{50\,\mu M}$
 17	<30% $^{50\,\mu M}$	 18	<30% $^{50\,\mu M}$

Among the group of 4-styrylpyrazoles, only two compounds, 1A and 16, were active against PGE_2 production with IC_{50} of 11 ± 1 µM and 4.9 ± 0.4 µM, respectively. This may suggest that these 2 chemical structures are conducive to inhibition of PGE_2 production.

Among the 5-styrylpyrazoles, only pyrazole 6A had inhibitory activity (IC_{50} = 9 ± 1 µM) on the production of PGE_2. Once again, it may indicate that this structure promotes the inhibition of PGE_2 production.

Lastly, concerning the miscellaneous group, almost all the compounds did not present inhibitory activity against PGE_2 production, with the exception of the pyrazole 14 which presented an IC_{50} of 22 ± 2 µM.

4.5. COX-1 Inhibition

The evaluation of COX-1 inhibitory activity by pyrazoles was measured using a fluorometric inhibitor screening kit according to the manufacturer's instructions. In the inhibition of COX-1 assay, the positive control used was SC-560, a selective COX-1 inhibitor, that presented an IC_{50} of 9 ± 1 nM (Figure 33).

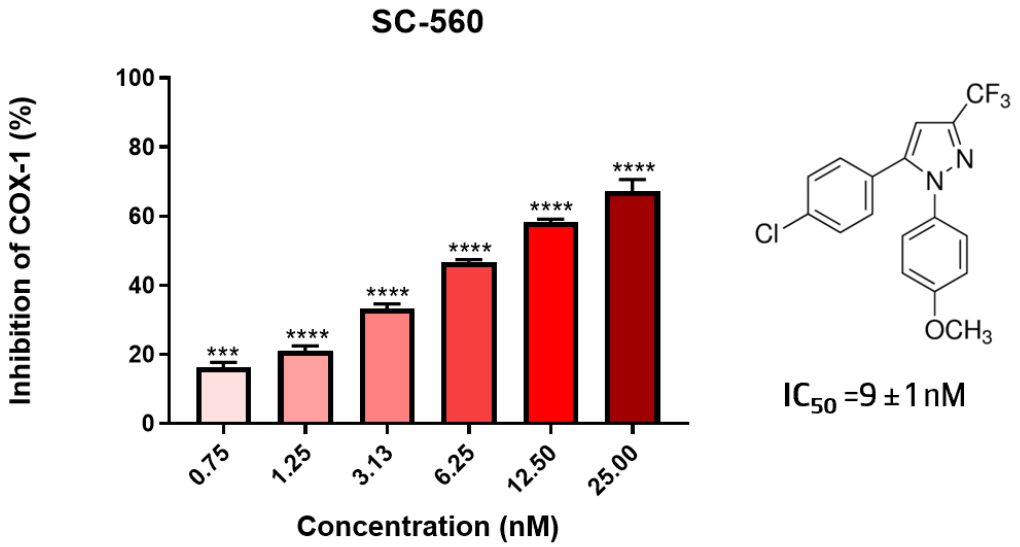


Figure 33: Inhibitory effect of SC-560 against the COX-1 activity. ***p < 0.001, ****p < 0.0001 when compared with control (without pyrazoles). The results are represented as mean ± SEM (n ≥ 3).

The inhibitory effect of the compounds against COX-1 activity is shown in table 6. It is important to highlight that only the pyrazoles that presented an IC_{50} less than 25 µM for COX-2 or PGE_2 inhibition, were tested against COX-1 activity.

Table 6: Chemical structures and *in-vitro* COX-1 inhibition by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

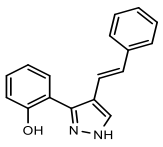
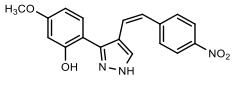
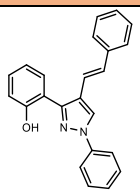
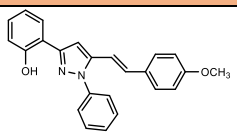
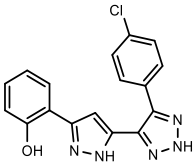
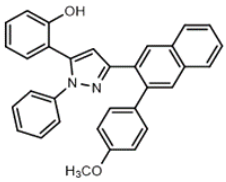
4-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 1A	26 ± 4 µM	 4	25 ± 3 µM

Table 6: Chemical structures and *in-vitro* COX-1 inhibition by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

<table>
<tr><th colspan="4">4-Styrylpyrazoles</th></tr>
<tr><th colspan="2">Pyrazole</th><th colspan="2">IC_{50} or % of Inhibition</th></tr>
<tr><td colspan="2"> 
16</td><td colspan="2"><30% $^{50\,\mu M}$</td></tr>
<tr><th colspan="4">5-Styrylpyrazoles</th></tr>
<tr><th colspan="2">Pyrazole</th><th colspan="2">IC_{50} or % of Inhibition</th></tr>
<tr><td colspan="2"> 
6A</td><td colspan="2"><30% $^{50\,\mu M}$</td></tr>
<tr><th colspan="4">Miscellaneous</th></tr>
<tr><th>Pyrazole</th><th>IC_{50} or % of Inhibition</th><th>Pyrazole</th><th>IC_{50} or % of Inhibition</th></tr>
<tr><td> 
11B</td><td>39 ± 2 µM</td><td> 
14</td><td>0.3 ± 0.1 µM</td></tr>
</table>

In fact, almost all compounds were active against COX-1 inhibition with exception of pyrazoles 16 and 6A. Among the 4-styrylpyrazole group, pyrazoles 4 and 16 had a similar inhibition of COX-1 with IC_{50} of 26 ± 4 µM and 25 ± 3 µM, respectively. Regarding the miscellaneous group, the pyrazole 14 was the most active with an IC_{50} of 0.3 ± 0.1 µM.

4.6. COX-2 expression on human leukocytes

Considering the COX-2 expression on human leukocytes, the positive controls used were apigenin and dexamethasone (6.25 and 25 μM). It is important to highlight that only the pyrazoles with IC_{50} less than 25 μM for COX-2 (4 and 11B) or PGE_2 (1A, 6A, 14 and 16) inhibition were tested their effect on COX-2 expression.

