Zingiber officinale Roscoe and *Bryonia dioica* Jacq: Promising anti-inflammatory tubers

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AAPH	2.2’-Azobis (2-methylpropionamidine) dihydrochloride
ACLY	ATP citrate lyase
AGS	Adenocarcinoma gastric cell line
AKI	Acute kidney injury
ATF3	Activating transcription factor 3
BL	Burkitt’s lymphoma
C	Component
CD	Crohn's disease
CE	Cholesteryl ester
CIC	CItrate carrier
CNS	Central nervous system
COX	Cyclooxygenase
CS	Citrate synthase
DAD	Diode array detector
DCF-DA	Dichlorofluorescin diacetate
2-DG	2-Deoxyglucose
DHA	Docosahexaenoic acids
DMEM	Dulbecco's modified eagle medium
DMM	Dimethyl malonate
DMSO	Dimethyl sulfoxide
OD	Optical density
DW	Dry weight
EAE	Experimental autoimmune encephalomyelitis
EC_{50}	Concentration achieving 50% of the Inhibition
ECACC	European collection of authenticated cell culture
EPA	Eicosapentaenoic
F	Fumarase
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI_{50}	Growth inhibitory dose 50%
GMP	Guanosine monophosphate

GluT-1	Glucose transporter-1
HAT	Histone acetyltransferase
HBSS	Hanks balanced saline solution
HDAC	Histone deacetylases
HPLC	High performance liquid chromatography
HeLa	Cervical carcinoma cell line
HepG2	Hepatocellular carcinoma cell line
Hif-1*α*	Hypoxiainducible factor 1-alpha
ICAM	Intercellular adhesion molecules
IDH	Isocitrate dehydrogenase,
IFN	Interferon
IL	Interleukine
***i*NOS**	*i*nducible nitric oxide synthase
IRG-1	Immune responsive gene-1
Ig	Immunoglobulin
L	Leukotriene
LOX	Lipoxygenase
LPS	Lipopolysaccharides
M-CSF	Macrophage colony stimulating factor
MCF-7	Breast carcinoma cell line
MCP-1	Monocutyes chemoattractant protein-1
MDH	Malate dehydrogenase
MS	Mass spectrometer
NCI-H460	Lung carcinoma cell line
ND	Not detected
NED	*N*-(1-naphthyl)ethylenediamine
NF-kB	Nuclear factor-kappa-B
NK	Natural killer
NO	Nitric oxide
NSAID	Non steroidal anti-inflammatory drugs
PAF	Platelet activating factor
PARP	Poly(adp-ribose) polymerase
PBS	Phosphate buffered saline

PDB	Protein data bank
PG	Prostaglandin
PKM2	Pyruvate kinase M2
PLP2	Porcine liver primary cell line
PTGS	Prostaglandin endoperoxide synthase
PUMA	P53 up-regulated modulator of apoptosis
RA	Rheumatoid arthritis
RMSD	Root mean square deviation
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
SD	Standard deviation
SDH	Succinate dehydrogenase
SLE	Systemic lupus erythematosus,
SRB	Sulforhodamine B
SUCNR1	Succinate receptor 1
TCA	Trichloroacetic acid
TEPP-46	Thieno pyrrole pyridazinones
TGF	Transforming growth factor
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-alpha
Tr	Traces
Th	T helper
UFC	Unit forming colony
UFLC	Ultra fast liquid chromatography
VCAM-1	Vascular cell adhesion molecule-1
Vero	Fibroblast-like from African green monkey kidney cell line

Abstract

Inflammation is a defense mechanism designed to eliminate microorganisms, among other agents, to protect living tissues from infections, injuries, and to enhance tissue repair mechanisms. Non-steroidal anti-inflammatory drugs and synthetic glucocorticoids are widely used, but unfortunately, they have been associated with many side effects, namely the increased risk of upper gastrointestinal complications.

Therefore, the discovery of new, efficient, and safer anti-inflammatory agents is crucial to prevent and treat inflammatory processes. Natural matrices, such as tubers, have been explored and described as having a huge diversity of bioactive molecules with anti-inflammatory capacity and with few undesirable effects. Thus, in the present work, the hydroethanolic extracts of two tubers, *Zingiber officinale* Roscoe (Ginger) and *Bryonia dioica* Jacq. Were studied as potential anti-inflammatory agents.

The main objective of this work was to validate the anti-inflammatory activity of these tubers through two anti-inflammatory assays in vitro: i) inhibition of the production of nitric oxide (NO) in a cell model, RAW 264.7 - stimulated rat macrophage cell line by lipopolysaccharide (LPS); ii) inhibition of the activity of the inducible nitric oxide synthase (*i*NOS) enzyme. To ensure the safe use of these extracts, cytotoxic activity in tumor and non-tumor lines and cellular antioxidant activity were evaluated. Cell cycle analysis and apoptosis were also assessed using flow cytometry techniques. In addition, these extracts were characterized in terms of organic acids and phenolic compounds through chromatographic techniques to establish a structure-activity relationship.

According to the results obtained, the two tubers revealed the presence of organic acids, well described bioactive molecules, and four phenolic compounds were also identified in *Z. officinale*. Both samples are promising anti-inflammatory agents, since they exhibited the ability to inhibit NO production with very low IC_{50} values. It should be noted that *B. dioica* showed an anti-inflammatory and cytotoxic activity more effective than the standards used. The results of antioxidant activity were less promising, as both samples were less effective than quercetin. The results of the cell cycle show that *B. dioica* suppresses the G0/G1 phase, while apoptotic analysis indicates that *Z. officinale* has a greater capacity to induce cell death by the apoptotic pathway. These results are very promising and highlight the anti-inflammatory

potential of these tubers, however further tests are needed to evaluate other inflammatory mediators and confirm in vivo their efficacy and safety.

Resumo

A inflamação é um mecanismo de defesa concebido para eliminar microrganismos,entreoutros agentes, para proteger os tecidos vivos de infeções, lesões, e para potenciar os mecanismos de reparação tecidular. Os anti-inflamatórios não esteroides e glucocorticoidessintéticos são muito utilizados, mas infelizmente têm sido associados a muitos efeitos secundários, nomeadamenteao risco acrescido de complicações gastrointestinais superiores. Portanto, a descoberta de agentes anti-inflamatórios novos, eficientes e mais seguros é crucial para prevenir e tratar processos inflamatórios. As matrizes naturais, como os tubérculos, têm sido exploradas e descritas como detentores de uma enorme diversidade de moléculas bioativas com capacidade anti-inflamatória e com poucos efeitos indesejáveis. Assim, no presente trabalho, os extractoshidroetanólicos de dois tubérculos, *Zingiber officinale* Roscoe (Ginger) e *Bryonia dioica* Jacq. foram estudadoscomo potenciais agentes anti-inflamatórios. O principal objetivo deste trabalho foi a validação da atividade anti-inflamatória destes tubérculos através de dois ensaios anti-inflamatórios *in vitro*: i) inibição da produção de óxido nítrico (NO) num modelo celular,RAW 264.7–linhacelularde macrófagos deratoestimulado por lipopolissacarídeo (LPS); ii) inibição da atividade da enzima óxido nítrico sintaseindutível (*i*NOS). Para garantir a segurança da utilização dos referidos extratos foi avaliada a atividade citotóxica em linha tumorais e não tumorais e a atividade antioxidante celular. A análise do ciclo celular e a apoptose também foram avaliadas usando técnicas decitometria de fluxo. Além disso, estes extratosforam também caracterizados em termos de ácidos orgânicos e compostos fenólicos através de técnicas cromatográficas a fim de estabelecer uma relação estrutura-atividade. De acordo com os resultados obtidos, ambas as amostras revelaram a presença de ácidos orgânicos, moléculas de reconhecida bioatividade, tendo sido ainda detetados 4 compostos fenólicos na amostra *Z. officinale*. Os dois tubérculos são agentes anti-inflamatórios promissores, já que exibiram capacidade para inibir a produção de NO com valores muito baixos de IC_{50}. De salientar que a *B.dioica* mostrou uma atividade anti-inflamatória e citotóxica mais eficaz do que os padrões utilizados. Os resultados daatividade antioxidante foram menos promissores, já que ambas as amostras foram menos eficazes do que a quercetina. Os resultados do ciclo celular mostram que a *B.dioica* suprime a fase G0/G1, enquanto a análise apoptótica indica que a *Z. Officinale* apresenta maior capacidade para induzir morte

celular pela via apoptótica. Estes resultados são muito promissores e realçam o potencial anti-inflamatório destes tubérculos, no entanto é necessário fazer mais ensaios para avaliar outros mediadores inflamatórios e confirmar *in vivo* a sua eficácia esegurança

1. Introduction

Inflammation is a defense mechanism that is designed to eradicate microbes or irritants to protect living tissues from infection, injuries, and to potentiate tissue repair**.** This process leads to changes in blood flow, an increase in permeability of blood vessels, and the migration of fluid, proteins, and white blood cell (leukocytes) from the circulation system to the site of the damaged tissue. If the inflammatory response last for few days it is called acute inflammation however if it last for longer time it is referred as chronic inflammation and can cause physiological decompensation, organ dysfunction and death[1].

The inflammation process is characterized by five major stages: i) *rubor* (redness, due to hyperemia); *tumor* (swelling caused by increased permeability of the microvasculature and leakage of protein into the interstitial space); ii) *calor* (heat associated with the increased blood flow and the metabolic activity of the cellular mediators of inflammation); iii) *dolor* (pain, in part due to changes in the peri-vasculature and associated nerve endings); and iv) *Functiolaesa* (dysfunction of the organs involved)[2].

The common anti-inflammatory drugs can calm the symptoms or limit the deleterious effects of inflammation in the organism. Usually, there are two types of anti-inflammatory drugs, the non-steroidal and glucocorticoids. These drugs can be applied in different forms, namely by oral treatment, suppository, inhalation, infusion or local by ointment, eye drops among others); however these anti-inflammatory drugs have serious side effects[3]. The use of oral steroids, aspirin and acetaminophen at doses of approximately 2 g each is associated with a double increase in the risk of upper gastrointestinal complications; non-steroidal anti-inflammatory drugs are associated with a nearly fourfold increase in risk [3].

Therefore, it is crucial to find new and efficient anti-inflammatory drugs that can eliminate the infection without undesired side effects. Natural products have been explored and described as possessing a huge diversity of bioactive molecules with anti-inflammatory capacity [4]. Thus, in this study we are going to evaluate the bioactivities of *B. dioica* and *Z. officinale* as promising anti-inflammatory agent susing the hydro-ethanolic extract.

Beside the anti-inflammatory activity, we are going to test the cytotoxicity, NOS activity, CAA, the cell cycle and the apoptosis and determine the phenolic compound

and the organic composition to understand better the mechanisms of action of both samples as anti-inflammatory agents.

1.1. Inflammatory mechanisms

1.1.1. Initiation of the inflammatory response

1.1.1.1 Vasodilatation, fluid exudation and leukocyte migration

Vasodilatation is a typical characteristic of acute inflammation, which is clinically characterized at the injury site by redness which warmth. The vasodilatory response aims at promoting the local delivery of soluble mediators and inflammatory cells. Inflammation-induced vasodilatation is mainly mediated by vasodilating prostaglandins and NO. Upon exposure to microbial products or pro-inflammatory cytokines, the activated leukocytes develop inducible NOS (*i*NOS). The NO provided by cyclic GMP-dependent mechanisms induces subsequent smooth muscle relaxation. Prostacycline (PGI2), PGD2, PGE2 and PGF2a are the main vasodilatory prostaglandins (**Figure1**) [1]. These prostaglandins are formed by the phospholipase and cyclooxygenase activities[5].

Vasodilatation caused by inflammation first requires arterioles accompanied by the formation of new micro-vascular beds. Widespread vasodilatation can induce systemic hypotension and shock in cases of severe systemic inflammation such as sepsis. Such physiological modifications are potentiated by myocardial depression caused by the sepsis stage, a disease triggered by the activities of NO and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) [6] (**Figure1**).

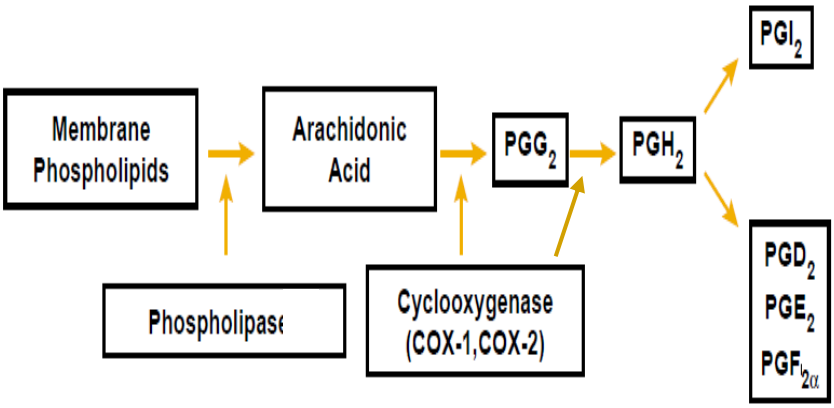


Figure 1: The production of vasodilatory prostaglandins through the actions of phospholipase and cyclooxygenase. The major vasodilatory prostaglandins are prostacyclin (PGI2) and the prostaglandins PGD2, PGE2 and PGF2a. COX, cyclo-oxygenase; PG, prostaglandin.[1]

At the same time, capillary hydrostatic pressure at the injury site is increased early during inflammation or damage triggered by local vasodilatation. Outpouring of protein-rich fluid induces erythrocyte aggregation in small vessels and increase blood viscosity. This flow of trans-vascular fluid gradually returns to normal intravascular pressure at the inflammation site. At the same time, plasma protein loss reduces oncotic pressure intravascularly[1].

The increase in vascular permeability, the temporary rise in capillary hydrostatic pressure and the decrease in plasma oncotic pressure work together to induce a trans-vascular fluid and protein flow into the inflamed interstitium. The role of these alterations is to facilitate the delivery to the injury site of soluble factors such as antibodies and acute-phase proteins. However, extreme systemic inflammation can cause excessive increases in vascular permeability, which can contribute to the development of oedema in the lungs and extremities. In critically ill patients, the accumulation of fluid in the lungs causes the acute respiratory distress syndrome, a significant source of morbidity and death [7].

Leukocyte margination, adhesion, and migration are followed by vasodilatation and fluid exudation. Neutrophils are the first and most numerous leukocytes to appear in an infection or inflammatory site. The process of neutrophil movement from intravascular space into the inflamed interstitium occurs mainly in the systemic circulation of postcapillary venules and in the lung's pulmonary capillaries. The process of transmigration is categorized into several distinct stages, namely

margination, spinning, adhesion, diapedesis and chemotaxis [8] (**Figure 2**).The process of neutrophil migration from the main bloodstream to the vessel's periphery is denomited marginalisation. Stasis after fluid exudation at the site of inflammation and physical contact between erythrocytes and neutrophils facilitates this process. A poor adhesive association occurs after margination between neutrophils and endothelial vascular cells, allowing neutrophils to linger in close proximity to the vascular endothelium. Neutrophil rolling is encouraged by the shear stress of moving erythrocytes, the rolling speed being proportional to the velocity of red cells [9].

These interactions are mediated by selectins and their ligands, promoting rolling of the neutrophils. The adherence to neutrophils is then potentiated by interactions with beta-integrins and intercellular adhesion molecules (ICAM). Neutrophils then move by contact with chemoattractant molecules such as chemokines and bacterial products to the site of the inflammation [10].

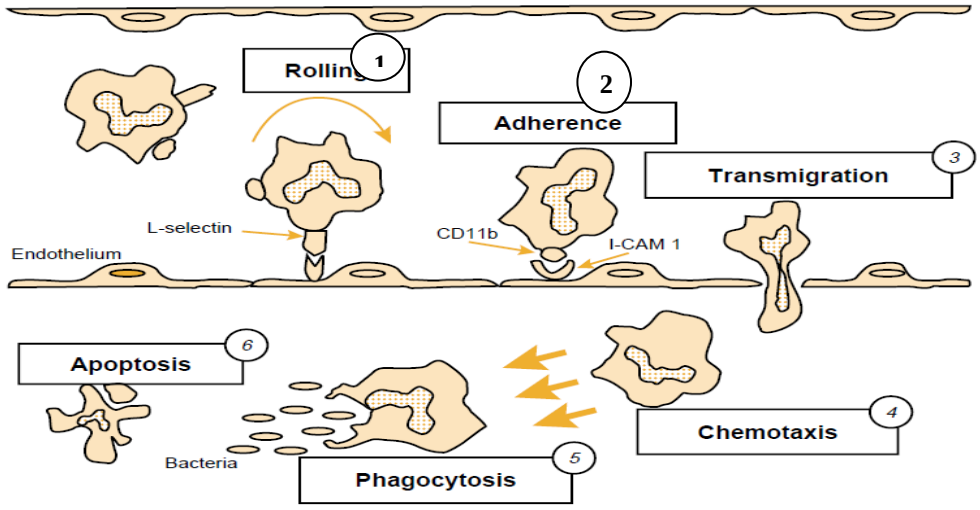


Figure 2: Neutrophil rolling processes, adhesion system, diapedesis and chemotaxis.The formation of loose connections between endothelial cells and neutrophils is caused by inflammation, which produces neutrophil margination. Selectins and their ligands moderate these connections, allowing neutrophils to roll more easily. Interactions between beta-integrins and ICAM then enhance neutrophil adherence. After interacting with chemoattractant molecules such chemokines and bacterial products, neutrophils move to the site of inflammation.[1].