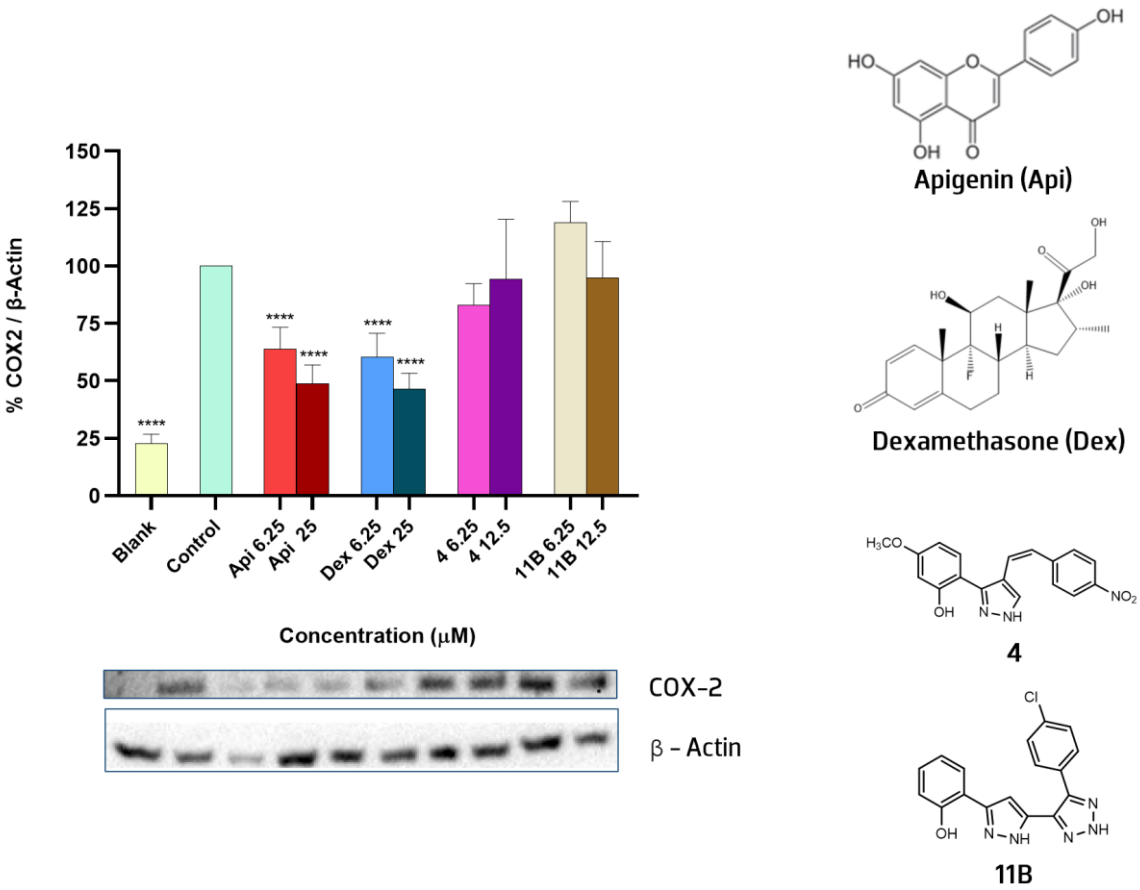


Figure 34: Effect of pyrazoles on COX-2 protein expression in human leukocytes. ****p < 0.0001 when compared with control (LPS 1 μg/mL). The results are represented as mean ± SEM (n ≥ 3).

As can be seen in the figure 34, none of the pyrazoles active in COX-2 inhibition, influence the expression of the COX-2 protein. In contrast, the positive controls showed similar reductions in COX-2 protein expression.

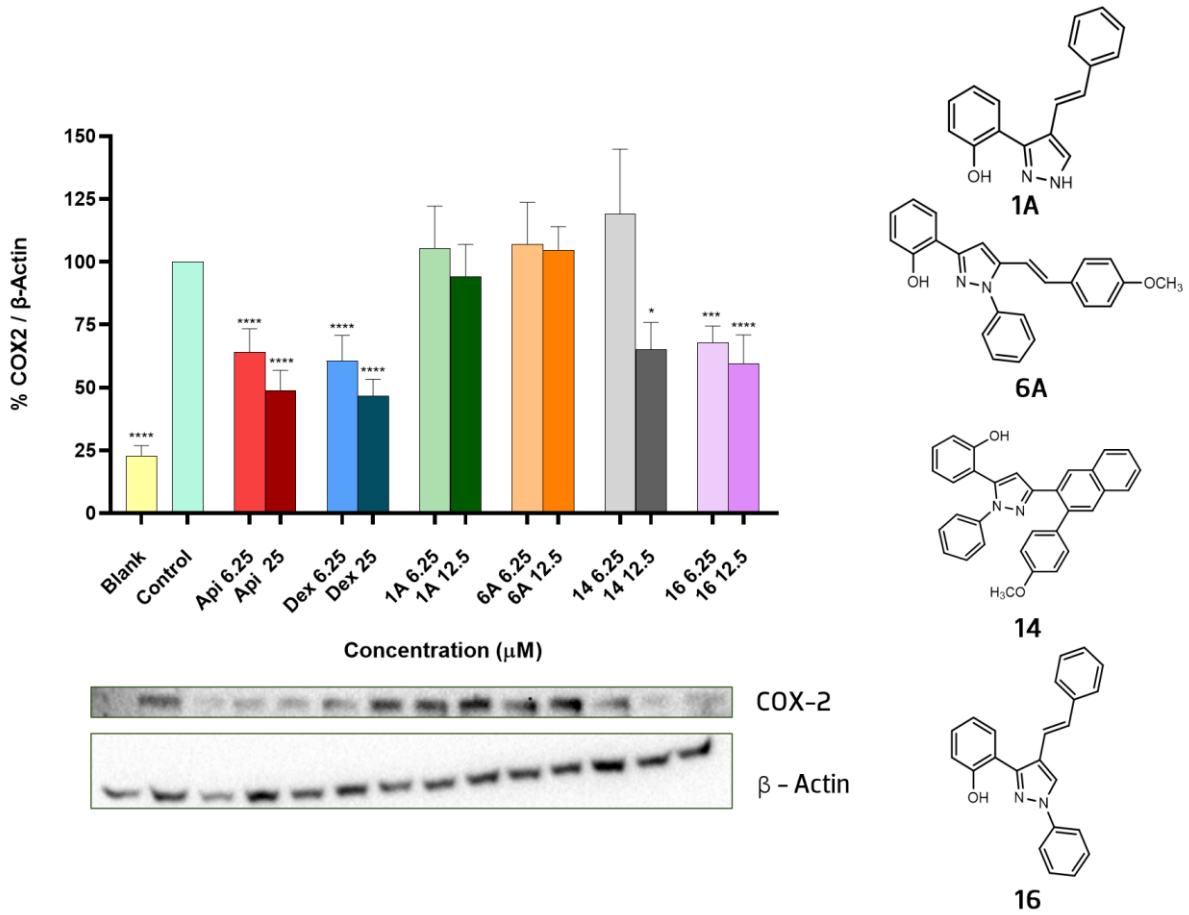


Figure 35: Effect of pyrazoles on COX-2 protein expression in human leukocytes. *p < 0.05, ***p < 0.001, ****p < 0.0001 when compared with control (with LPS 1 μg/mL). The results are represented as mean ± SEM (n ≥ 3).

In turn, the compounds active in inhibiting the production of PGE_2, the pyrazole 16 showed the highest reduction in COX-2 protein expression (40.5 ± 20.1 % at 12.5 μM). The compound 14 at a concentration of 12.5 μM also shows a reduction of 35 ± 18 %.

4.7. Inhibition of human leukocytes' oxidative burst

In the detection of human leukocytes' oxidative burst, the Vas-2870, a known inhibitor of NADPH-oxidase, was used as positive control and presented an IC_{50} of 1.6 ± 0.1 μM (Figure 36).

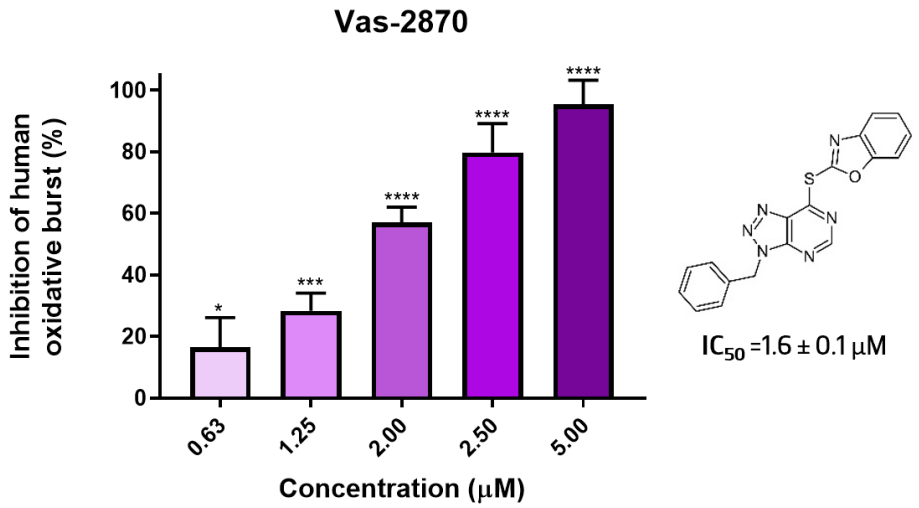


Figure 36: Inhibitory effect of Vas-2870 against the human leukocytes' oxidative burst. *p < 0.05, ***p < 0.001, ****p < 0.0001 when compared with control (without pyrazoles). The results are represented as mean ± SEM (n ≥ 3).

The inhibitory effect against the human leukocytes' oxidative burst by pyrazoles is shown in Table 7.

Table 7: Chemical structures and human leukocytes' oxidative burst inhibition by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

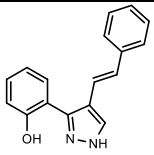
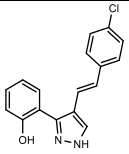
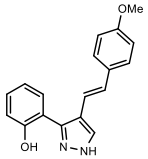
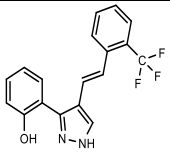
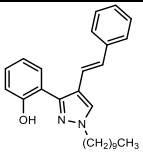
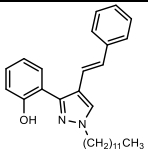
4-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 1A	<30% $^{50\ \mu M}$	 1B	17 ± 2 μM
 1C	34.8 ± 0.3 μM	 1D	31 ± 3 μM
 2A	<30% $^{25\ \mu M}$	 2B	<30% $^{25\ \mu M}$

Table 7: Chemical structures and human leukocytes' oxidative burst inhibition by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