1.1.1.2 Activation of the coagulation cascade during inflammation

Inflammation is closely associated with coagulation. The cascade of coagulation occurs after skin damage and during an infection process. It is divided into two pathways which converge and ultimately cause thrombin activation with the subsequent fibrinogen cleavage into fibrin (**Figure 3**). The intrinsic pathway is a sequence of plasma proteins regulated by Hageman factor (factor XII), a synthesized protein in the liver that is regulated by binding to collagen, basement membrane or activated platelets [11]. Enabled Hageman factor activates a cascade of proteins to start resulting in thrombin production. Guided tissue pain most commonly stimulates the intrinsic pathway.

The extrinsic pathway, by comparison, is triggered by the development of factor tissue. Recent studies suggest that the extrinsic pathway is the primary coagulation pathway that is triggered during infection and systemic inflammation, particularly during sepsis and the systemic inflammatory response syndrome [12].Tissue factor is exerted on surfaces of tissues that are not usually exposed to the vascular compartment, such as subcutaneous tissues and blood vessel adventitial layers. In addition, endothelial cells and activated monocytes develop tissue factor in response to TNF-a, IL-1, IL-6 and C-reactive protein during cycles of inflammation [13] . The presence of tissue factor triggers the activation of factor VII, which then forms a complex with tissue factor and eventually induces the production of thrombin by stimulating a variety of coagulation factors (**Figure 3**).

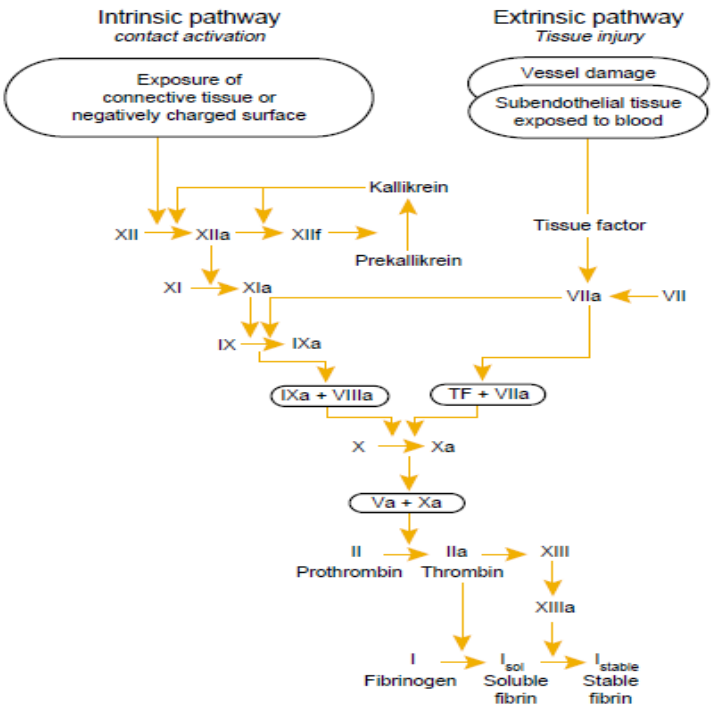


Figure 3 : The coagulation cascade. The extrinsic and intrinsic coagulation pathways combine to form a final common pathway. Tissue factor, which is released in response to tissue damage and macrophage activity, activates the extrinsic pathway. The intrinsic pathways are activated when sub-endothelial collagen and activated platelets are accessible. The development of a fibrin clot is the final outcome.[1].

Coagulation cascade activation is not only important for the development of fibrin clots but it also has major effects on the pro-inflammatory response. Pro-inflammatory activity has been shown to be caused by factor Xa, thrombin, and tissue factor – VIIa complex In particular, thrombin and the tissue factor – VIIa complex that induce mononuclear and endothelial cells to produce pro-inflammatory cytokines such as TNF- α[14]. This influence tends to be mediated by binding certain factors on the surface of target cells to protease-activated receptors. Acute inflammation then allows the coagulation cascade to start, which can then potentiate the inflammatory response further.

1.1.2. Complement system

The complement system is a collection of microbial-activated proteins that help to facilitate inflammation and microbial degradation. The nutrient cascade is also likely to be activated during tissue injury, which plays a role in cell damage associated with severe injuries which burns [15]. There are three methods that trigger the

complement cascade: the classical pathway, the alternative pathway, and the lectin pathway, each pathway is activated through different mechanism, the classic pathway is activated by IgM or IgG antibodies that attach to the surface of microbes, the alternative pathway is activated directly by microbial surface molecules that bind the C3 component supplement, the lectin pathway is activated by mannose-binding lectin that interacts with microbial glycoproteins and glycolipids. (**Figure 4**). [1].

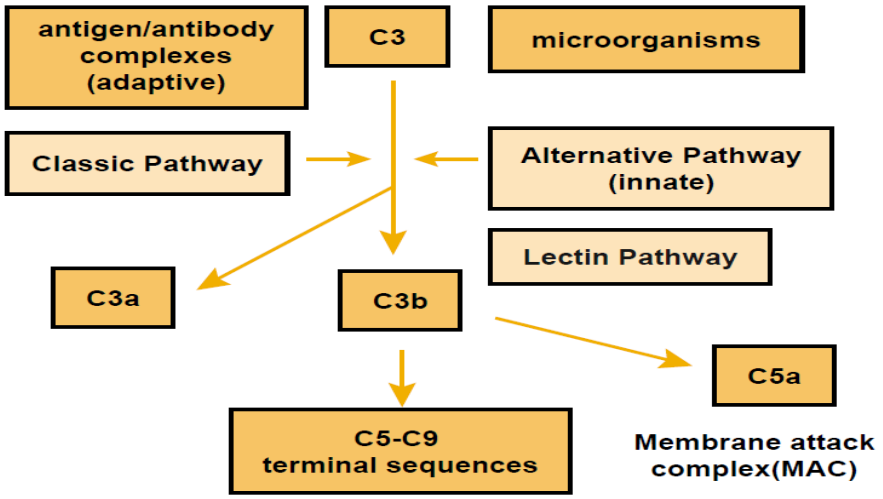


Figure 4: The complement system Several mechanisms, including the classic, alternative, and lectin pathways, activate the complement cascade. C3a and C3b, which act as pro-inflammatory mediators, are the most important mediators of the complement system. Membrane disruption is also caused by the development of the membrane attack complex (C5-C9)[1].

Any of these paths will enable the cleavage into C3a and C3b of the complement portion. The C3a acts as chemo-attractant for neutrophils and C3b binds to the surface of the microbes to promote phagocyte detection and enable phagocytosis[16] . Furthermore, C3b forms a proteolytic complex with other complementary components to allow C5 to be cleaved into C5a and C5b [17]. C5a is a chemotactic driver for neutrophils and also affects vascular permeability at the inflammatory site. The C5b attaches to the microbial surface and promotes the development of the membrane attack complex consisting of C6, C7, C8 and C9 [18] . Complex membrane attack induces microbial cell membrane destruction and eventual death.

1.1.3. Amplification of the inflammatory response

1.1.3.1 Innate immune system

Immune response may be split into natural and adaptive responses to tissue injury or infection. The primary response to tissue invasion is supported by the innate immune system. The phases of vasodilatation described above, increased vascular permeability and cellular infiltration form part of the innate immune response. Macrophages, dendritic cells, natural killer cells (NK) and neutrophils are the main cell components of the innate immune system. Besides these cellular elements, circulating effectors proteins such as complement, acute-phase reactants and the cascade of coagulation play important roles in innate immunity [1].

The extent of innate reaction is primarily determined by the production of cytokines and non-cytokine inflammation mediators. Cytokines are polypeptides formed by immune system cells in response to an infection or damage to the tissue. They work to control inflammatory and immune reactions. Generally speaking, cytokine production is self-limited although certain cytokines can remain in circulation for long periods of time. What's more, cytokine reactions are pleiotropic and repetitive. TNF- α is prototypical pro-inflammatory cytokine [19]. TNF- α is mainly activated by macrophages within minutes of local or systemic damage, and modulates a number of immunological and metabolic events. TNF- α initiates an inflammatory response at sites of local infection or inflammation, which stimulates anti-microbial resistance mechanisms and replaces tissue until the infection has been eradicated [20]. It is a potent neutrophil and mononuclear phagocyte activator that also acts as a growth factor for fibroblasts and an angiogenesis factor.

These PAF, a phospholipid autocoid produced by endothelial cells that controls the release of cytokines and amplifies the pro-inflammatory response, has been implicated in many non-cytokine inflammatory processes[21] . The adhesion of neutrophils to endothelial cells seems to be an important factor. PAF's extended involvement in serum of SIRS patients has associated with negative outcome. Eicosanoids are metabolites of arachadonic acid which regulates many aspects of the immune response. Leukotrienes (LTC4–LTE4) allow the endothelial cells to contract and promote capillary leakage. Thromboxane A2, a catalyst originating from the macrophage and platelets, causes platelet accumulation, vasoconstriction, and likely tissue thrombosis [1].

1.1.3.2 Acquired immune response

Often, the innate immune response helps to activate and enhance the immunity acquired. This influence is mainly mediated by IL-12, which induces T-cell activation and facilitates the segregation of T-cells naive into the Th1 phenotype[22] . However, the adaptive immune response is mainly triggered by the introduction of foreign antigens to T-cells CD4 + and CD8+ T. Activation of CD4+ T-cells triggers further development of cytokines and amplifies the innate and acquired immune systems. At the time of antigen presentation, the different cytokines generated by CD4+ cells are dependent upon the immunological microenvironment.

CD4 T-cells are the best-defined subsets of Th1 and Th2 cells. Those subsets are characterized primarily by the cytokines they generate. IFN-γ is the principal cytokine formed by Th1 cells, In fact, IFN- γ amplifies the pro-inflammatory reaction by inducing activation of the macrophage and activating the cytolytic functions of CD8 T-cells [23] . IFN- γ also activates B-cells to develop IgG1 and IgG3 antibodies that are opsonizing and complement binding [24] . Helminths and the susceptibility to allergens induce the differentiation of Th2. Such stimuli induce sustained stimulation of T-cells without an significant innate immune response or activation of the macrophage.

Two more T-cell subsets are Th3 and T-regulatory 1 (Tr1) cells. Th3 cells produce TGF-b and play an important role in the development of immune tolerance, particularly after exposure to antigens supplied via the gastrointestinal tract [25].

1.2. Relationship between inflammatory process and chronic diseases

1.2.1. A generic model of chronic inflammatory disease

Several diseases such as type 2 diabetes, obesity, Alzheimer's disease, heart disease and allergies have few common factors; first and foremost, they are all primarily lifestyle diseases and are usually related to chronic inflammation.

A generic model of chronic inflammatory disease is proposed to change the traditional thoughts that have claimed the chronic diseases of various organ systems as their own, and probed the pathophysiology in a reductionist manner, however these chronic diseases have more mechanisms in common than usually recognized so this model highlights these shared phathophysiologic mechanisms[26] .

The epithelial and mesenchymal cells of the affected organs are two prototypic cell types induced by signals resulting from interactions between the innate and adaptative immune systems (**Figure 5**). These signals induce tissue responses such as recruitment of leukocytes involved in chronic inflammation, extracellular matrix remodeling, cellular proliferation or death, and angiogenesis (**Figure 5**)[26].

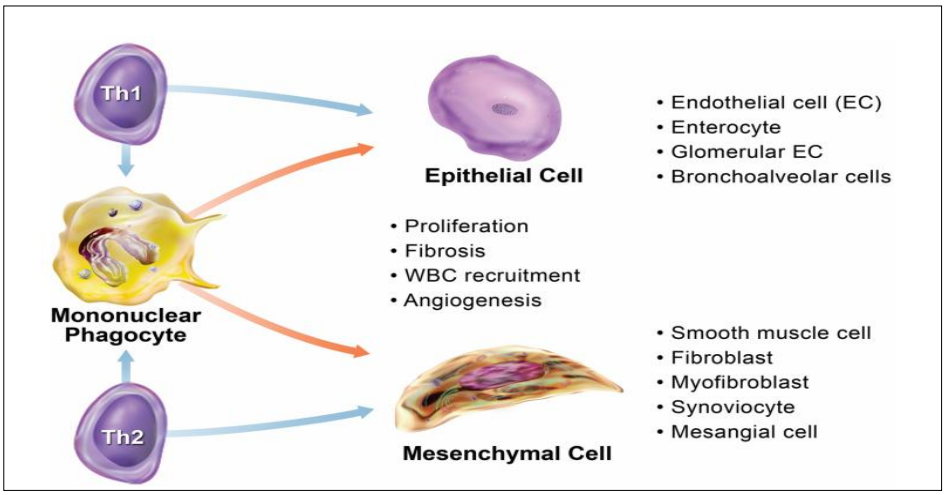


Figure 5: Common inflammatory and immune processes on different cell types.Inflammatory and immune activity alters the function of various types of epithelial cells such as endothelial cells, synoviocytes, enterocytes, glomerular/tubular epithelial cells, and bronchoalveolar cells, and each cell type characteristically participates in the development of a different disease (atherosclerosis, arthritis, inflammatory bowel disease, kidney disease, and lung disease, respectively). Inflammatory and immune processes may also act on different types of mesenchymal cells (e.g., smooth muscle cells, fibroblasts, myofibroblasts, mesangial cells, or pericytes), leading to the development of chronic disease depending on the specific cells targeted.[26].

Atherosclerosis, interstitial lung disease, rheumatoid arthritis and cirrhosis share the same fundamental mechanisms and mediators drive the disease process; however they act in totally different ways [27,28]. In many organs, helper T-cell bound in the lesions of chronic inflammation including atherosclerotic plaques, forms of chronic hepatidites, rheumatoid synovium and in a number of pulmonary diseases. Mononuclear phagocyte, histocyte, microglia, or alveolar macropages are also found in such lesions. Every tissue involved has specific epithelial cells: the vascular endothelial cells in atherosclerosis; the glomerular or tubular epithelial cell in renal disease; and enterocytes in inflammatory bowel diseases. Similarly, depending on the organ involved, inflammatory and immune mechanisms have different types of

mesenchymal cells, arterial smooth muscle cells, fibroblasts, myofibroblasts, synoviocytes, pericytes or mesangial cells. The first step of inflammation involves selective and sequential migration of blood cells into tissues and the second step is the local activation and interaction of these blood-based cells with resident tissue cells. Only some limited elements of this classic inflammatory process can be displayed by some conditions. However, the key inflammatory mediators dominate but without the context of the classic inflammatory mechanisms.

In osteoporosis, mediators such as IL-1, IL-6 and TNF-α are primarily furnished by resident stromal cells to regulate bone turnover unaccompanied by other components of the classic innate immune response. A normal host defense mechanism can coax into an injurious response due to a persistent stimulus. Infection due to a pyogenic microbial pathogen engenders an acute leukocyte response to clear the invading organism.

1.2.2. Examples of chronic diseases related with inflammation

There are numerous chronic diseases related with inflammation (**Table1**). Nevertheless, some diseases are considered more relevant as they cause irreversible damage to the human organisms.

1.2.2.1 Relationship between inflammation and atherosclerosis

Atherosclerosis is a disease in which the inside of an artery narrows due to the buildup of plaque (**Figure 6**). In initial stages there are generally no symptoms, but in advanced stages, depending on the type and place of the affected arteries, it can result in different complication such as: coronary artery disease, stroke, peripheral artery disease, or kidney problems[28].

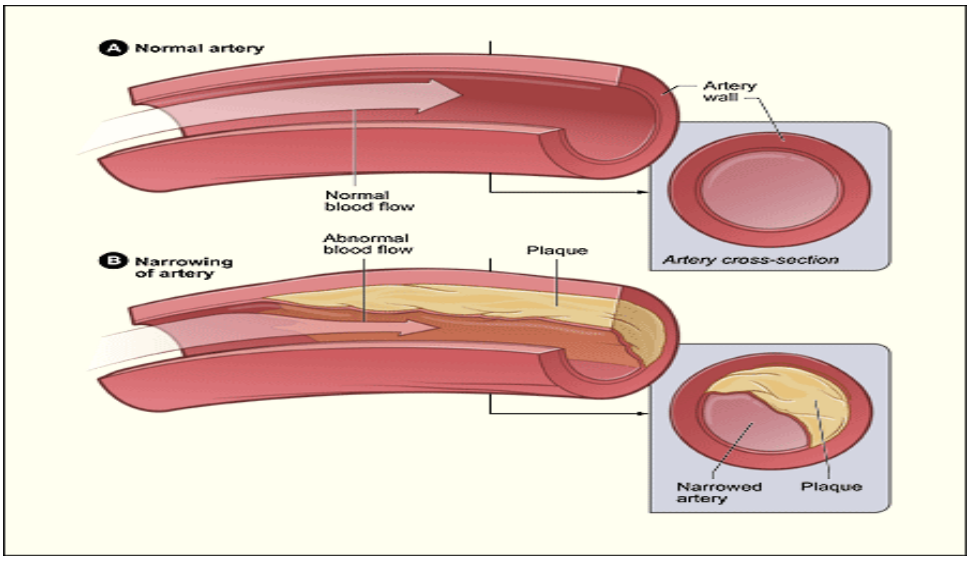


Figure 6: Figure A shows a normal artery with normal blood flow. The inset image shows a cross-section of a normal artery. Figure B shows an artery with plaque buildup. The inset image shows a cross-section of an artery with plaque buildup[28].