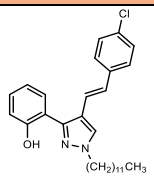
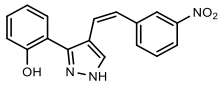
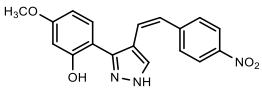
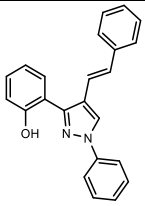
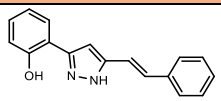
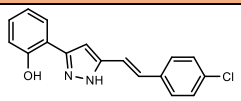
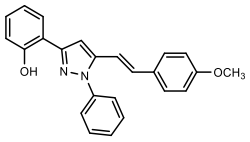
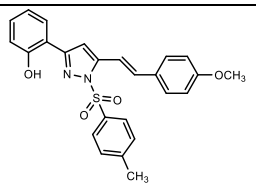
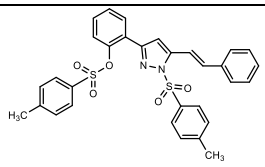
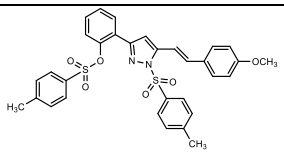
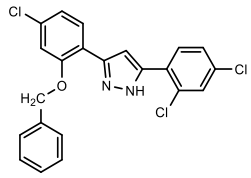
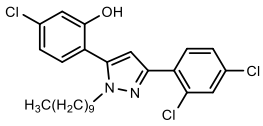
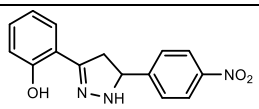
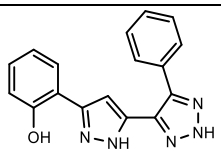
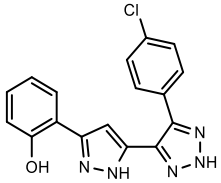
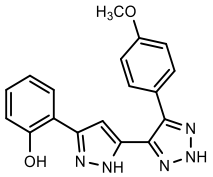
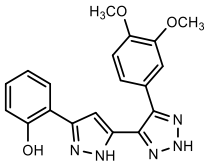
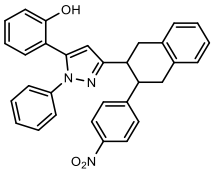
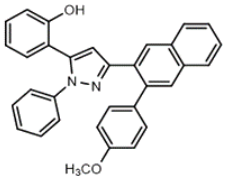
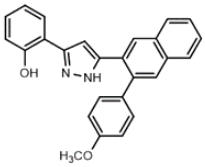
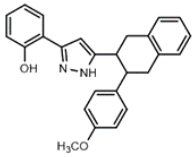
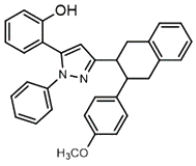
4-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 2C	<30%$^{12.5\ \mu M}$	 3	34 ± 3 µM
 4	4.5 ± 0.2 µM	 16	<30%$^{50\ \mu M}$
5-Styrylpyrazoles			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 5A	<30%$^{50\ \mu M}$	 5B	<30%$^{50\ \mu M}$
 6A	<30%$^{50\ \mu M}$	 6B	34 ± 3%$^{50\ \mu M}$
 7A	<30%$^{50\ \mu M}$	 7B	<30%$^{50\ \mu M}$

Table 7: Chemical structures and human leukocytes' oxidative burst inhibition by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

Miscellaneous			
Pyrazole	**IC_{50} or % of Inhibition**	**Pyrazole**	**IC_{50} or % of Inhibition**
 8	<30% $^{50\,\mu M}$	 9	<30% $^{50\,\mu M}$
 10	8 ± 1 µM	 11A	34 ± 2 µM
 11B	19 ± 2 µM	 11C	24 ± 2 µM
 11D	21 ± 1 µM	 12	31 ± 4 µM
 14	<30% $^{50\,\mu M}$	 15	<30% $^{50\,\mu M}$
 17	<30% $^{50\,\mu M}$	 18	<30% $^{50\,\mu M}$

Regarding the 4-styrylpyrazoles, it was observed that all the compounds belonging to subgroup 1 (1A-1D), except for 1A, showed inhibitory activity. The most active compound was the pyrazole 1B, with an IC_{50} of 17 ± 2 μM. This result showed that the chlorine substitution at position 4 of the benzene of the styryl group is favourable for the inhibitory effect against human leukocytes' oxidative burst. On the other hand, in subgroup 2 (2A-2C), none of the pyrazoles showed inhibitory activity at the maximum tested concentration. Considering the remaining compounds of this group, the pyrazole 4 has a high activity as it can be seen by the low IC_{50} (4.6 ± 0.2 μM), and compound 3 showed some activity with a IC_{50} of 34 ± 3 μM. Pyrazole 16 did not reveal an inhibitory activity, at the maximum tested concentration.

None of the compounds of 5-styrylpyrazoles group reach the IC_{50} towards human leukocytes' oxidative burst, which indicate that the presence of the styryl group in position 5 of the pyrazole does not promote the inhibition of the production of RS.

Regarding the miscellaneous group, the most active pyrazole was 10 with an IC_{50} of 8 ± 1 μM. It was observed that all the compounds belonging to the subgroup 11 showed inhibitory activity. The most active compound was 11B, presenting an IC_{50} of 19 ± 2 μM. This result suggests that the substitution with chloro in position 4 of the phenyl attached to the triazole group is more favorable than the presence of methoxyl, for the inhibition of human leukocytes' oxidative burst. Among the remaining group, only the pyrazole 12 had activity with an IC_{50} of 31 ± 4 μM.

5. Discussion

The inflammation is our natural response to an injury or infection. However, this response inevitably becomes a problem when it is excessive or chronic, thus contributing to the onset and progression of various diseases (autoimmune diseases, rheumatoid arthritis, and type 2 diabetes *mellitus* (Krishnamoorthy & Honn, 2006). Consequently, understanding the mechanisms underlying inflammation and how we can control this process is extremely important for medical as well as pharmacological research.

Pyrazoles are aromatic heterocyclic compounds composed of a doubly unsaturated 5-membered ring with 2 nitrogen atoms at the 1 and 2 positions of the ring. Pyrazole derivatives have received a lot of attention as potential anti-inflammatory, analgesic, and antipyretic agents (Küçükgüzel Ş & Şenkardeş, 2015). In this sense, a large number of pyrazoles have been synthesised with a variety of biological activities such as anti-inflammatory, antimicrobial, anticancer, cytotoxic, analgesic and hypertensive, among others. In fact, some of drugs containing pyrazoles, such as celecoxib, antipyrine, phenylbutazone, rimonabant, and others, are already on the market (Naim *et al.*, 2016). In this context, the study of pyrazoles as potential modulators of inflammation is high relevant and promising, since it could lead to the development of new drugs and the reduction of side effects, which would significantly improve the lives of patients with chronic inflammatory diseases, as well as personalising treatment, adapted to the specific needs of each patient.

COX-2 is an enzyme that plays a central role in the body's inflammatory response. It is responsible for the synthesis of prostaglandins, especially PGE_2, that play a crucial key in inflammatory process (Stiller & Hjemdahl, 2022). Currently, the existing COX-2 inhibiting drugs, NSAIDs, can have several adverse effects, particularly gastrointestinal ones. Although NSAIDs with more selectivity to COX-2 are known to decrease these gastric complications, their long-term use negatively affects the six main organs (heart, brain, lungs, stomach, kidneys and, liver) (Bindu *et al.*, 2020).

The oxidative burst is a metabolic process that occurs in leukocytes, mainly neutrophils and macrophages, as part of the inflammatory response. Nonetheless, the excessive production of RS, can also cause damage to surround tissues, leading to inflammatory symptoms, such as pain and swelling. Therefore, its regulation is essential as a part of modulation of inflammatory process (Peluso *et al.*, 2012).

Despite the existence of several studies on the effect of pyrazoles in modulating inflammation, the different experimental conditions of the *in vitro* or *in vivo* studies (*e.g.* cellular models/tested animals, substrates, enzymes origin, concentrations, pyrazoles origin, time of exposure, route of administrations, detection probes, reading equipment) and the limited number of studies in each research difficult or even avoid the comparison among studies and, consequently, the establishment of an accurate structure-activity relationship (SAR). Considering these difficulties, in the present work it was evaluated the modulatory effect of a panel of twenty-eight pyrazoles against inflammation, through the inhibition of COX-2 activity and human leukocytes' oxidative burst. Afterwards, it was also evaluated the modulatory effect of the most active pyrazoles (IC_{50} < 25 μM to COX-2 or PGE_2 inhibition) against the COX-1 and COX-2 expression. The SAR was established, whenever possible. In order to achieve the main objectives, this work has been divided into several interrelated sections.

Firstly, in order to avoid any kind of interference by pyrazoles with the following methodologies, the effect of pyrazoles on the viability of blood cells (erythrocytes and leukocytes) was studied. The maximum concentration chosen for the compounds was 50 μM. Although, due to solubility limitations, for pyrazoles 2A, 2B and 2C the maximum concentration tested was 25 μM. Regarding the viability of red blood cells based on the hemolysis values (%), it was observed that the pyrazoles, at the tested concentrations did not affect the viability of erythrocytes, with the exception of the compound 2C, which showed a significant decrease at concentration of 25 μM. Meanwhile, to access the effects of the compounds on leukocytes viability, the nuclear marker PI was used to detect necrotic cells, as it is able of binding with DNA if the cell membrane is damaged. The results indicated that none of the concentrations tested affect the percentage of PI positive cells, indicating that the pyrazoles did not affect the viability of the leukocytes. In general, these results were not surprising, since there are drugs with pyrazole derivates in their composition, so at these concentrations, it would not be expected that these pyrazoles would affect the viability of blood cells (Karrouchi *et al.*, 2018).

Following this, the effect of pyrazoles on the inhibition of isolated human COX-2 was evaluated. The compounds were divided into 3 groups based on their chemical structure: the 4-styrylpyrazoles from the compound 1 to the 4 and 16 which have a styryl substituent at position 4 of the pyrazole structure; the 5-styrylpyrazoles (5 to 7B) that have a styryl group in position 5 of the pyrazole, and the miscellaneous compounds from 8 to 18, except the pyrazole 16, which have chemical structures that are very diverse of each other and have been grouped in a single

cluster. Considering the results obtained, the styryl group in position 4 of ring seems to be more favourable for COX-2 inhibition than the same group in the position 5 of the ring, since none of the pyrazoles belonging to the 5-styrylpyrazoles group was able to inhibit the COX-2 enzyme. Regarding the subgroups 1 and 11, from the 4-styrylpyrazoles and miscellaneous respectively, which have similar chemical structures differing only in the substituent groups, it might be concluded that the presence of a chloro substituent in the styryl group, appears to be fundamental for their inhibitory activity, as shown in compounds 1B and 11B. Dube *et al.* (2014) reported that structurally simple pyrazole derivates with a phenyl group proved, through *in vitro* assays, to be potent selective COX-2 inhibitors. Murahari *et al.* (2019) also concluded that pyrazoles derivates with nitro ligands or halogenated groups such as chloro and fluoro in ortho as well as in meta positions promoted an anti-inflammatory activity, particularly by COX-2 inhibition. Moreover, Wulan *et al.* (2023) documented by computational techniques that the chloro group in thienyl pyrazole derivates is important to inhibit the COX-2 enzyme, as it increases the affinity the protein's active core.

Subsequently, the effect of pyrazoles on PGE_2 production was determined. It would be expected that the results obtained would be similar to those of the previous methodology, since the determination of PGE_2 is an indirect method of assessing COX-2 inhibition. However, it is not the case, only one pyrazole, 1A, was able to simultaneously inhibit the COX-2 enzyme and the production of PGE_2. Besides this, three other compounds were active, namely pyrazoles 6A,14 and 16. Considering that PGE_2 was determined using a whole blood, that is a complex matrix when compared with the isolated enzyme that was used to detect the COX-2 inhibition, this could result in a less effective inhibition of PGE_2 production. Also, in whole blood assay, the compounds might bind to proteins or other components of blood, reducing its availability to interact with the target enzyme responsible for PGE_2 production. The compound's bioavailability within whole blood may be also limited, meaning it does not reach the target enzyme or site of action in sufficient quantities to inhibit PGE_2 production effectively, when compared with the isolated enzyme. Furthermore, the compounds that were active against COX-2 may not have a lipophilic-hydrophilic equilibrium sufficient to enter into blood cells, particularly leukocytes. In contrast, pyrazoles 6A, 14 and 16 only inhibited the production of PGE_2. This seems to suggest that although these pyrazoles did not inhibit COX-2, they were able to decrease PGE_2 production by inhibiting another enzyme, involved in the PGE_2 production (*e.g.* phospholipase A_2, PGEs), or even by affecting the expression of the COX-2, as will be discussed later. As Park *et al.* (2006) pointed

out, inhibiting PGEs rather than COX-2 could be positive, since COX-2 inhibitors besides reduce the production of pro-inflammatory PG, they might as well affect the synthesis of beneficial prostaglandins with cardiovascular protective effects and therefore only inhibit the production of pro-inflammatory PGE_2, leaving aside the homeostatic mechanisms could be beneficial.