From a deep point of view, vascular endothelial cells resist to prolonged contact with leucocytes; however they express a palette of vascular cell adhesion molecule-1 (VCAM-1) and members of the selectin family, P- and E-selectin, when they are exposed to an activating stimulus such as modified lipoproteins, microbial constituents or pro-inflammatory cytokines[29]; Monocytes migrate directly into artery wall after adhesion to the endothelial surface; this directed migration is mediated by chemokines such as monocutyes chemoattractant protein-1 (MCP-1)[30]. Once resident in the arterial intima, these monocytes differentiate into macrophages and proliferate after exposition to such activating and co-mitogenic mediators as macrophage colony-stimulating factor (M-CSF)[31],. These macrophages over-express scavenger receptors and through endocytosis they engulf modified lipoprotein particles, then they accumulate cholesteryl ester (CE) in cytoplasmic droplets creating foam cells, considered a hallmark of the nascent atherosclerotic plaque[32].

The inflammatory and immune process contribute to acute thrombotic which is one o the ultimate complications o atherosclerosis[30]. Inflammation within the arterialintima leads to signal exchange among the T-cell, mononuclear phagocyte, and cells of the vessel wall. This pathway leads to a weakening of the fibrous plaque through decreased collagen synthesis or increased degradation. IFN- α mediates decreased synthesis while increased breakdown is a result of proteinases induced by

inflammatory signaling. CD40 ligation augments the thrombogenecity of the lipid core by expression of tissue factor, a major trigger of thrombus formation (**Figure7**)[33].

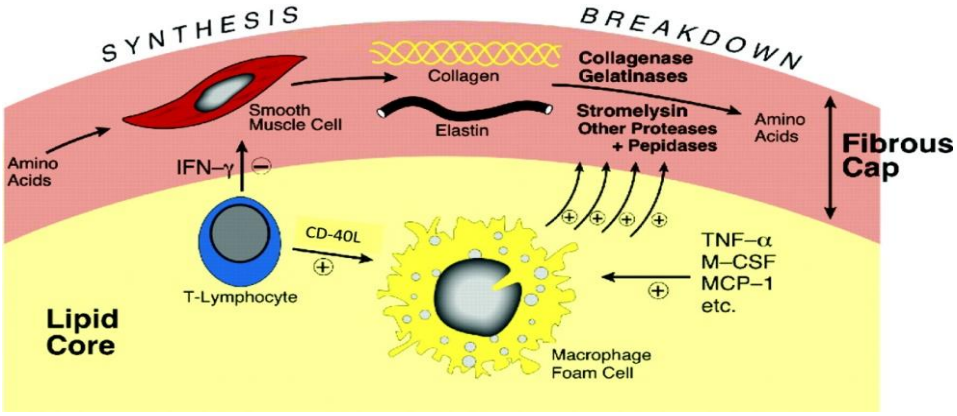


Figure 7: Simplified model illustrating the effects of inflammation on the integrity of the plaque fibrous cap signaling between T cells, mononuclear phagocytes, and vessel wall cells occurs when the arterial intima is inflamed. Through decreased collagen synthesis or increased breakdown, this mechanism causes the fibrous plaque to weaken. IFN-γmediates decreased synthesis while increased breakdown is a result of proteinases induced by inflammatory signaling. The production of tissue factor, a key activator of thrombus formation, is enhanced by CD40 ligation, which increases the thrombo-genecity of the lipid core[26].

1.2.2.2 Relationship between inflammation and Alzheimer's disease

Alzheimer's disease is a progressive chronic neurodegenerative disease that get worsens over time. This chronic disease is characterized by cognitive decline and the presence of two core pathologies, amyloid β plaques and neurofibrillary tangles [34].

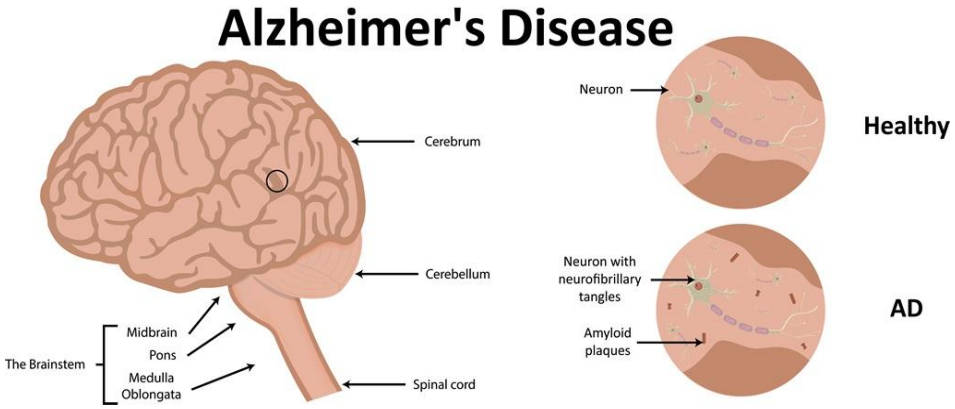


Figure 8: Comparison between healthy and Alzheimer affected brain; the neuron of the affected brain contain neuro-fibrillary tangles and amyloid plaques [34].

In pathologically vulnerable regions of Alzheimer's disease, clearly local peripheral inflammatory responses with full complexity were found. The inflammation is stimulated by degenerating tissue and deposition of highly insoluble abnormal materials, likewise damaged neurons, and neurites and highly insoluble amyloid β peptide deposits and neurofibrillary tangles provide obvious stimuli for inflammation. These stimuli are discrete, microlocalized, and present from early preclinical to terminal stages of Alzheimer's disease that's why local upregulation of complement, cytokines, acute phase reactants, and other inflammatory mediators are also discrete, microlocalized, and chronic. Direct and bystander damage from Alzheimer's disease inflammatory mechanisms are cumulated over many years and are likely to significantly give rise to pathogenic processes. Clinical studies and animal models strongly suggest that Alzheimer's disease inflammation significantly contributes to Alzheimer's disease pathogenesis [35].

Table 1: Examples of chronic diseases related with inflammation.

Disease	Description	Inflammatory mechanism	Treatment	References
Chronic obstructive pulmonary disease (COPD)	A chronic inflammatory lung disease that causes obstructed airflow from the lungs.	Both innate (macrophages/neutrophils) and adaptive inflammatory immune cells (CD4, CD8 and B lymphocytes) which developed lymphoid follicles increased the tissue volume of the bronchial wall characterized by infiltration of the wall.	Phosphatidylcholine (PC)(naturel) 18:1 PC (cis), 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (synthetic)	[36]
Alcoholic fatty liver disease (AFLD)	a build-up of fats in the liver caused by drinking a large amount of alcohol.	Expression of the following inflammatory molecules in the liver: tumor necrosis factor α (TNF-α), monocyte chemotactic protein 1 (MCP-1), chemokine (C-X-C motif) ligand 1 (CXCL-1) and interleukin 1 beta (IL-1β).	Phosphoesterase complex (Pho)	[37]
Obesity	a complex disease involving an excessive amount of body fat.	Abnormal cytokine production, increased synthesis of acute-phase reactants and activation of inflammatory signaling pathways	Weight lost physical activities	[38]
Chronic kidney disease	a long-term condition where the kidneys don't work as well as they	Activation of the prototypical proinflammatory signaling pathway, the best characterized being NF-κB and AP-1, mainly based on the stimulation of multiple mediators, including proinflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor α	Ramipril, Enalapril, Lisinopril. Atorvastatin ,Simvastatin, VitaminD, furosemide, cyclophosphamide.	[39]

	should.	(TNF-α).		
Autoimmune diseases (SLE, RA, Sjogren's Syndrome)	A condition in which the immune system mistakenly attacks the body.	Substitution of the AU-rich element (ARE) in the IFN- 3 'un-translated region (called ARE-Del) with random nucleotides which results in a weak but chronic expression of IFN-γ.	Nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen (Motrin, Advil) and naproxen (Naprosyn).	[40]
Myocardial infarction (MI)	A heart attack, occurs when blood flow decreases or stops to a part of the heart, causing damage to the heart muscle.	inhibition of the signaling pathways of nuclear transcription factor κB (NF-κB), p38, c-Jun NH2-terminal kinase (JNK) and transforming growth factor β (TGF-β).	Thrombolytics, Aspirin, Nitroglycerin, Beta-blockers, ACE inhibitors.	[41]
Psoriasis	A skin condition that causes red, flaky, crusty patches of skin covered with silvery scales.	Heightened innate and adaptive immune activation. T helper (Th)1 and Th17 cells drive pro-inflammatory cytokines including TNFα, interferon-γ, IL17A, and IL23	Anti-TNFα therapy,Topical – creams and Ointments, Phototherapy.	[42]

1.3. Treatment of inflammatory process

1.3.1. Corticosteroids

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex of vertebrate. Two main classes of corticosteroids, namelly glucocorticoids and mineralo-corticoids, are involved in the regulation of inflammatory response.

This class of drugs represents a natural starting point for empirical anti-inflammatory therapy. The most broadly active anti-inflammatory/immunosuppressive agents in clinical use are the glucocorticoids[43]. Suppression of neutrophil adherence, aggregation, phagocytosis and accumulation, suppression of monocyte accumulation, inhibition of prostaglandin and leukotriene production, lympholysis, inhibition of immunoglobulin production, and impairment of delayed hypersensitivity are known as specific actions of corticosteroids.

Steroids have been widely used in the treatment of idiopathic inflammatory diseases of the central nervous system (CNS), including lupus cerebritis and temporal arteritis, as also to treat multiple sclerosis. Nevertheless, there are serious drawbacks to clinical trials of this class of drugs in Alzheimer's disease.

A high doses of corticosteroids can cause toxic effects such hypertension, hyperglycemia, fluid retention and exacerbation of con- gestive heart failure, psychiatric syndromes including psychosis, mania, and depression, gastrointestinal ulceration or perforation, increased susceptibility to infection, osteoporosis with vertebral compression fractures, aseptic necrosis of bone, myopathy, truncal obesity, ac- celerated atherogenesis, glaucoma, adrenal suppression, and cataracts[44].

1.3.2. Non-steroidal Anti-Inflammatory Drugs

These drugs are members of a drug class that reduces pain, decreases fever, prevents blood clots, and decreases inflammation in higher doses.

The first line drugs for inflammatory diseases are non-steroidal anti-inflammatory drugs such as rheumatoid arthritis and gout. Their main role is the inhibition of neutrophil function and prostaglandin synthesis[45]. Non-steroidal anti-inflammatory drugs interact primarily with pro-inflammatory cytokines interleukin (IL)-1a, IL1b, IL-6 and tumor necrosis factor (TNF-α)[46]. The cardinal signs of inflammation occur because of the increase of TNF-α concentration; they also

stimulate white cell phagocytosis and the production of inflammatory lipid prostaglandin E2 (PGE2). The major mechanism that leads to the success of these medications is their ability to interfere with the production of prostaglandin during the inflammatory cascade (**Figure 9**)[47].

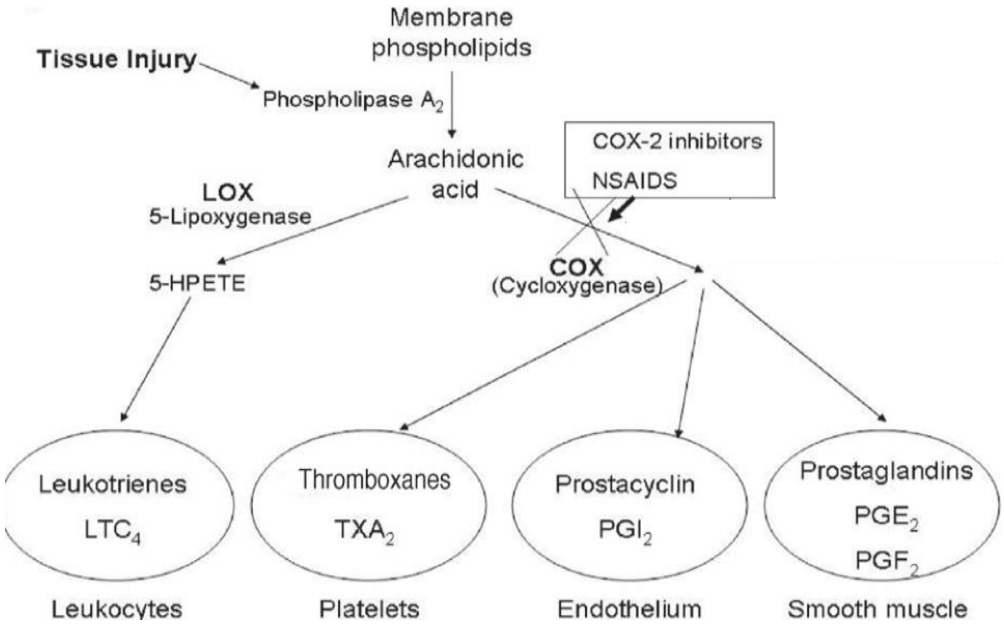
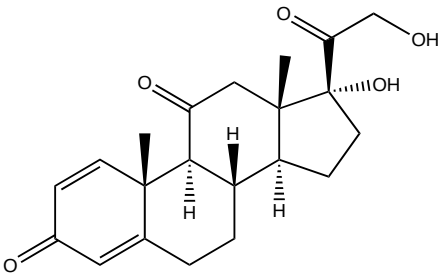
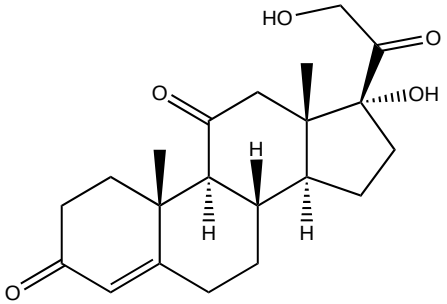
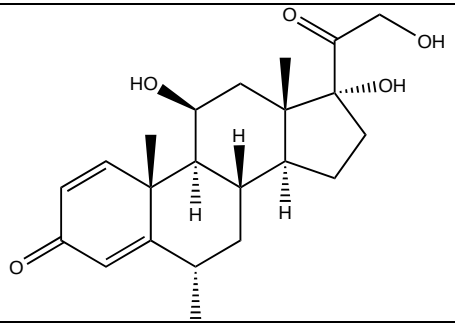
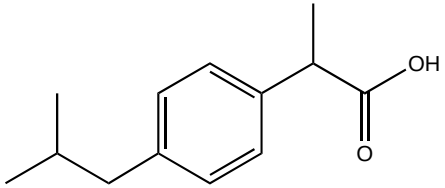
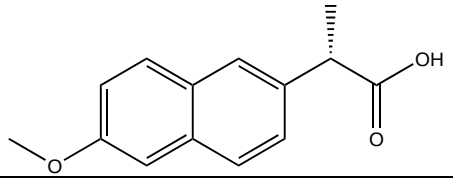


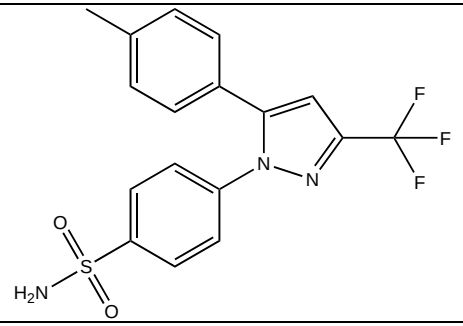
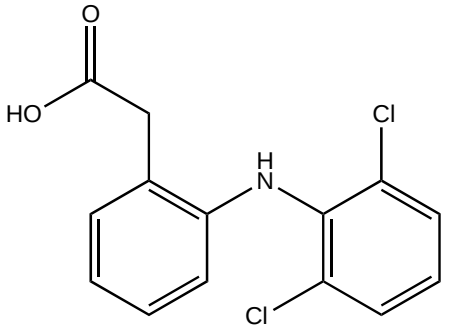
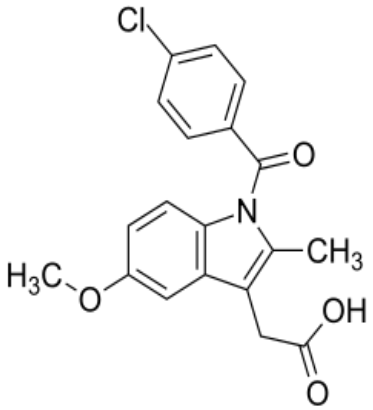
Figure 9: Block of COX action by the non-steroidal anti-Inflammatory.The NSAIDs can block COX action and thereby prevent the formation of the COX-derived inflammatory mediators. 5-HPETE = 5-hydroperoxyeicosatetraenoic acid; LTC4 = leukotriene C4; PGE2 = prostaglandin E2; PGF2 = prostaglandin F2; PGI2 = prostacyclin; TXA2 = thromboxane.[4]

Steroidal and non-steroidal anti-inflammatory medications have a significant side effect profiles; there is a greater interest in natural compounds, such as dietary supplement and herbal remedies, which have been used for centuries to reduce inflammatory response [48].

Table 2: Mechanism of action, Structure and Side effects of some steroidal and non-steroidal anti-inflammatory drugs.

Drug type	Mechanism of action	Chemical structure	Side effects	Reference
		Corticosteroid drugs		
Prednisone	Decreases inflammation through suppression of the migration of polymorphonuclear leukocytes and reversing increased capillary permeability. It also suppresses the immune system by reducing the function and the amount of the immune system.		Nausea, vomiting, loss of appetite, heartburn, trouble sleeping, increased sweating, acne.	[49]
Cortisone	Switching off multiple activated inflammatory genes through inhibition of HAT and recruitment of HDAC2 activity to the inflammatory gene transcriptional complex.		Confusion, Excitement, Restlessness, Headache, Nausea, Vomiting, Skin problems including: acne thin skin heavy sweating redness trouble Sleeping.	[50]
Methylprednisolone	The methylprednisolone-glucocorticoid receptor complex binds and blocks promoter sites of pro-inflammatory genes, promotes expression of anti-inflammatory gene products, and inhibits the synthesis of inflammatory cytokines, mainly by blocking the function of transcription factors, such as nuclear factor-kappa-B (NF-kB).		Upset stomach, Stomach irritation, Vomiting, Headache, Dizziness Insomnia, Restlessness, Depression, Anxiety, Acne,	[51]

			Increased hair growth, Easy bruising, Irregular or absent menstrual periods	
Non-steroidal Anti-Inflammatory Drugs NSAIDs				
Ibuprofen	Non-selective, reversible inhibition of the cyclooxygenase enzymes COX-1 and COX-2 (coded for by PTGS1 and PTGS2, respectively).		Headaches, feeling dizzy, feeling sick (nausea), being sick (vomiting), wind, indigestion, swollen ankles.	[52]
Naproxen	Blocks arachidonate binding to competitively inhibit both cyclooxygenase (COX) isoenzymes, COX-1 and COX-2, resulting in analgesic and anti-inflammatory effects.		Confusion, headache, ringing in the ears, changes in vision, tiredness, drowsiness, dizziness and rashes.	[53]
Celecoxib	Selective inhibition of cyclooxygenase-2 (COX-2), which is responsible for prostaglandin synthesis, an integral part of the pain and inflammation pathway.		Stomach pain, heartburn, gas, diarrhea, constipation, nausea, vomiting; swelling in the hands or feet; dizziness, cold symptoms.	[54]

				
Diclofenac	Inhibition of prostaglandin synthesis by inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) with relative equipotency.		Headaches, dizziness, stomach pain, feeling or being sick, diarrhoea and rashes.	[55]
Indomethacin	Inhibition of prostaglandin synthesis by inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2)		Vomiting, upset stomach, heartburn, diarrhea, a feeling of bowel fullness, constipation, bloating, gas, rectal irritation, dizziness, drowsiness, nervousness.	[56]
Piroxicam	Inhibition of cyclooxygenase (COX-1 and COX-2). Piroxicam is a potent inhibitor of prostaglandin (PG) synthesis in vitro.		Abnormal liver function tests,	

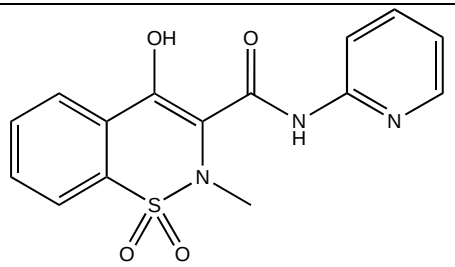
			urination problems, upset stomach, heartburn, loss of appetite, stomach pain, nausea, vomiting, gas, diarrhea, constipation; dizziness, headache; itching, rash, ringing in the ears.	[57]

Table 2 shows that corticosteroid and non-steroidal anti-inflammatory drug molecules contain at least one chemical group in their composition, such as hydroxyl, methyl, and ketone. This property will assist us in finding a natural matrix that will substitute these synthetic drugs with lower side effects.

1.3.3. Natural anti-inflammatory agents

Most anti-inflammatory medications were linked with an elevated risk of severe upper gastrointestinal problems. The level of risk for anti-inflammatory drugs has been estimated through epidemiological studies. Using oral steroids or low-dose aspirin, the likelihood of upper gastrointestinal tract leakage or perforation rises by almost twice as high as with the use of non-aspirin, non-steroidal, anti-inflammatory medicines. Overall, with more than one anti-inflammatory drug administered concurrently, the risk is dose dependent and is higher. Therefore, in order to minimize the risk of severe upper gastrointestinal complications, anti-inflammatory drugs should be prescribed during mono-therapy and at the lowest effective dose whenever possible [3].

Owing to the substantial side-effect profiles of steroidal and NSAID drugs, there is a greater interest in natural compounds, such as nutritional supplements and herbal medicines, which have been used for centuries to reduce pain and inflammation. Many of these natural compounds often act in a similar fashion to NSAIDs by inhibiting inflammatory pathways. Many natural compounds function to inhibit the inflammatory pathways of nuclear factor-kB (NF-kB) in addition to the COX pathway [4].

One of the most effective natural anti-inflammatory agents available is the omega-3 polyunsaturated fatty acids[58]. The American Heart Association for the prevention of this disease recommends the consumption of fish and fish oil supplements after the discovery that vascular inflammation is the underlying cause of coronary artery disease[59].

Eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) are the active ingredients in fish oil and can enhance the conversion of COX to prostaglandin E3 that is a natural anti-inflammatory agent. Prostaglandin E3 competitively inhibits the effects of the arachidonic acid conversion to prostaglandin E2, a highly inflammatory substance. It also inhibits the synthesis of TNF-α and IL-1b, both of which are inflammatory cytokines. The 5-LOX pathway can be inhibited by the EPA and DHA, which converts arachidonic acid to inflammatory leukotrienes, by competitive inhibition as well[4]. Omega-3 EFA, found in fish oil, can reduce the inflammation in

synovial cartilage by the direct reduce of the degenerative enzymes, aggrecanase and matrix metalloproteinase, as well as IL-1, TNF-α, and COX2[60].

One of the oldest herbal remedies for pain and inflammation is bark from the white willow tree, dating back to ancient Egyptian, Roman, Greek, and Indian civilizations, as an analgesic and antipyretic agent. White willow bark and aspirin have a similar mechanism of action which is the block of inflammatory prostaglandins by nonselective inhibitor of COX-1 and COX-2[61]. Various studies comparing white willow bark with non-steroidal anti-inflammatory drugs have shown an interesting activity, comparable to the synthetic agents. White willow bark can lead to few side effects because of the conversion of salicin to salicylic acid by the liver; but these side effects are much fewer than the ones caused by aspirin and other agents. However, it is costlier than aspirin, and should not be used in children, or in patients with peptic ulcer disease, poorly controlled diabetes, hepatic or renal disorders, or other conditions in which aspirin would be contraindicated[62].

Besides its efficacy in the prevention form cardiovascular diseases and cancer, green tea has been used in the treatment of arthritic disease as an anti-inflammatory agent. Polyphenolic compounds called catechins, and epigallocatechin-3 galate are the most abundant constituents of green tea. Epigallocatechin-3 galate leads to proteogly can release and type 2 collagen degradation in cartilage explants by the inhibition of IL-1[46], it also suppresses IL-1b and attenuates activation of the transcription factor NF-kβ In human in vitro human models. The aggrecanases which degrade cartilage it's also inhibited by green tea[4] .

In ancient civilizations, herbal practitioners relied on herbs to strengthen the body's immune systems. In several countries, ginger and its derivatives have improved the immune system. Gingerol, shogaol, and other structurally related compounds in ginger block biosynthesis of prostaglandin and leukotriene by blocking 5-lipoxygenase or prostaglandin synthetase. In addition, they can also inhibit pro-inflammatory cytokine synthesis such as IL-1, TNF-α, and IL-8.Studies have shown that ginger extract in liver cancer-induced rats has been able to suppress elevated expression of NFκB. Similarly, elevated expression of TNF-α was also inhibited by the treatment with ginger extract in liver cancer rats. It is obvious that ginger can serve as an anti-cancer and anti-inflammatory agent by blocking NFκβ activation through the inhibition of TNF-α, a pro-inflammatory cytokine [63,64].

B. dioica a perennial growing herb with tuberous roots that occurs in temperate Europe, North Africa, and West Asia. *B. dioica* aqueous extract can induce apoptosis in Burkitt's lymphoma cells line BL41 by triggering the mitochondria mediated pathway (the disruption of mitochondria, the activation of caspase-9 and -3, the cleavage of PARP and degradation of PUMA). The phytochemical screening indicated the existence of bioactive compounds which may contribute to the *B. dioica* aqueous extract's apoptogenic function, such as flavonoids, triterpens and sterols. *B. dioica* may also be deemed a potential avenue for the production of new therapies against Burkitt's lymphoma [65,66].

A lot of other plants are known for their anti-inflammatory activity, **table 3** shows the technique used, the active compound and its anti-inflammatory effect, and the anti-inflammatory activity which is presented by the EC_{50}(µg/mL - corresponding to the sample concentration giving 50% of NO production inhibition)for various studied plants, a low EC_{50} concentration means a high anti-inflammatory activity.

Table3: *In vitro* anti-inflammatory activity of natural matrices.

Plant species	Anti-inflammatory assay	Active compounds	Mechanism of action	Anti-inflammatory activity EC_{50} (µg/mL)	References
***Acacia tortilis* (Forssk.)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	(epi)-gallocatechin derivatives	Inhibition of cyclooxygenase-1 (COX-1) and COX-2 enzymes that are involved in the inflammatory response.	88 ± 4*	[67]
***Acanthus montanus* (Nees)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	verbascoside	-	91.50±0.95	[68]
	inhibition of COX-2 expression			92.55±0.64	
***Aloe Vera* (Carl Linnaeus)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	aloe-emodin	active against the human colon cancer cell lines DLD-1 and HT2.	8.6 ± 0.1	[69]
***Ammodaucus leucotrichus* (Coss. & Dur.)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	perilla Aldehyde and limonene	active against skin pathologies	11.70	[70]
***Asterace aeannua*L. (C.Winkl.)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated	Artemisininscopoletin, chrysosplenetin,eupa	-	87.43	[71]

	RAW 264.7 murine macrophage-like cell line	tin, sitosterol-3-*O*-*β*-d-glucopyranoside			
***Asteraceaeherba-alba* Asso (Bercht. & J.Presl)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	-	-	60	[72]
***Bauhinia variegate* L.**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line cultured in DMEM medium	phenolic acids and flavonoid glycoside	-	255 ± 16	[73]
***Biophytumum braculum* (Welw.)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	-	-	39.6±6.8	[74]
***Brillantaisiao wariensis* P.**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	-	-	71.01±0.65	[68]
	inhibition of COX-2 expression			71.01±0.65	
***Buddleja salviifolia* (Lam)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	Acteoside	-	42	[75]
***Cassia fistula* L. (Collad.)**	inhibition of nitric oxide (NO) production in a cell-based model of	-	-	83	[75]

	lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line				
***Eucalyptus camaldulensis* (Dehnh)**	lipoxygenase inhibition activity (LOX)	Essential oils	-	36.79	[76]
***Jacaranda arborea* (Urban)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	methyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)acetate (jacaranone) and its ethyl ester	Inhibition of the production of TNF-a in LPS-treated macrophages with low toxicity	0.99 (uM)	[77]
***Morinda citrifolia* (Carl Linnaeus)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	-	-	67	[75]
***Nigella sativa* (Mill.)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	Essential oils,Trans-sabinene hydratemethyl ether,1,2-epoxy-menth-4-ene	-	6.3	[78]
***Rauvol fiavomitoria* (Afzel)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line	peraksine derivatives	-	17.52 to 20.99	[79]
***Rubus rosifolius* (Sm.)**	inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated	Essential oils	-	56	[75]

	RAW 264.7 murine macrophage-like cell line	

As stated in **Table 3**, aloe-emodin, perilla aldehyde, phenolic acids, flavonoid glycoside, flavan-3-ols, peraksine derivatives, methyl (1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl), acetate (jacaranone), ethyl ester , verbascoside , artemisinin , scopoletin , chrysosplenetin, eupatin , sitosterol-3-O-β-d—glucopyranoside, essential oils, trans-sabinene hydrate, methyl-ether, 1,2-epoxy-menth-4-ene21, acteoside terpenoids , alkaloids, and triterpenoids are the most important bioactive molecules found in the natural matrices that have chemical features responsible for the anti-inflammatory properties. These molecules present in their structure chemical groups such as hydroxyl, methyl, and ketone, similar to the synthetic drugs and thus corroborating the efficacy of these functional groups in the treatment of inflammation processes. Hydroxyl and ketone groups are required for glucocorticoid activity in the immune system, glucocorticoids are part of the feedback process that decreases certain facets of immune function, such as inflammation. Therefore, they are used in medicine to combat conditions such as allergies, asthma, inflammatory diseases, and sepsis that are caused by an overactive immune system.

1.4. Methods for assessing anti-inflammatory activity

1.4.1. *In vivo* assessment

There are several *in vivo* anti-inflammatory methods commonly used by the scientific community such as: i) *"Cotton Pellet Method of Meier, Schuler and Desaulles"*, based in the fact that anti-inflammatory medication decreases the deposition of granulation tissue. This assay basically consists of the insertion of pellets in mice that are further injected with anti-inflammatory agents. Afterwards the mice are sacrificed, the pellets extracted, the extraneous tissue is cut off and the pellets are dried overnight. The pellets are measured again and the volume of granulation tissue is calculated [80]; ii) "*Inhibition of the Tuberculin Reaction in B.C.G. Sensitized Guinea Pigs"* method, also in use, is based on the findings that some fractions of liquor ice extract were as active as cortisone. In this assay, white guinea pigs are sensitized to tuberculin, and are subcutaneously injected with the anti-inflammatory agent with the aim at analyzing the inflammation reducing capacity [81], iii) "*Rat Foot Test",* in which formaldehyde solution is injected into the right rear foot plantar aponeurosis and the degree of swelling is measured. The difference between the amount of the injected and the none injected foot can be contrasted with the percentage change of the regulation as measured as a

percentage of the none injected foot[82], iv) "*Granuloma Pouch Method*", in which a volume of air is gradually inserted under the skin of the rat's back through a fine hypodermic needle. Anti-inflammatory medicine reduces pouch wall thickening and fluid exudation into the sac [83], v) "*Topical anti-inflammatory activity"* was tested in mice as an inhibition of the ear oedema caused by croton oil. Croton oil suspended in aqueous ethanol is applied to the inner surface of the right ear. The anti-inflammatory activity was demonstrated as a proportion of the decrease in oedema in treated mice. Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), was used as a guideline[84]; vi) "*Carrageenan-induced rat paw edema assay",* uses carrageenan-induced rat paw edema. By subplantar injection of carrageenan (1 percent w/v), edema was briefly caused on the right hind paw. The amount of injected and contra lateral paws was measured after inflammatory activation using a plethysmometer (Orchid Scientific Laboratory). The value is calculated as a decrease in volume percentage compared to the control group at various time intervals[85], vii) "*Detection of ROS and NO production in an established zebrafish model;* ROS is often over-released as inflammatory response happens, in addition to extreme NO as an indication of inflammation response. A zebrafish model is commonly used as a convenient and economical animal screening model to test anti-inflammatory effects *in vivo*, where the output of NO and ROS is calculated [86].

1.4.2. *In vitro* assessment

Regarding the *in vitro* assessments, there are also a wide range of assays used to perform a first screening to select the most promising anti-inflammatory agents.

"*PhagoBurst Assay"*, consists in kits that use flow cytometry to examine the phagocytic activity of granulocytes and monocytes in the whole blood. Phagotest allows for the quantitative evaluation of leukocyte phagocytosis (bacterial uptake). It specifies the percentage and activity (number of bacteria per cell) of phagocytes that ingest fluorescein-isothiocyanate (FITC) labeled opsonized bacteria[87].