The COX-1 inhibitory activity by pyrazoles was determined to evaluate their selectivity. It is important to highlight that only the pyrazoles with IC_{50} < 25 μM for COX-2 or PGE_2 inhibition were tested against COX-1 ovine activity. Although ovine COX-1 was used, it has been reported that human and ovine COX-1 are 96% similar, therefore COX-1 ovine is a reliable model for evaluating the possible action of pyrazoles on human COX-1 (Lee *et al.*, 2007). Among the tested compounds, the pyrazole 11B was the only one that showed selectivity for COX-2 inhibition with a selectivity index of 3 (IC_{50} of COX-1/IC_{50} of COX-2). On the other hand, only analysing the COX-1 and COX-2 results, the pyrazole 1A showed selectivity to COX-1. However, looking to PGE_2 results, the compound presented a lower IC_{50} to PGE_2 when compared to COX-1. However, based in these results it is not possible to attribute the inhibition of PGE_2 production to the direct inhibition of COX-2 since this inhibition might have the contribution of other enzymes that contribute to the production of PGE_2, as already mentioned. Additionally, the pyrazole 14 presented high selectivity to COX-1 with a selectivity index of 0.01. According to the literature, the selective inhibition of COX-2, as it was observed for pyrazole 11B, is the ideal scenario, in a context of inflammation considering that non-selective compounds have a variety of severe side effects, particularly gastric and duodenal damage (Zarghi & Arfaei, 2011). Hence, by selectively inhibiting the COX-2 enzyme, which plays a primary role in the inflammatory process, we can effectively target the inflammatory process. However, it is worth noting that the observed selective inhibition of COX-1 in pyrazole 14, while not typically desired for inflammation modulation, could hold significant value. Dvorakova *et al.* (2021) have mentioned that COX-1 upregulation was discovered in a multiple of cancers (skin, breast, colorectal) and also atherosclerosis. Therefore, the specific inhibition of COX-1 is a potentially attractive target for the treatment of neurodegenerative diseases and cancers. Indeed, Calvello *et al.* (2017) have demonstrated that the selective COX-1 inhibitors reduced PGE_2 derived from COX-1 in LPS-activated mouse BV-2 microglial cells. Furthermore, Perrone *et al.* (2012) had already mentioned that 5-(furan-2-yl)-1-(4-mehoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole selectivity inhibited COX-1 through a COX (ovine) inhibitor assay kit.

In order to elucidate the mechanism of action of the pyrazoles, their effect on COX-2 expression was also evaluated. Once again, only the pyrazoles with IC_{50} < 25 μM for COX-2 or PGE_2 inhibition were tested. Among the tested pyrazoles, only two were active in reducing COX-2 expression, the pyrazoles 14 and 16, which belong to the group of compounds that only inhibited the production of PGE_2. It appears to suggest that the reduction in PGE_2 production observed in these two pyrazoles is not a result of COX-2 activity inhibition but rather a consequence of reduced enzyme expression. In fact, there is no literature available that links pyrazoles to COX-2 expression in human leukocytes, which demonstrates the pertinence and importance of this assay. In addition to decreasing COX-2 expression, apigenin also inhibited PGE_2 production with an IC_{50} of 8 ± 1 μM. This is corroborated by Yi Lau and Leung (2010) who already found a decrease in PGE_2 in breast cells containing apigenin as well as a reduction in COX-2 expression. This seems to suggest that pyrazoles 14 and 16 might have a similar mechanism of action to apigenin since they also inhibited PGE_2 production and affected the COX-2 expression. Kim *et al.* (2018) observed that celecoxib paradoxically did not cause a reduction but rather an increase in COX-2 expression in human monocytes. This may indicate that the pyrazole 1A, which inhibited both COX-2 activity and PGE_2 production, and 11B, which showed selectivity for COX-2 inhibition, may have a similar mechanism to celecoxib, once there were no significant differences in COX-2 expression.

Lastly, the effect of pyrazoles on human leukocytes' oxidative burst was evaluated. In order to induce the RS production in human leukocytes, the cells were activated with PMA, a chemical compound that acts by mimicking the action of DAG, a specific activator of PKC, which is responsible for triggering the activation of NADPH oxidase. In consequence, NADPH oxidase generates a large amount of RS (Belambri *et al.,* 2018). The concentration of PMA (160 nM) adopted was based on previous studies of our research group (Freitas, Porto, *et al.*, 2009; Ribeiro *et al.*, 2013). In view of the results obtained, the styryl group in position 4 of ring seems to be more favourable for human leukocytes' oxidative burst inhibition than the same group in the position 5 of the ring, since none of the pyrazoles belonging to the 5-styrylpyrazoles group was able to inhibit the RS production. Considering the results of the compounds in subgroup 1, the presence of a chloro substituent in position 4 of the benzene of the styryl group seems to be more favourable for the inhibition of human leukocytes' oxidative burst than the methoxy substituent or no substituent in the same position. In contrast, in subgroup 11, the results may indicate that the presence of chloro or methoxy substituent in the position 4 of the phenyl attached to the

triazole group is indifferent to effect on oxidative burst, since they presented similar IC_{50}. Indeed, Costa *et al.* (2006) referred that the pyrazoles derivatives, pyrazolones, were able to inhibit the production of HOCl and $HO^{\cdot}$ in human neutrophils, namely the pyrazole derivate substituted at position 4 by methyl(sulfonatomethyl)amino group. In another publication, Costa *et al.* (2008) suggest that the pyrazoles derivates, particularly dipyrone and aminopyrine are potent scavengers of $^1O_2$ when compared to other NSAIDs studied, presenting IC_{50} values in the low micromolar range. It is worth emphasising the lack of information in literature linking the inhibitory activity of pyrazoles on the oxidative burst.