In the "*Anti-inflammatory bioassay",* a reaction mixture consisting of egg albumin, phosphate buffered saline and varying concentrations of the anti-inflammatory agent is incubated with egg albumin under controlled experimental conditions. Then, the absorbance is measured and the percentage inhibition of protein denaturation is calculated by using the following formula [88].

$$\% \text{ inhibition} = 100 \times [\ Vt / Vc - 1]$$

Where, Vt = absorbance of test sample, Vc = absorbance of control.

The "*Cyclooxygenase inhibitors",* is also an*in vitro* approach used for more than two decades and is based in the incubation of arachidonic acid with COX-1-containing sheep seminal vesicle microsomes. However, distilled COX-2 and COX-1 were used in 2000 and 2004, respectively, by the same community. To identify the COX inhibitory effect of plant extracts, various parameters were used. To be considered active, some researchers set a minimum COX inhibition of 50 percent for water extracts and 70 percent for organic solvent extracts. The standards where the inhibition was below 20 percent were considered negligible. COX inhibition activity is known to be marginal between 20 and 40%, mild between 40 and 70% and strong anti-inflammatory activity above 70[89].

About the "*Lipoxygenase inhibitors",* it is interesting to point out that leukotrienes are another class of inflammatory mediators formed by LOX enzymes from arachidonic acid metabolism. It has been documented that leukotrienes are responsible for binding white blood cells to the endothelium of weakened blood vessels and function as phagocyte chemoattractant. In addition, leukotriene development has been linked to clinical symptoms of pathological disorders such as asthma and anaphylaxis. Leukotrienes, formed by 5-LOX in inflammatory cells such as poly-morphonuclear neutrophils, basophils, mast cells, eosinophils and macrophages, are the most actively examined [89].

Finally, the "*Inhibition of nitric oxide (NO)",* maybe the most used one, is based on the ability of anti-inflammatory agents to suppress inflammatory processes through the inhibition of nitric oxide (NO) production, in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line [67].

1.5. Importance of new methods/compounds for the treatment of these processes

1.5.1. Targeting glycolysis with small molecules to elicit an anti-inflammatory effect

Several models of infection and inflammation have shown an anti-inflammatory response after the inhibition of hexokinase by 2-DG and GAPDH by Heptelidic acid as well as tetramerization and the activation of the pyruvate kinase activity of PKM2 by

TEPP-46 [90]. Targeting the glucose transporter Glut1 is one of the novels promising targeted therapy to limit inflammation (**Figure 10**).

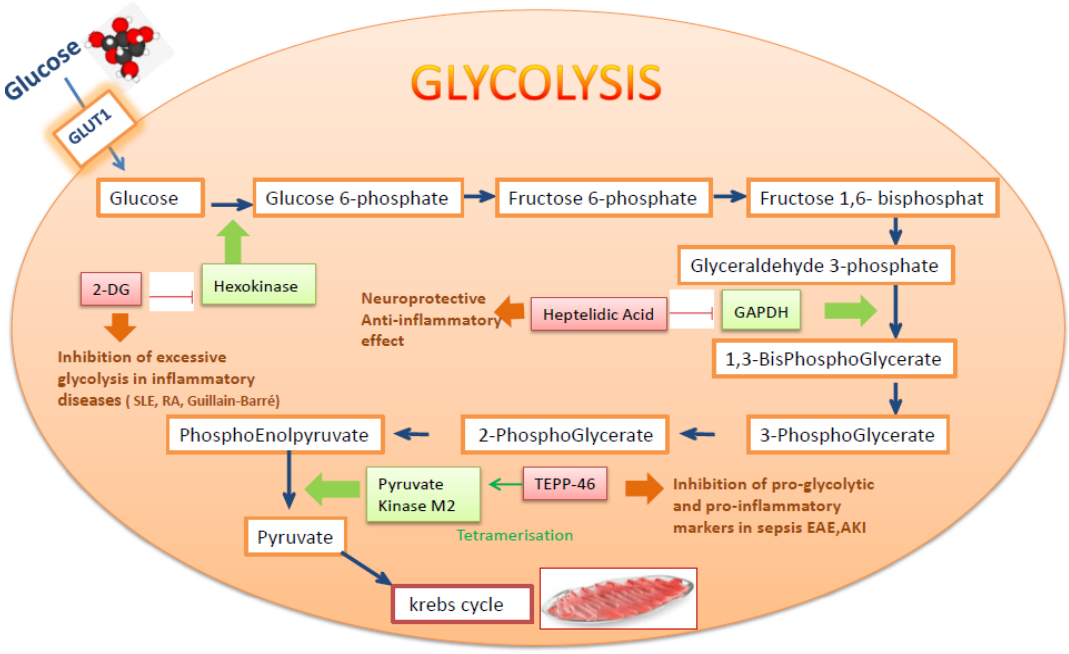


Figure 10: Targeting enzymes of glycolysis as an anti-inflammatory strategy. Glut-1 glucose transporter-1, 2-DG 2-deoxyglucose, SLE systemic lupus erythematosus, RA rheumatoid arthritis, GAPDH Glyceraldehyde 3-phosphate dehydrogenase, EAE experimental autoimmune encephalomyelitis, AKI acute kidney injury.

1.5.2. Modulating the Tricarboxylic acid cycle (TCA cycle) therapeutically to limit inflammation

The TCA cycle is located in the mitochondrial matrix; this cycle serves to break down nutrients such as glycolysis-generated pyruvate. Succinate, itaconate and citrate are metabolic intermediates that play an important role in inflammation (**Figure 11**).

Succinate has several inflammatory process; the accumulation of succinate in LPS-activated macrophages resulted in activation of Hif-1α, which is a key mediator in both innate and adaptive immune responses, subsequent upregulation of the pro-inflammatory cytokine IL-1β [4], a synergistic effect on TLR signaling, as well as increasing the antigen presenting ability of dendritic cells[91]. For example, scientists observed a SUCNR1 (Succinate Receptor 1)-dependent increase in the expression of IL-1β in synovial fluid from rheumatoid arthritis patients with high levels of succinate, providing more evidence of inhibition of SUCNR1 as a potentially interesting anti-inflammatory target [92].

Itaconate is recently described as immuno-modulatory derivative of the TCA cycle; it inhibits pro-inflammatory markers in classically activated macrophagesc (**Figure 11**). Mechanistically, itaconate has been shown to exert its anti-inflammatory actions through a broad range of mechanisms. It inhibits succinate dehydrogenase (SDH) and thereby block IL-1β production similar to DMM, and regulate IkBζ and IL-6 expression via the transcription factor ATF3[93,94]. This ability to influence key immune-modulators makes boosting cellular itaconate levels an attractive therapeutic. *In vivo* studies shows that itaconate is an important mediator of the resolution of inflammation, potentially providing a novel therapeutic opportunity and furthermore, as an endogenous metabolite there is strong evidence to predict good clinical tolerability [95].

Citrate is another TCA cycle metabolite that accumulates in activated immune cells which is converted into isocitrate by aconitase enzyme (**Figure 11**). Activated macrophages and dendritic cells have an increased ratio of isocitrate to αKG and furthermore, reduced expression of Idh1 which, in combination with increased expression of the citrate carrier (CIC) and ACLY, is thought to contribute to the accumulation of the detected citrate[96,97]. Multiple processes play an important role in activated macrophages and may provide an attractive target for limiting excessive inflammation. Hence, the citrate metabolic pathways play central roles in regulating immune responses[95].

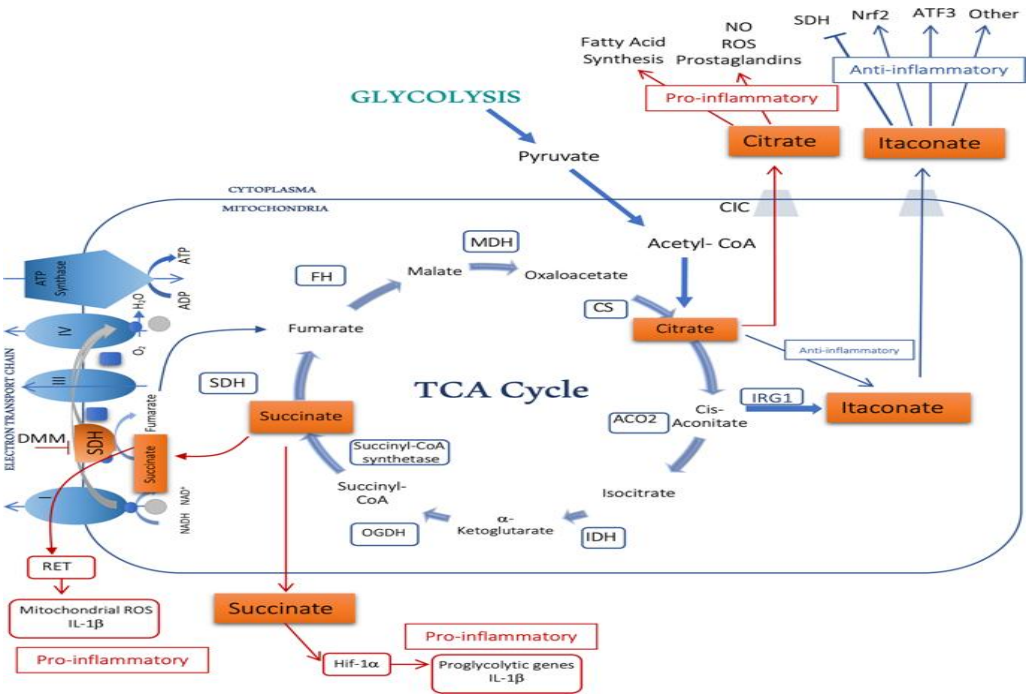


Figure 11: Modifying intermediates of the TCA cycle to drive an anti-inflammatory response. Enzymes include citrate synthase (CS), isocitrate dehydrogenase (IDH), aconitase (ACO2), α-ketoglutarate dehydrogenase (OGDH), succinyl-CoA synthestase, succinate dehydrogenase (SDH) which makes up Complex II of the electron transport chain, fumarase (FH), malate dehydrogenase (MDH) and Immuneresponsive gene-1 (IRG-1), also known as Aconitate Decarboxylase 1 (ACOD1), the enzyme responsible for the production of itaconate [95]

1.5.3. Molecular docking; in silico study

Molecular docking has been consistently used to highlight ligand molecules' molecular interactions and is thus a valuable method in the direction of drug discovery and development. It is one of several computational methodologies developed to help researchers classify candidates for new drugs and investigate them. Molecular docking analysis was then used to elucidate the structural criteria for interaction of compounds with various anti-inflammatory drug targets.

The Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB, http://www.rcsb.org/pdb/home/home.do) will obtain three-dimensional coordinates of various inflammation-related drug targetssuch as cyclooxygenase-2, tumour necrosis factor-α, inducible nitric oxide synthase and galectin-3. The graphical user interface of the Biovia Exploration Studio Visualizer and MGLTools packages can be used to prepare all input files for docking. It is possible to extract native co-crystallized ligands, water molecules and cofactors from each PDB and Gasteiger file and process and use them to assign partial atomic charges. After combining non-polar

hydrogens, each rotatable bond of a ligand can be allocated. The 2D-structure can be transformed by the MM2 process implemented in ChemBio3D Ultra 12.0 into its 3D-coordinate and energy reduced. For molecular docking simulation using the Lamarckian genetic algorithm technique, AutoDock 4.2 and AutoDock Vina 1.1 are included. The default docking protocol needs to be introduced with 100 individual runs for a rigid protein and a fluid ligand [98].

Compared to the co-crystallized ones, the observed root mean square deviation (RMSD) of the docked ligands with minimal binding energy should be around 2.0 Å, suggesting that the scoring parameters implemented in both AutoDock 4.2 and AutoDock Vina 1.1 are accurate [98].

1.6. Cellular cycle and apoptosis

The cell cycle is a strictly mediated mechanism responsible for sufficient cell division, growth, and differentiation. To ensure equal distribution of duplicated genetic material in the daughter cells, it combines DNA replication with chromosomal segregation. The phases of the cell cycle are classified into four sequential phases, which proceed from quiescence (phase G0) to proliferation (phases G1, S, G2, and M) and back to phase G0 as seen in **(Figure 12).** A characteristic of tumor cell growth is known to be the deregulation of this process. Therefore, the development of drugs targeting cell cycle phases has been heavily focused on both academic and commercial drug research projects. Researchers can easily and accurately identify various phases of the cell cycle using sophisticated flow cytometry[99]. A disregulation of the cell cycle components may lead to tumor formation; when certain genes, such as cell cycle inhibitors, RB, and p53, change, they might cause the cell to grow uncontrollably, resulting in the creation of a tumor. Although tumor cells have a cell cycle that is equivalent to or longer than normal cells, the proportion of cells involved in active cell division is substantially higher in tumors than in normal tissue. As a result, the number of cells that die through apoptosis or senescence remains constant, resulting in a net increase in cell population[100].

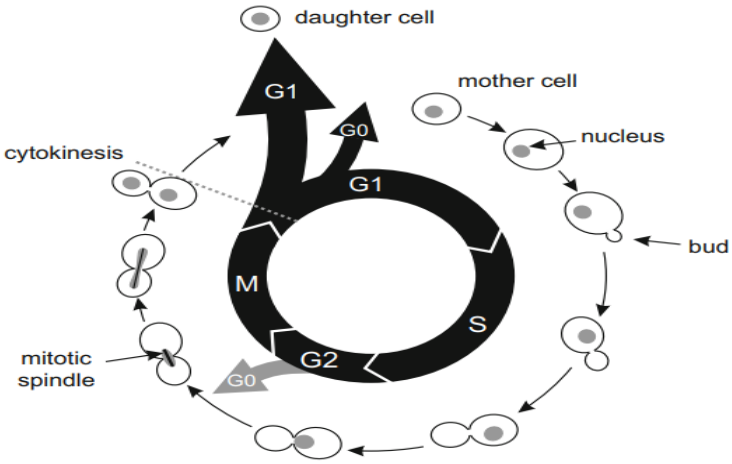


Figure 12: Inner circle: Phases of generic eukaryotic cell cycle. Outer circle: characteristic phenotype of budding yeast cells in distinct cell cycle phases.[102]

Apoptosis is a type of programmed cell death seen in multi-cellular organisms; it's a characteristic cell changes (morphology) and death that are the result of biochemical processes. Cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA degradation are manifestations of these alterations. It's important to note that the apoptosis kills between 50 and 70 billion cells a day in the ordinary adult person[101].

2. Objectives

The aim of this study was to characterize the bioactive compounds and bioactive activities of *Z. officinale* and *B. dioica* as potential natural matrice. It was also planned to research the health-promoting properties of its hydroethanolic extracts in order to see whether they could be used as functional ingredients.

The specific objectives were:

- Obtaining hydroethanolic extracts and assessing bioactive properties such as anti-inflammatory activity, NOS activity, cytotoxicity in tumor and non-tumor cells, antioxidant activity, cell cycle analysis and apoptosis.
- Phenolic compound and organic acid characterization of both samples.

3. Materials and methods

3.1. Preparation of the samples

The tuberculoses *Z. officinale* was bought at a local supermarket and *B. dioca* were collected in Bragança in late summer of 2020, Both samples used in this research were frozen and lyophilized. The samples after the lyophilization process are shown in **Figure 13**.

Figure 13: Lyophilized samples; a: *Z. officinale.*, b: *B. dioica*

After ensuring that both samples were fully dehydrated, they were grounded (20 mesh) and stored at 4°C in a light-protected environment as shown in **Figure 14**.

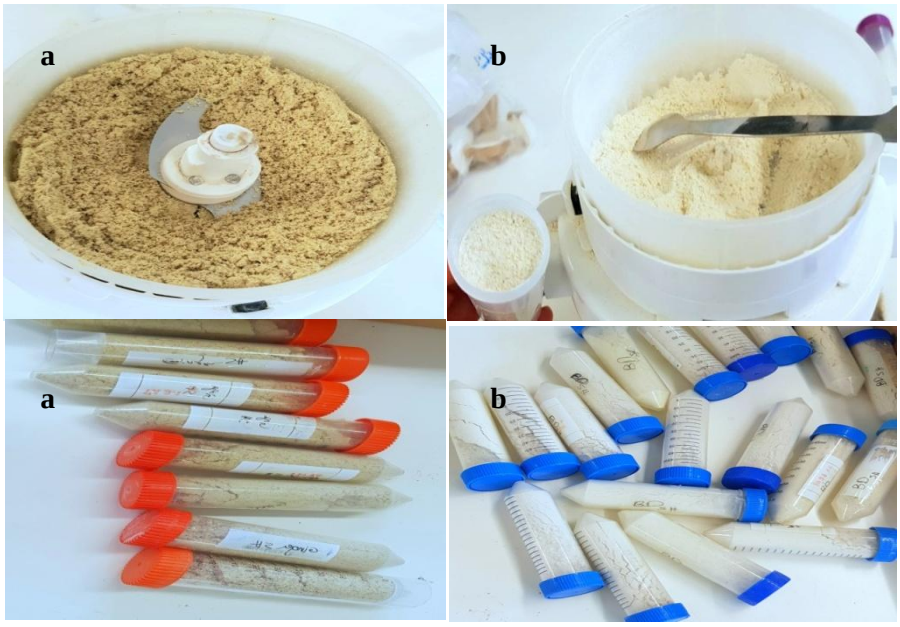


Figure 14: Grounded samples; a: *Z. officinale*, b: *B. dioica*

3.2. Preparation of the hydroethanolic extracts

1 g of each sample was mixed with 30 ml of ethanol: water (80:20 v/v) in a 30 ml flask. After that, the samples were macerated for 1 hour at room temperature in a magnetic stirrer plate (LBX H20D Labbox, 550W, Barcelona, Spain). The samples were then filtered through a Whatman paper N^{o} 4 filter and the ethanol was evaporated under reduced pressure Büchi rotary evaporator R-210, Flawil, Switzerland). The samples were lyophilized (47 ºC, 0.045 bar; Freezone 4.5, Labconco, Kansas City, MO, USA)once the ethanol was removed (**Figure 15**).