6. Conclusions

The results obtained in the present work allowed us to draw several conclusions about the modulatory effect of a panel of structure related pyrazoles against inflammatory process, through the inhibition of COX-2 activity and human leukocytes' oxidative burst.

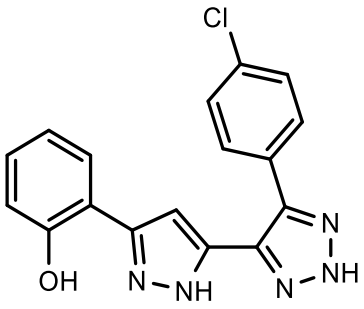
Firstly, several pyrazoles were able to inhibit the COX-2, mainly the 4-styrylpyrazoles. Pyrazoles 4 and 11B are particularly noteworthy as they had IC_{50} values below 25 μM. It was also observed that only compound 1A was active against COX-2 activity as well as production of PGE_2.

Furthermore, regarding the inhibition of COX-1, the pyrazole 11B showed selectivity to COX-2 with a selectivity index of 3. On the other hand, the pyrazole 14 presented a high selectivity to COX-1 (selectivity index of 0.01). In turn, pyrazoles 14 and 16 exhibited a substantial reduction in COX-2 expression levels, even though they did not directly inhibit COX-2 activity.

Considering the human leukocytes' oxidative burst, a wide range of pyrazoles inhibited the RS production, with particular emphasis on pyrazole 1B, 4 and 11B which also have the ability to inhibit the COX-2 activity.

In sum, considering all the results obtained the pyrazole that seems to be more interesting is 11B, since it promoted the selective COX-2 inhibition, and also inhibited the human leukocytes' oxidative burst, acting as a dual modulator of the inflammatory process (Figure 37).

This study provided important considerations about the pyrazoles and their anti-inflammatory as well as their antioxidant properties and subsequently their promising modulatory effects towards the inflammatory process. These results might also contribute for the design of novel compounds that can be useful in the management of inflammation.



11B

Figure 37: Chemical structure of the pyrazole 11B.

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Attachments

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

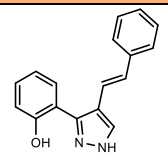
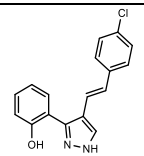
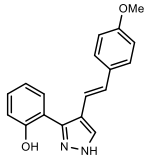
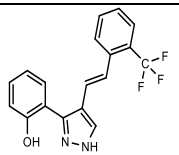
4-Styrylpyrazoles				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 1A	$IC_{50} = 58 \pm 2\ \mu M$	$IC_{50} = 11 \pm 1\ \mu M$	$IC_{50} = 26 \pm 4\ \mu M$	$<30^{50\ \mu M}$
 1B	$IC_{50} = 43 \pm 4\ \mu M$	$<30^{50\ \mu M}$	----	$IC_{50} = 17 \pm 2\ \mu M$
 1C	$IC_{50} = 62 \pm 4\ \mu M$	$<30^{50\ \mu M}$	----	$IC_{50} = 34.8 \pm 0.3\ \mu M$
 1D	$IC_{50} = 62 \pm 3\ \mu M$	$<30^{50\ \mu M}$	----	$IC_{50} = 31 \pm 3\ \mu M$

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

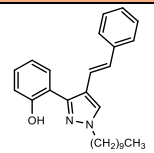
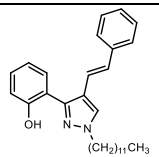
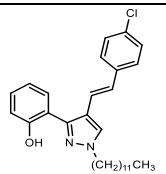
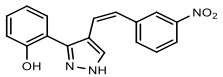
4-Styrylpyrazoles				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 2A	<30% $^{25\,\mu M}$	<30% $^{25\,\mu M}$	----	<30% $^{25\,\mu M}$
 2B	<30% $^{25\,\mu M}$	<30% $^{25\,\mu M}$	----	<30% $^{25\,\mu M}$
 2C	<30% $^{12.5\,\mu M}$	<30% $^{12.5\,\mu M}$	----	<30% $^{12.5\,\mu M}$
 3	42 ± 1% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	IC_{50} = 34 ± 3 µM

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

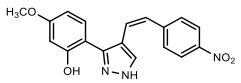
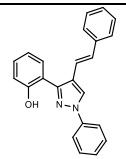
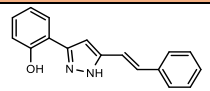
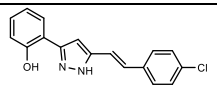
4-Styrylpyrazoles				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 4	IC_{50} = 23 ± 3 µM	<30% $^{50\ \mu M}$	IC_{50}= 25 ± 3 µM	IC_{50} = 4.6 ± 0.2 µM
 16	<30% $^{50\ \mu M}$	IC_{50} = 4.9 ± 0.4 µM	<30% $^{50\ \mu M}$	<30% $^{50\ \mu M}$
5-Styrylpyrazoles				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 5A	<30% $^{50\ \mu M}$	<30% $^{50\ \mu M}$	----	<30% $^{50\ \mu M}$
 5B	<30% $^{50\ \mu M}$	<30% $^{50\ \mu M}$	----	<30% $^{50\ \mu M}$

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

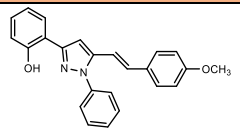
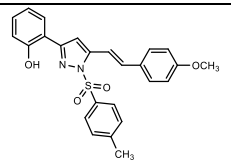
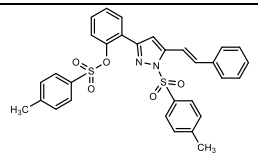
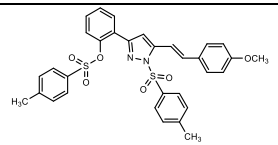
5-Styrylpyrazoles				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 6A	<30% $^{50\,\mu M}$	IC_{50}= 9 ± 1 µM	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$
 6B	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	34 ± 3% $^{50\,\mu M}$
 7A	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	<30% $^{50\,\mu M}$
 7B	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	<30% $^{50\,\mu M}$