Figure 15: Preparation of the hydroethanolic extracts.

3.3. Chemical characterization of the extracts

3.3.1. Phenolic compounds

A Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, San Jose, CA, USA) fitted with a diode-array detector and coupled to a mass detector (LC-DAD-ESI/MSn) was used to analyze the extracts (**Figure 16**). A quaternary compressor, an autosampler held at 5°C, a degasser, a photodiode-array detector, and an automated thermostatic column compartment made up the chromatographic device. Waters Spherisorb S3 ODS-2 C18 (3 m, 4.6 mm 150 mm, Waters, Milford, MA, USA) column thermostated at 35 °C was used for the chromatographic separation. (A) 0.1 percent formic acid in water, (B) acetonitrile were used as solvents. The elution gradient was isocratic: 15% for 5 minutes, 15% B to 20% B for 5 minutes, 20-25 percent B over 10 minutes, 25-35 percent B over 10 minutes, 35-50 percent for 10 minutes, and re-equilibration of the column at 0.5 mL/min.

Double online detection was performed in the DAD using chosen wavelengths of 280, 330, and 370 nm, as well as in a mass spectrometer (MS) connected to the HPLC device through the DAD cell outlet. The mass spectrometer, a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) fitted with an ESI source, was used in negative ion mode. The nitrogen sheath gas pressure was 50 psi, the spray voltage was 5 kV, the source temperature was 325 °C, the capillary voltage was -20 V, and the tube lens offset was -66 V. The entire mass spectrum was scanned, from m/z 100 to 1500. 35 kilojoules of collision energy is included (arbitrary units). The Xcalibur® computer framework was used to collect data (Thermo Finnigan, San Jose, CA, USA).

When available, the phenolic compounds were classified by comparing their retention times, UV-vis, and mass spectra to those of standard compounds. Otherwise, compounds were tentatively described by comparing the collected data to publicly available records. For quantitative analysis, the UV-Vis signal was used to create a calibration curve for each available phenolic standard (apigenin-6-*C*-glucoside (y = 107,025x + 61,531, R^2= 0.9989, LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL, peak 4) and quercetin-3-*O*-glucoside (y = 34,843x − 160,173, R^2= 0.9998, LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL, peaks 1, 2, and 3).Quantification of the identified phenolic compounds for which there was no commercial norm was done using the calibration curve of another compound from the same phenolic community. The results were given in milligrams per gram of extract[102].

Figure 16: Equipment (HPLC-MS) used in the determination of the phenolic compounds.

3.3.2. Organic acids

The analysis of organic acids was performed according to Silva AR et al[103] .1 g of each sample was combined with 25 mL of metaphosphoric acid in a simple procedure (4.5 percent). The mixture was kept out of direct sunlight and macerated for 20 minutes at room temperature. Following this, the sample was purified using a 0.2 m nylon filter

(Whatman). The organic acids were calculated using a photodiode array detector and ultra-fast liquid chromatography (UFLC) Shimadzu 20A sequence (Shimadzu Corporation, Kyoto, Japan) (PDA) (**Figure 17**).

The organic acids were separated using a SphereClone reverse phase C18 column (5 m, 250 mm, 4.6 mm i.d- internal diameter.) thermostated at 35°C (Phenomenex, Torrance, CA, USA). Using sulphuric acid (3.6 mM) and a flow rate of 0.8 mL/min, the elution was carried out. The chosen wavelengths for detection were 215 and 245 nm (for ascorbic acid). Calibration curves with established concentrations of industrial requirements were generated for the quantitative study, and the organic acids found in the two samples were calculated by comparing peak areas at 215 nm and 245 nm (for ascorbic acid).

Figure 17: UFLC-PDA equipment**.**

The calibration curve equations and R^2 of the found organic acids were:

$y = 1E+07x + 231891$ $R^2 = 0.9999$ for oxalicacid

$y = 950041x + 6255.6$ $R^2 = 0.9999$ for malicacid

$y = 1E+06x - 10277$ $R^2 = 0.9997$ for citricacid

$y = 1E+08x + 614399$ $R^2 = 0.9986$ for fumaricacid

3.4. Bioactive properties

3.4.1. Anti-inflammatory activity

Following the procedure described by Taofiq et al[104], RAW 264.7 mouse macrophages were used to assess anti-inflammatory activity. Cell cultures was collected in European

Collection of Authenticated Cell Culture(ECACC) and grown in DMEM medium supplemented with 10% heat-inactivated bovine serum and L-glutamine and held at 37°C with humidified air and 5% CO_2. According to the Trypan Blue exclusion method, cells with successful growth were scraped and balanced to an experimental density of $5x10^5$ cells/mL, with a dead cell ratio of less than 5%.Cells were spread (300 L/well) into 96-well microplates and incubated at 37° C and 5% CO_2 for 24 hours to adhere and multiply. After that, they were incubated for 1 hour with various extract solutions at final concentrations ranging from 400 to 1,56 μg/mL. Afterwards, they were provided with lipopolysaccharides (LPS, 1 g/mL, 30 μL) for 18 hours. Negative controls were made without the addition of LPS to see whether they could induce changes in nitric oxide levels at baseline (NO). Dexamethasone (50 mM) was used as a positive control. A Griess Reagent Kit (Promega) containing sulfanilamide, N- (1-naphthyl) ethylenediamine hydrochloride (NED), and nitrated solutions are used to assess the presence of nitric oxide. The supernatant cell solution (100 μL) was transferred to a microplate, along with sulfanilamide and NED solution, and mixed at room temperature for 5 to 10 minutes each. A reference curve for $NaNO_2$ (100 μM at 1.6 μM, $y = 0.0066x + 0.1349$; $R^2 = 0.9986$) was prepared in a 96-well microplate (**figure 18**). The amount of nitric oxide released was calculated by comparing the absorbance at 540 nm in an ELX800 Biotek microplate reader to the calibration curve. Finally, the extract concentration needed to inhibit NO output by 50% (EC_{50}, μg/ml) was calculated.

Figure 18: Microplate used in the anti-inflammatory activity assay.

3.4.2. Cytotoxicity

The Sulforhodamine B (SRB) assay was used to assess the cytotoxic effect of the various extracts on various human tumor cell lines as well as a non-tumor cell line. As

tumoral cell lines; NCI-H460 (non-small cell lung cancer), MCF-7 (breast carcinoma), Caco(colon carcinoma) and AGS(adenocarcinoma gastric cell)were obtained from DSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen. As non-tumoral cell lines; vero (fibroblast-like from African green monkey kidney) and A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to an existing protocol, for hepatotoxicity testing, and it was planned as PLP2 (porcine liver primary culture)[105].The cells were incubated at 37 ° C with humidified air and 5% CO2 in RPMI-1640 containing heat-inactivated FBS (10%), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/L). Prior to the assays, trypsin (a proteolytic enzyme) was applied to adherent cell cultures to ensure cell detachment and deagglomeration. The cells were pelleted after shedding by adding RPMI and centrifuging (5 min, 1200 rpm), then collected and resuspended in RPMI medium. Each cell line was prepared in 96-well plates at the required density (1.0 x 10^4 cells/well) and incubated for 24 hours to achieve cell attachment. The amounts of the extracts to be examined were applied and incubated for another 48 hours. After that, the cells were fixed with cold trichloroacetic acid (10%, 100 μL) and left to stand for 1 hour at 4 °C. After that, the plates were washed three times in deionized water and air dried. SRB solution (0.1 percent in 1% acetic acid, 100 μL) was added and incubated for 30 minutes at room temperature. To extract excess SRB, the plates were washed with acetic acid (1%) and allowed to air dry. Finally, adhered SRB was solubilized by adding tris-HCl (10 mM, 200 μL) to a microplate reader (BiotekElx800) and reading the absorbance at 540 nm **(Figure 19)**.Dose-response curves were obtained for each cell line studied, and GI_{50} values, corresponding to the concentration of extract that inhibited 50% of cell growth, were determined. Two separate tests were carried out for each compound, each in duplicate, with the effects expressed as mean values and standard deviation (SD). Ellipticine was used as a positive control [106].

Figure 19; Microplate reader for the cytotoxic activity evaluation.

3.4.3. *i*NOS activity

During the work of this master dissertation, a new method was optimized to assess the anti-inflammatory activity. This method is based in the inhibition of the enzyme responsible for the production of nitric oxide using ab211083 NOS Activity Assay Kit bought from Abcam.

NOS Reaction: 40 µL of Reaction Mix was prepared by mixing 10µL of diluted NOS Cofactor 1, 20µL of NOS Cofactor 2 (1X), 5µL of NOS Substrateand 5 µL of Nitrate Reductase, this mixture was added into standard, positive control, and samples well, after pipetting up and down they were Incubated at 37°C for 1 hour. Then 95 µL of NOS Assay Buffer and 5 µL of Enhancer were added to standard, positive control, and sample well without adding them to the background control wells, all this mixture was pipette up and down then incubated at room temperature for 10 minutes.

For the measurement,50 µL of Griess Reagent 1 and 50 µL of Griess Reagent 2 were added to the standard, positive control, and sample microplate wells, without adding reagents 1 and 2 to the background control well. After well homogenised, the mixture was incubated at room temperature for 10 minutes and the absorbance was measured immediately (for all wells) on a microplate reader at OD 540 nm.

Nitrite activity (pmol/min/µg or mU/mg) in the test samples was calculated according to the following formula:

$$iNOSSpecificActivity = \frac{B}{(TxC)}$$

Where: B = Nitrite amount in sample calculated from the Standard Curve (pmol); T = Reaction time (minutes) – 60 minutes. C = amount of protein (μg) [107] .

The calibration curve equation of the NOS was:

$$\boldsymbol{Y = 0.0002\ X - 0.0031} \text{and} \qquad \boldsymbol{R^2 = 0.9977}$$

3.4.4. Cellular antioxidant activity (CAA)

RAW264.7 cells were incubated with antioxidant compounds and AAPH, a compound that induces oxidative stress and promotes the production of free radicals, to determine intracellular ROS. As a fluorescent marker, DCFH-DA was used[108]. (DCF-DA is a compound that is readily oxidized by peroxide radicals in the cell medium to produce DCFH-DA, a fluorescent compound. When DCF-DA enters the cell, it is recognized by esterase enzymes, which cleave the diacetate present in the molecule. The molecule is oxidized by ROS, resulting in DCFH-DA, which has fluorescence. The activity of antioxidant compounds will prevent oxidation by lowering the antioxidant ability of free radicals (ROS). The antioxidant activity of the compounds studied is shown by the decrease in fluorescence emission as compared to control cells[109].

The method of Wolfe and Liu (2007) was modified, and cellular antioxidant analyses were performed. RAW cells were washed twice with sterile HBSS (pH 7.4) after reaching confluence in the culture flasks, and then isolated from the surface with 0.05 percent trypsin-EDTA. In 96-well black background culture plates, cells were plated (3.0 x 10^4) in 100 μL of cell/well culture medium and incubated until confluency (24-48 h). To minimize any difference caused by the plate's position, the perimeter wells were left empty. After confluence, the growth medium was separated, and the cells were washed with HBSS. Then, in triplicate, 200 μL of various extract concentrations were added to each well with 50 μM DCFH-DA prepared in ethanol and diluted in HBSS. A negative control of 200 μL of DCFH-DA was introduced in triplicate as a negative control. At 37°C, the cells were incubated for 1 hour. As a positive control, quercetin was used. The cells were then washed three times with HBSS to ensure that any antioxidant results seen later in the experiment were solely attributable to the compounds incorporated by the cells. After that, 100μL of AAPH was added. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

In terms of CAA quantification, the effectiveness of antioxidant therapies for both cell lines was found by observing the percentage of reduction in fluorescence. The 14

fluorescence reaction readings were used to establish curves over the course of a 40-minute assay. Excel and Integral Calculator (https://www.integral-calculator.com/) were used to quantify the area under each curve. Since the DCFH-DA reaction was not inhibited, the highest fluorescence was seen in the control wells.

The percent reduction (or the CAA unit) was calculated from the formula described by Wolfe and Liu[110],shown below:

$$\boldsymbol{CAA = \%\, reduction = 1 - \frac{\text{AUC sample}}{\text{AUC control}} \times 100}$$

Three separate assays listed with mean were used to calculate the percent reduction in quercetin.

3.4.5. Cell cycle analysis

NCI-H460 with GI_{50} concentration determined from the cytotoxicity assay cells were seeded in 6-well plates ($4x10^5$ cells/well) and incubated with the GI_{50} concentration for each sample, for 72 h. Cell cycle analysis was performed using propidium iodide (PI) staining and flow cytometry. This assay is established on the measurement of the DNA content of nuclei labeled with PI.

Following, the cells were staining according to the protocol PI/RNASE Solution (Immunostep, Spain, Salamanca). Were harvested cells corresponding to 2×10^5 to $1 \times$ 106, and centrifuged for 5 minutes at 300 g, and removed the supernatant. The cells were fixed and added 200 µl of 70% ethanol and left in ethanol at 4 ºC for 30 minutes. Following, the cells were washed once in 2 ml phosphate buffered saline (PBS) and centrifuged for 5 minutes at 300 g and resuspended in 500 µL of PI solution (PI/RNASE) and incubated in the dark at room temperature for 15 minutes. Cell cycle phase distribution was evaluated using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

The DNA content of at least 20,000 cells was counted per sample and the percentage ofcells in different phases (G0/G1, S and G2/M phases) of cell cycle was evaluated using BD Accuri C6 software (BD Biosciences, San Jose, CA, USA).

3.4.6. Apoptosis

The Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Kit (BD Biosciences, San Jose, CA, USA) and flow cytometry were used to detect apoptosis. The percentage of cells in a population that are successfully undergoing apoptosis are determined using FITC Annexin V staining. The NCI-H460with GI_{50} concentration determined from the cytotoxicity assay cells were washed twice in PBS and resuspended at 1 106 cells/ml in 1 x binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$).Following that, 100 µL of the solution (1 105 cells) was transferred to a 5 ml culture tube, 5 µL of FITC Annexin V (BD Biosciences, San Jose, CA, USA) and 5 µL of PI (BD Biosciences, San Jose, CA, USA) were introduced to each tube, and incubated for 15 minutes in the dark at room temperature. Finally, each tube received 400 µL of 1x binding buffer. The Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) was used to collect 30,000 cells, and the percentage of cell distribution was calculated using the Accuri C6 software (BD Biosciences, San Jose, CA, USA)[111].

3.5. Statistical analysis

Throughout this work, results are expressed as mean ± standard deviation. An analysis os variance (ANOVA) was used to determine the statistical difference among the samples. When two independent samples were analyzed, a Student's T test was used to classify them, placing an asterisk to discriminate between two statically different samples. When three independent samples were analyzed, a Tukey's test was used for classification, using different letters to classify statistically different samples. The equality of variance of the samples was confirmed using a Levene’s test. Throughout the whole work, the p-value for all statistical classifications was set at 0.05.

4. Results and discussion

4.1. Chemical characterization of the extracts

4.1.1. Phenolic compounds

Phenolic compounds are a kind of small molecules with at least one phenol unit in its structure. They are classified into many subgroups based on their chemical compositions, including phenolic acids, flavonoids, tannins, coumarins, and others. Phenolic compounds are important in defensive responses in humans, such as anti-aging,

anti-inflammatory, antioxidant, and anti-proliferative activities. As a result, it is advantageous to consume plant foods that are rich in antioxidant compounds, as these can reduce the prevalence of some chronic diseases, such as diabetes, cancer, and cardiovascular disease, by reducing oxidative stress for these reasons we determined the phenolic compounds profile of our both samples; *Z. officinale* and *B. dioica*[112].

The phenolic compounds composition for *Z. officinale* is presented in **Table 4**, **Figure 20** and **Figure 21**, where it can be seen the chromatographic characteristics of retention time, UV-vis maximum absorption, pseudo-molecular ion, and fragmentation pattern, tentative identification, and quantification (mg/g extract) of the phenolic compounds present in the hydroethanolic extracts of *Z. officinale*.

Four compounds were tentatively identified in the sample of *Z. officinale.* Three *O*-glycosylated isorhamnetin derivatives and one *C*-glycosylated derivative (aglycone unknown).