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

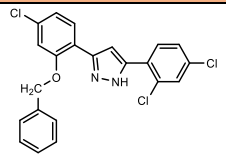
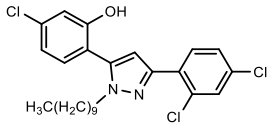
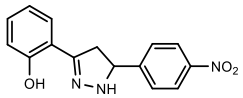
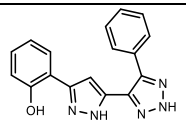
Miscellaneous				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 8	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	<30% $^{50\,\mu M}$
 9	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	<30% $^{50\,\mu M}$
 10	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	$IC_{50} = 8 \pm 1\,\mu M$
 11A	$34 \pm 3\%^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	$IC_{50} = 34 \pm 2\,\mu M$

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

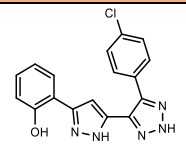
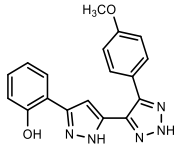
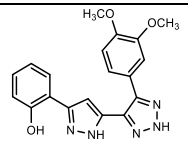
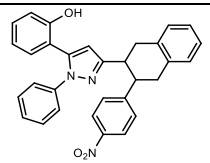
Miscellaneous				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 11B	IC_{50} = 15 ± 2 µM	<30% $^{50\,\mu M}$	IC_{50} = 39 ± 2 µM	IC_{50} = 19 ± 2 µM
 11C	IC_{50} = 41 ± 2 µM	<30% $^{50\,\mu M}$	----	IC_{50} = 24 ± 2 µM
 11D	37 ± 3% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	IC_{50} = 21 ± 1 µM
 12	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	IC_{50} = 31 ± 4 µM

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

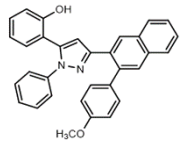
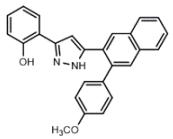
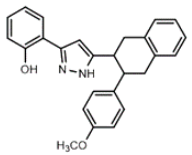
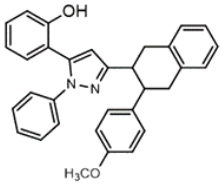
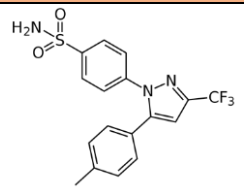
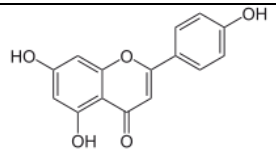
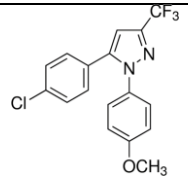
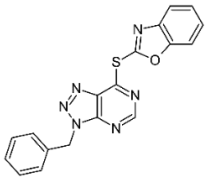
Miscellaneous				
Pyrazole	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 14	<30% $^{50\,\mu M}$	IC_{50} = 22 ± 2 µM	IC_{50} = 0.3 ± 0.1 µM	<30% $^{50\,\mu M}$
 15	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	<30% $^{50\,\mu M}$
 17	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	<30% $^{50\,\mu M}$
 18	<30% $^{50\,\mu M}$	<30% $^{50\,\mu M}$	----	<30% $^{50\,\mu M}$

Table 8: Chemical structures and the *in vitro* tested inhibitory activities by pyrazoles (IC_{50} or % of inhibition at the highest tested concentration, indicated superscript).

Positive Controls				
Compound	**COX-2 Inhibition**	**PGE_2 Inhibition**	**COX-1 Inhibition**	**Oxidative Burst**
 Celecoxib	$IC_{50} = 0.3 \pm 0.1\,\mu M$	$IC_{50} = 0.4 \pm 0.1\,\mu M$	----	----
 Apigenin	----	$IC_{50} = 8 \pm 1\,\mu M$	----	----
 SC-560	----	----	$IC_{50} = 9 \pm 1\,nM$	----
 VAS-2870	----	----	----	$IC_{50} = 1.6 \pm 0.1\,\mu M$

Attachment 2: Certificate of the ethical commission CHUP/ICBAS regarding the collection of blood from humans.

REPÚBLICA PORTUGUESA
SAÚDE

SNS SERVIÇO NACIONAL DE SAÚDE

centro hospitalar do Porto

COMISSÃO DE ÉTICA CHUP/ ICBAS

Presidente da Comissão

Dr.ª Luisa Bernardo

Constituição

Prof.ª Doutora Carla Margarida Coelho Marques Abrantes Teixeira Claro da Fonseca

Dr.ª Cármen Dolores Moreira de Carvalho

Dr.ª Fernanda Manuela Pereira da Costa

Dr. Gonçalo Senhorães Senra

Prof. Doutor José António Pinho da Silva

Dr.ª Maria Helena Silva Ramos

Prof.ª Doutora Maria Manuel Araújo Jorge

Prof.ª Doutora Maria Strecht Monteiro Mata de Almeida

Dr.ª Paulina Maria de Carvalho Araújo Fernandes Ferreira Aguiar

Prof. Doutor Paulo Manuel Rodrigues Martins da Costa

Secretariado Técnico

Dr.ª Maria de Jesus

Exm.ª Senhora
Prof.ª Dr.ª Eduarda Fernandes

Porto, May 30, 2019

STATEMENT

To whom it may concern,

The Ethics Committee of the *Centro Hospitalar do Porto* analysed the request of Professor Eduarda Fernandes for the "Use of blood samples provided by the *Serviço de Hematologia Clínica do Hospital Geral de Santo António*" for investigation purposes. Taking into account that blood donors are correctly informed, sign the informed consent, and the confidentiality and anonymity of identification is guaranteed, this Ethics Committee approved the request, due to the absence of material that offends ethical principles.

Dr.ª Luisa Bernardo
President of the Ethics Committe, CHUP / ICBAS.

Comissão de Ética CHUP / ICBAS
Hospital de Santo António
Largo Professor Abel Salazar
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Telf. 222077500 - Ext 1429
email: secretariado.etica@chporto.min-saude.pt