Peaks 1 and 2 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 769 with a unique MS^2 fragment at *m/z* 315 (isorhamnetin aglycone), corresponding to the loss of one hexosyl moiety (162 u) and two deoxyhexosyl moieties (146 u + 146 u), being tentatively identified as isorhamnetin-*O*-hexosyl-di-deoxyhexoside. Peak 3 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 623 with a unique MS^2 fragment at *m/z* 315), corresponding to the loss of one hexosyl (162 u) and one deoxyhexosyl moieties (146 u), being tentatively identified as isorhamnetin-*O*-deoxyhexoside-hexoside.

Finally, peak 4 $(M-H]^-$ at *m/z* 723) presented the same chromatographic characteristics as previously described by Prakash, Baskaran &Kudachikar[113],which led to the tentative identification of this peak as an unknown *C*-glycosil derivative. This compound was the major one found, with 1.33±0.04 mg/g of extract.

The chromatogram obtained for this sample revealed the presence of more peaks. However, the tune method used to break the compounds, for later identification, is not the most appropriate and therefore did not allow the tentative identification of what we think are gingeriol-related compounds. These type of compounds have been extensively studied in this samples by other authors[114-115], and will be properly studied when a more appropriate tune method is developed.

Regarding the *B. dioica* sample, it was not possible to identify phenolic compounds in the sample. It is possible to enumerate a series of reasons for which this happened, among which is highlighted: *i)* the type of extraction used for phenolic compounds was not the most adequate; *ii)* the sensitivity of the HPLC apparatus did not allow a response

to the type of compounds present in the sample; *iii)* the MS method was not the most suitable for breaking the compounds present in the sample and, consequently, their tentative identification; and, finally, *iv)* the hydroethanolic extract would need an extra purification step to remove proteins, sugars, and other types of cellular constituents that may be co-eluting with the phenolic compounds.

Since it was not possible to repeat the extractions and/or to improve the chromatographic method, these steps are foreseen as future work for samples of *B. dioica*, but also for *Z. officinale* as its crucial to reveal the phenolic compound´s composition to better understand the mechanism of action of both samples and to correlate these compounds with the exhibited bioactivity, especially for *B. dioica* considering its important activities.

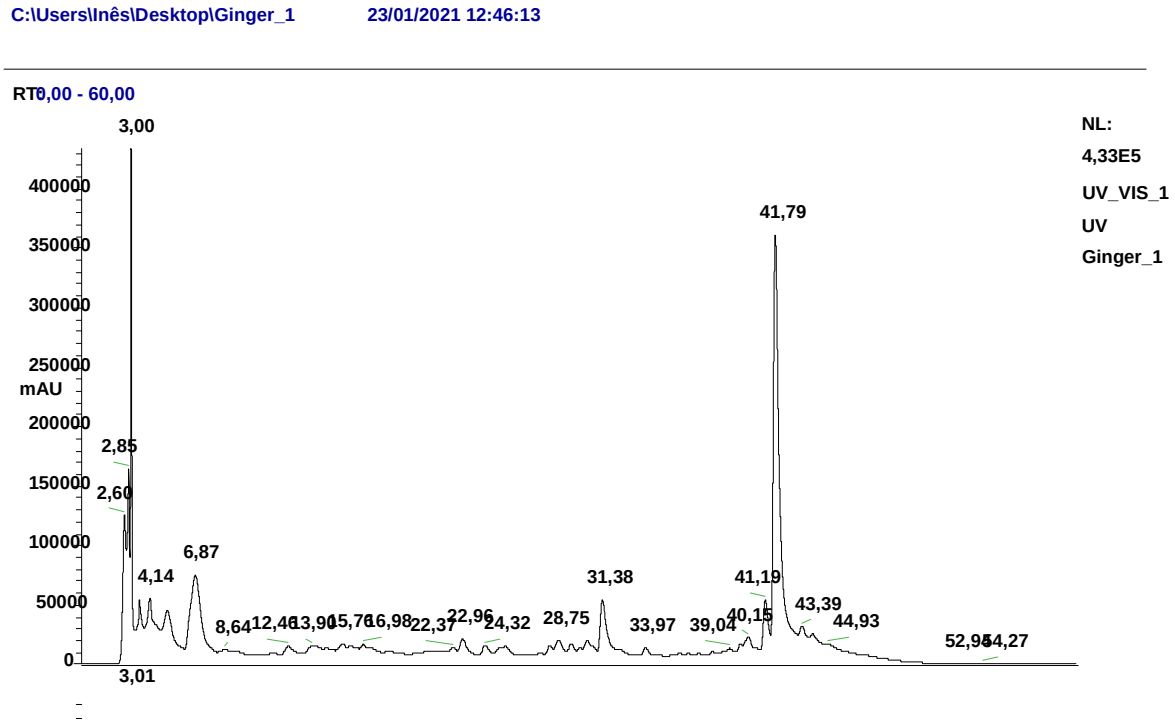


Figure 20: Phenolic compound´s profile of *Z. officinale*

Table 4: Chromatographic responses, tentative identification, and quantification (mg/g extract) of the phenolic compounds found in the hydroethanolic extracts of *Z. officinale* (Mean±SD).

Peak	Rt (min)	λmáx (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	mg/g extract
1	15.82	345	769	315(100)	Isorhamnetin-*O*-hexosyl-di-deoxyhexoside	0.485±0.001
2	16.14	345	769	315(100)	Isorhamnetin-*O*-hexosyl-di-deoxyhexoside	0.479±0.002
3	20.72	337	623	315(100)	Isorhamnetin-*O*-deoxyhexoside-hexoside	0.476±0.001
4	31.38	281/323	723	677(100),451(20)	Unknown C-glycosil derivative	1.33±0.04
					Total identified phenolic compounds	2.772±0.035

Standard calibrations curves used: apigenin-6-*C*-glucoside (y = 107,025x + 61,531, R^2= 0.9989, LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL, peak 4) and quercetin-3-*O*-glucoside (y = 34,843x − 160,173, R^2= 0.9998, LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL, peaks 1, 2, and 3).

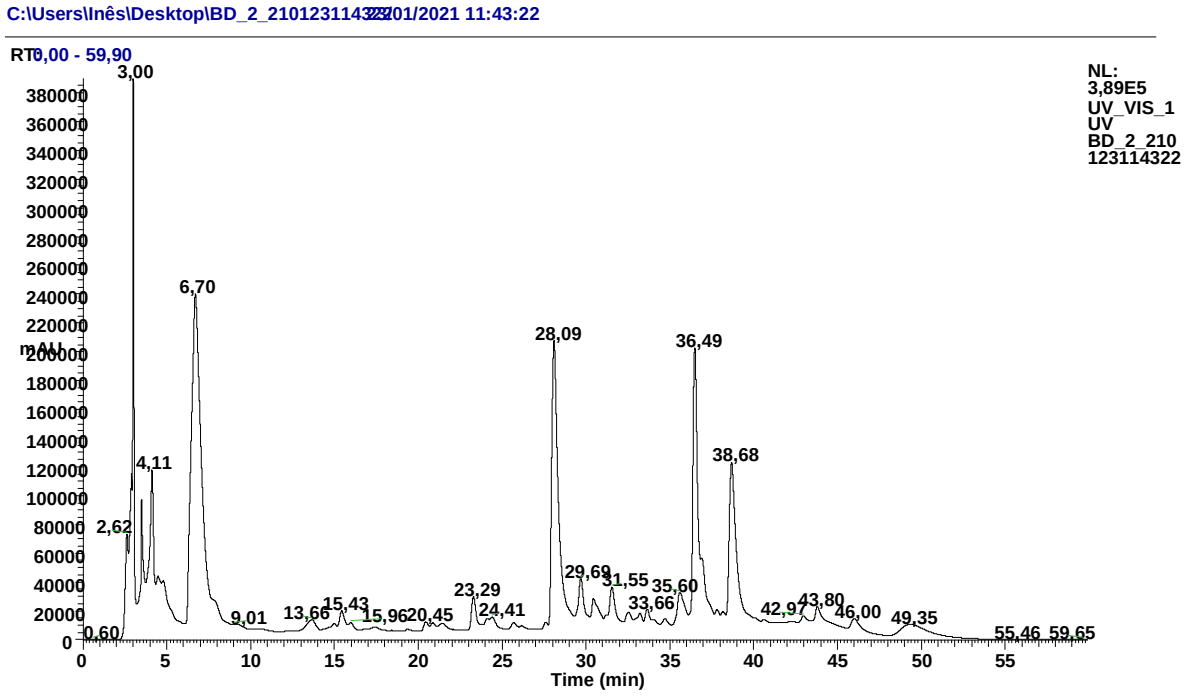


Figure 21: Phenolic compound´s profile of *B. dioica.*

4.1.2. Organic acids

Organic acids play a role in a variety of essential cellular processes, such as cellular pH regulation and redox state regulation. As a result, it's not surprising that these compounds are involved in the regulation of different biochemical and physiological *in vivo* processes [116]. For these reasons, in this work, organic acid´s composition of *Z. officinale* and *B. dioica* were assessed.

Oxalic, malic, citric, and fumaric acids were detected in *Z. officinale*, with a total organic acids content of 6.67±0.03g/100g dw. Oxalic, citric, and fumaric acids were detected in *B. dioica*, with a total organic acids content of 7.4±0.2g/100g dw. as seen in **Table 5**. The major organic acid found in *Z. officinale* was oxalic acid, which had a concentration of 4.77±0.04g/100g dw. Malic acid was also present with a significant concentration of 1.74±0.07g/100g dw, while fumaric and citric acids had low concentrations of 0.10g/100g w and 0.05g/100g dw, respectively. Citric acid was the most abundant one in *B. dioica* extract, with a concentration of 4.9±0.2g/100g dw, followed by oxalic acid with a quite important concentration equal to 2.5±0.02g/100g dw and fumaric acid which was quantified only in trace amount equal to 0.0015±0.0001g/100g dw..

When comparing these findings with the literature, Aicha Jelled et al[117] determined the organic acid profile of *Z. officinale* and discovered the same composition, but with different quantifications; the total organic acids was equal to 3.03±0.01g/100g dw and the oxalic acid was the most abundant one with a concentration equal to 1.63±0.01g/100g.It's possible that the concentration differences are attributable to the fact that different extraction methods were used.

For *B. dioica* there is no available data in the literature reporting the organic acids concentration of this sample, highlighting the importance of these characterizations of *B. dioica*.

Table 5: Organic acid´s composition (g/100g dw) of *Z. officinale* and *B. dioica*.

	Z. officinale	***B. dioica***
Oxalic Acid	4.77±0.04*	2.5±0.02
Malic Acid	1.74±0.07	nd
Citric Acid	0.05±0.001*	4.9±0.2
Fumaric Acid	0.01±0.001*	0.0015±0.0001
Total Organic Acids	**6.67±0.03***	**7.4±0.2**

nd - not detected. In each row, an asterisk means statistically significant differences between the two samples with a p-value<0.05

4.2. Bioactivity

4.2.1. Anti-inflammatory activity

In terms of anti-inflammatory properties, NO is a signaling molecule that plays a key role in the inflammation response[118]. Its physiological levels are essential for a variety of processes, including vasodilatation and neurotransmission. As a result, excessive NO output may be a toxic and pro-inflammatory mediator, triggering inflammation [119]. As a result, NO inhibition is related to the production of anti-inflammatory drugs.

The capacity of hydroethanolic extracts of *Z. officinale* and *B. dioica* their combination to modulate the synthesis of inflammatory mediator NO in RAW 264.7 macrophages was tested in this study, and the EC_{50} values (μg/mL) were determined. According to recent studies, *Z. officinale* contain gingerol, shogaol, and other structurally related compounds that block biosynthesis of prostaglandin and leukotriene by blocking 5-lipoxygenase or prostaglandin synthetase, In addition, they can also inhibit pro-inflammatory cytokine synthesis such as IL-1, TNF-α, and IL-8[63,64]. *B. dioica* activate the mitochondria-mediated cascade in Burkitt's lymphoma cells line BL41. The presence of bioactive compounds such as flavonoids, triterpenes, and sterols may be the reason of the apoptogenic role of the *B. dioica* aqueous extract[65].As anti-inflammatory responses are usually associated with cell proliferation, in the present dissertation, the effects of *Z. officinale* and *B. dioica*, extracts and their combination on the proliferation of RAW 264.7 cells were achieved.

As shown in the **Table 6** and **Figure 22**, the different extracts suppressed the growth of RAW 264.7 cells in a dose-dependent manner with different values of EC_{50}.

B. dioica extracts presented the lowest EC_{50} value equal to 1.187±0.18μg/mL, which prove the high anti-inflammatory potential comparing to *Z. officinale* and their combination, it also shows an EC_{50} value even lower than the positive control (dexamethasone: EC_{50} equal to 1.6 μg/mL). *Z. officinale* showed a value equal to 2.806±0.28μg/mL, while the combination of the 2 samples showed a value equal to 2.556±0.33μg/mL. The mixture of the two extracts did not increase the anti-inflammatory activity, thus for the further assays the samples were analyzed separately, since no synergism was verified, and no additional benefit was obtained by mixing the two samples.

A recent research used various *in vitro* and *in vivo* approaches to test the anti-inflammatory capacity of *Z. officinale* and found that the extract has important anti-inflammatory properties through inhibiting macrophage and neutrophil activation, as well as monocyte and leukocyte migration. The decline in pro-inflammatory cytokines and chemokines, as well as the replenishment of total antioxidant ability, corroborates that[120].

Once again, to the best of the authors knowledge, there are no reports on this subjected about *B. dioica*.

Table 6: Anti-inflammatory activities of the *Z. officinale* and *B. dioica*, and their combination.

	Anti-inflammatory activity(EC_{50}; μg/mL)		
	Z. officinale	***B. dioica***	***B. dioica* + *Z. officinale***
RAW264.7	2.806±0.28[b]	1.187±0.18[a]	2.556±0.33[b]

EC_{50} values correspond to the extract concentration achieving 50% of the inhibition of NO-production. EC_{50} values for dexamethasone (positive control): 1.6 μg/mL.In each row, different letters mean statistical differences among samples.

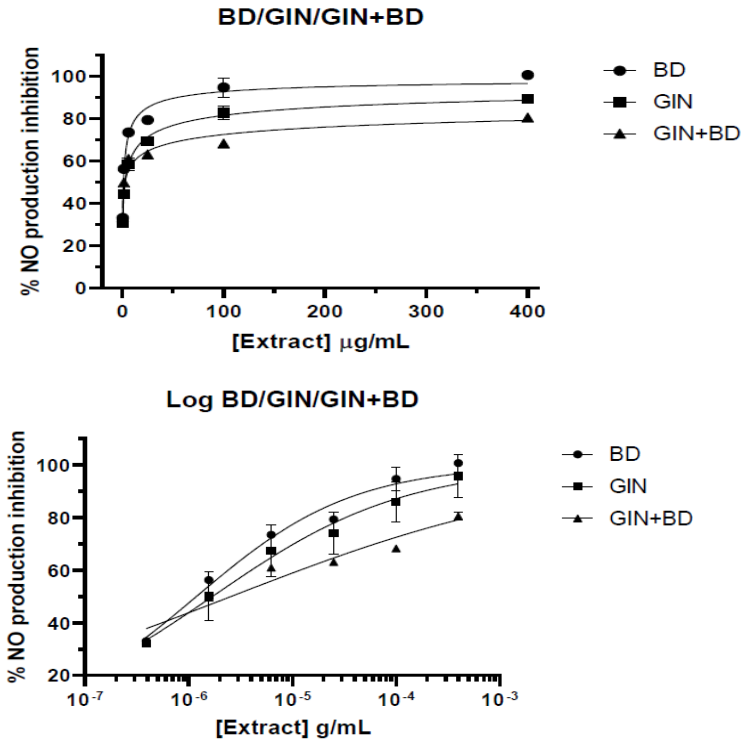


Figure 22: Anti-inflammatory activities of the *Z. officinale* and *B. dioica*, and their combination.

4.2.2. Cytotoxic activity

The cytotoxic activity of the hydroethanolic extracts was tested against four human cancer cell lines: NCI-H460 (non-small cell lung cancer), MCF-7 (breast carcinoma), Caco2 (colon carcinoma), AGS (adenocarcinoma gastric cell); and two non-tumor cell lines: PLP2 (porcine liver primary cell line) and Vero (fibroblast-like from African green monkey kidney). Ellipticine was used a positive control.

The hydroethanolic extracts of *Z. officinale* and *B. dioica* were active against the different cell lines, as seen in **Table 7.** It is possible to note that *Z. officinale* has a much higher GI_{50} than *B. dioica* and ellipticine for all cell types, meaning that it has a lower cytotoxicity effect. *Z. officinale* has the highest cytotoxic activity against NCI H460 with the lowest GI_{50} value of 29.28±1.78 µg/mL, followed by Caco2 with GI_{50} equal to55.23±4.43 µg/mL, MCF-7 with GI_{50} of 60.71±3.26 µg/mL, and AGS with GI_{50} of 90.26±7.81µg/mL. The cytotoxic activity of *Z. officinalis* PLP2 is the lowest, with a GI_{50} value of 133.4±3.9 µg/mL. Regarding the activity exhibited for the non-tumor cells PLP2 and Vero, it was observed that besides the samples presents some cytotoxic effects on these cells, the concentration needed to cause toxicity is much higher than the one needed to inhibit the tumor cells.

A similar research was carried out by Kottarapat et al[121], but this time with Ginger essential oil (GEO). The authors found that both Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines demonstrated were strongly inhibited when exposed toGEO. The concentration of ginger essential oil needed to kill 50% of DLA cell lines (IC_{50}) was found to be 11 μg/mL for DLA cell line and 18 μg/mL for EAC cell line respectively. The differences in these findings are due to the different types of extract used and the different types of cells used.

The analysis of *B. dioica* data revealed that MCF-7 and NCI H460 have a very similar GI_{50} values equal to 0.121±0.004 and 0.135±0.003 μg/mL, respectively, consecutively followed by Caco2 which has a GI_{50} value of 0.43±0.05 μg/mL. The GI_{50} of the positive control ellipticine was higher than that of *B. dioica* for these three cancer cell lines, indicating that *B. dioica* is more active against these cell lines. The GI_{50} for AGS was 1.35±0.04 g/mL for *B. dioica,* which is slightly higher than it was for ellipticine, indicating that ellipticine is more effective against the AGS cell line. PLP2 findings indicate that *B. dioica* was more active against cancer cell lines than normal cell line, with a GI_{50} value 4.3±0.2 μg/mL, which is a positive signal since it is possible to inhibit cancer cell lines without harming normal cell line simply by lowering the concentration below 4.3±0.2 μg/mL. Ellipticine was more active against the PLP2 cell line than *B. dioica,* making it more difficult to regulate their cytotoxicity against normal cell lines, being more dangerous.

No similar study was performed to evaluate the cytotoxic activity of *B. dioica*, once again highlighting the importance of analyzing the bioactive properties of *B. dioica*.

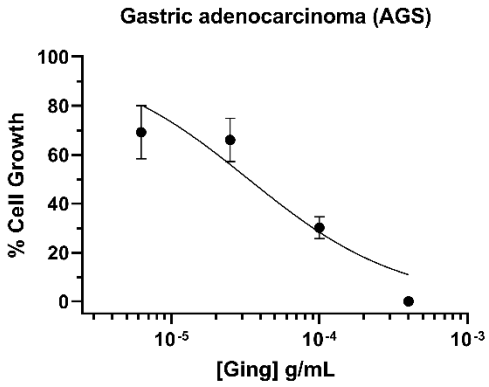
Table 7: Cytotoxic activities (GI_{50}; µg/mL) of *Z. officinale* and *B. dioica*.

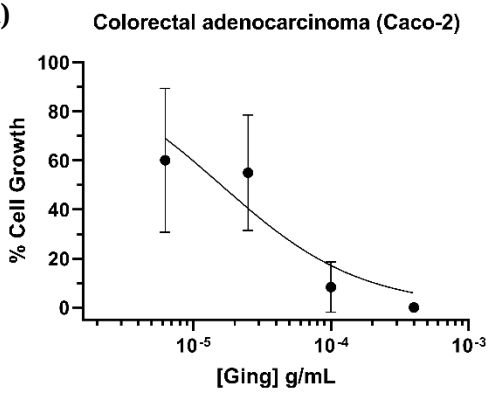
	Z. officinale	***B. dioica***	**Ellipticine**
NCI H460 (non-small cell lung cancer cell line)	29.28±1.78	0.135±0.003*	1.01 ± 0.01
MCF-7 (breast carcinoma cell line)	60.71±3.26	0.121±0.004*	1.02 ± 0.02
Caco2 (colon carcinomacell line)	55.23±4.43	0.43±0.05*	1.21± 0.02
AGS (adeno-carcinoma gastric cell line)	90.26±7.81	1.35±0.04*	1.23 ± 0.03
PLP2 (Porcine liver primary cell line)	133.4±3.9	4.3±0.2*	1.4 ± 0.1
Vero (fibroblast-like from African green monkey kidney cell line)	196.3±11.8	2.1±0.2*	1.41±0.06

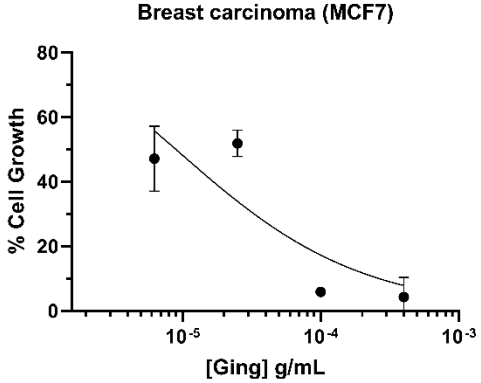
GI_{50} (µg/mL) values, corresponding to the concentration of extract that inhibited 50% of cell growth. nd - not detected. In each row, an asterisk means statistically significant differences between the two samples with a p-value<0.05

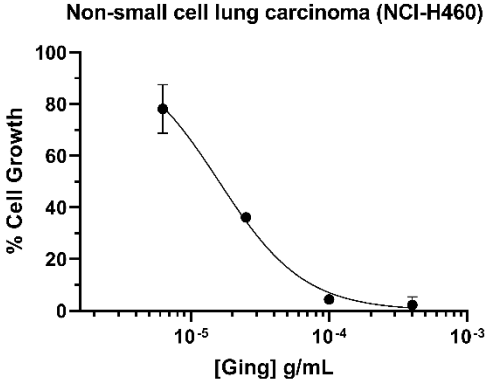
The analysis of (**Figure 23)(A)** shows that for Vero, the percentage of inhibition remains at 100% even though the concentration *of Z. officinale* is increased, then drops suddenly to 0% when the concentration reaches $10^{-4.5}$ log (concentration, µg/ml), and for all the other cell lines the percentage of inhibition decreases as the concentration of *Z. officinale* increases. For *B .dioica* **(B)** all the cell line shows the same graph in which the percentage of inhibition decreases as the concentration of the extract increases with the exception of PLP2 cell line in which the percentage of inhibition remains at 100% then drops to 0% when the concentration of the extract reaches 10^{-4} log (concentration, µg/ml), this indicates that the dose response varies depending on the cell type.

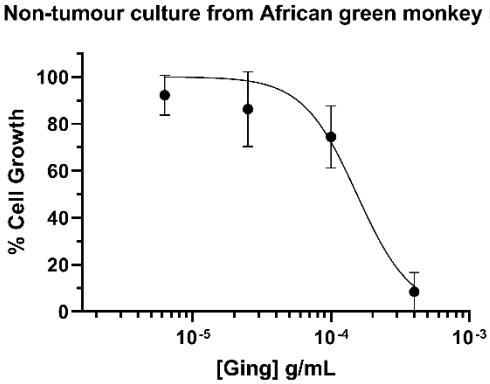
(A)


Gastric adenocarcinoma (AGS)
% Cell Growth
100
80
60
40
20
0
10-5
10-4
10-3
[Ging] g/mL


Colorectal adenocarcinoma (Caco-2)
% Cell Growth
100
80
60
40
20
0
10-5
10-4
10-3
[Ging] g/mL


Breast carcinoma (MCF7)
% Cell Growth
80
60
40
20
0
10-5
10-4
10-3
[Ging] g/mL


Non-small cell lung carcinoma (NCI-H460)
% Cell Growth
100
80
60
40
20
0
10-5
10-4
10-3
[Ging] g/mL


Non-tumour culture from African green monkey (Vero)
% Cell Growth
100
80
60
40
20
0
10-5
10-4
10-3
[Ging] g/mL

(B)

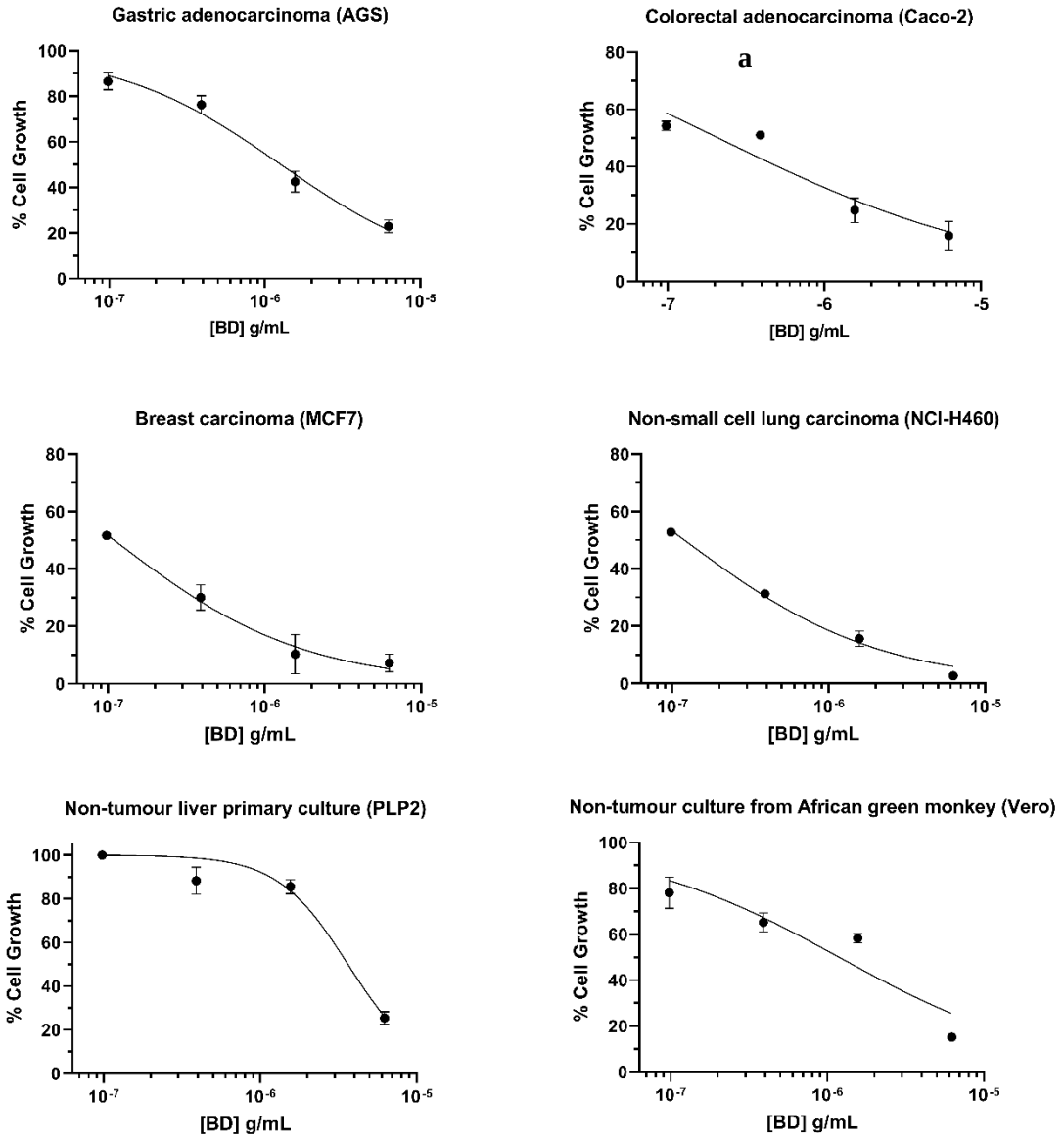


Figure 23: Dose-response curve for *Z. officinale* (A)and *B. dioica* (B) hydroethanolic extract.

4.2.3. *i*NOS activity

NO is a signalling molecule that plays an important role in inflammatory pathogenesis. Under normal physiological conditions, it has an anti-inflammatory effect. NO, on the other hand, is a pro-inflammatory mediator that causes inflammation when produced in excess in irregular circumstances. Both *Z. officinale* and *B. dioica* have anti-inflammatory properties, but we need to know at what concentrations they prevent the synthesis of oxide nitric enzyme to further understand how these two samples function.

Table 8 indicates that *Z. officinale* has a higher IC_{50} value (equal to 101.68±7.59µg/mL)than *B. dioica* (IC_{50} equal to 42.17±0.55µg/mL), assuming that *B.dioica* has a higher NOS activity and inhibits the synthesis of nitric oxide more efficiently.

A study made by Nobuko Imanishiat al[122]showed that *Z. offifinale* has the ability to cause the expression of macNOS mRNA and NO development by itself. According to the findings of the dose-dependent assay, 100 µg/ml of *Z. offifinale* was necessary for induction (the inducible effects of 10 µg/ml and 1 µg/ml were small and negligible, respectively). Once again, no studies were found in the literature to evaluate the NOS activity of *B. dioica.*

Table 8: *i*NOS activity (IC_{50}; µg/mL) of *Z. officinale* and *B. dioica*.

	Z. officinale	***B. dioica***
iNOS activity	**101.68±7.59**	**42.17±0.55***

IC_{50} (µg/mL) values, corresponding to the concentration of extract that inhibited 50% of iNOS activity.In each row, an asterisk means statistically significant differences between the two samples with a p-value<0.05

4.2.4. Cellular antioxidant activity (CAA)

The antioxidant activity of *Z. officinale* and *B. dioica* was measured using a cellular antioxidant activity (CAA) assay since this test is biologically relevant when compared to the common chemical antioxidant activity analysis, as it covers some aspects of cell consumption, metabolism, and location of antioxidant compounds.

Table 9 indicates that *Z. officinale* has a low percentage of oxidation inhibition at the maximum concentration of 1000 µg/mL equal to 27±3% indicating that we can't calculate the IC_{50} values since this percentage did not rich 50%. B. dioica has a high percentage of oxidation inhibition at the maximum concentration of 1000 µg/mL equal to 85±4 % so we can say that it has a good antioxidant activity .When compared to quercetin (positive control), both samples have a lower efficiency, which is attributed to the fact that quercetin is a synthetic and pure compound, whereas these samples are mixtures of molecules that may not always have a synergistic effect or a high bioactivity. *B. dioica* has an IC_{50} equal to 65418 µg/mL and quercetin (IC_{50} equal to = 0.08 µg/mL), these

findings show that Quercetin has the highest antioxidant activity, followed by *B. dioica* and *Z. officinale*.

Other *in vivo* study performed by Qian-Qian Mao et al123evaluated the antioxidant activity of *Z. officinale* using C57BL6/J mice with50 mg/mL dose. The authors found that the extract of *Z. officinale* has a strong antioxidant activity and induce the inhibition of TNF-α production, Akt activation, and NF-κB activation.

Until now no study has been performed to evaluate the antioxidant activity of *B. dioica.*

Table 9: Cellular antioxidant activity of *Z. officinale* and *B. dioica*

	Z. officinale	***B. dioica***
% oxidation inhibition at the maximum concentration of 1000 µg/Ml	27±3	85±4*
IC_{50} value (µg/mL)	**>1000**	**654±18*1**

Quercetin (positive control): % oxidation inhibition at 0,3 ug/ml inhibits 95 %; GI_{50} = 0.08µg/mL.nd - not detected. In each row, an asterisk means statistically significant differences between the two samples with a p-value<0.05

4.2.5. Cell cycle Analysis

Cell cycle analysis is a very common flow cytometry application. A DNA profile can be determined using a DNA-specific stain, such as finding the percentage of the population in G0/G1, S, and G2/M. This data may be used to monitor the effectiveness of an anticancer drug, for example.

The comparison of the results of *Z. officinale* and the control test (cells without treatment) as seen in **table 10** shows that there is a slight difference in the G0/G1 phase (60.3% for *Z. officinale* vs 53.1% for control test) and in S phase (7.7% for *Z. officinale* vs 8.7% for control test).This difference is not significant, the G2 phase is the same for both (20.1%), thus, *Z. officinale* don't have a significant effect on all the phases of the cell cycle. The analysis of *B. dioica* data shows that there is no significant difference between the S and G2 phases as compared to the control test 9.6 % vs 8.7 % for S and 21.9 % vs 20.1 % for G2 for *B. dioica* and control test consecutively. The percentage of the cells in G0/G1 phase for *B. dioica* was lower than it was in thecontrol test 22.4% vs 53.1%, indicating that *B. dioica* has an effect on the cell cycle by suppressing the G0/G1 phase.

According to the published literature, no research has been done to examine if *Z. officinale* or *B. dioica* interfere with the cell cycle, hence this approach is important and usefull.

Table 10: Cell cycle analysis of *Z. officinale*, *B. dioica* and control test

Sample	**control test**	***Z. officinale***	***B. dioica***
G0/G1 (%)	53.1	60.3	22.4
S (%)	8.7	7.7	9.6
G2 (%)	20.1	20.1	21.9

Figure 24 confirms that the cell cycle profiles of *Z. officinale* (b) and control test (a) are likely the same, but there is a significant difference between them and *B. dioica* (c) in the G0/G1 phase, demonstrating that *B. dioica* has a G0/G1 phase suppressing effect as discussed previously.

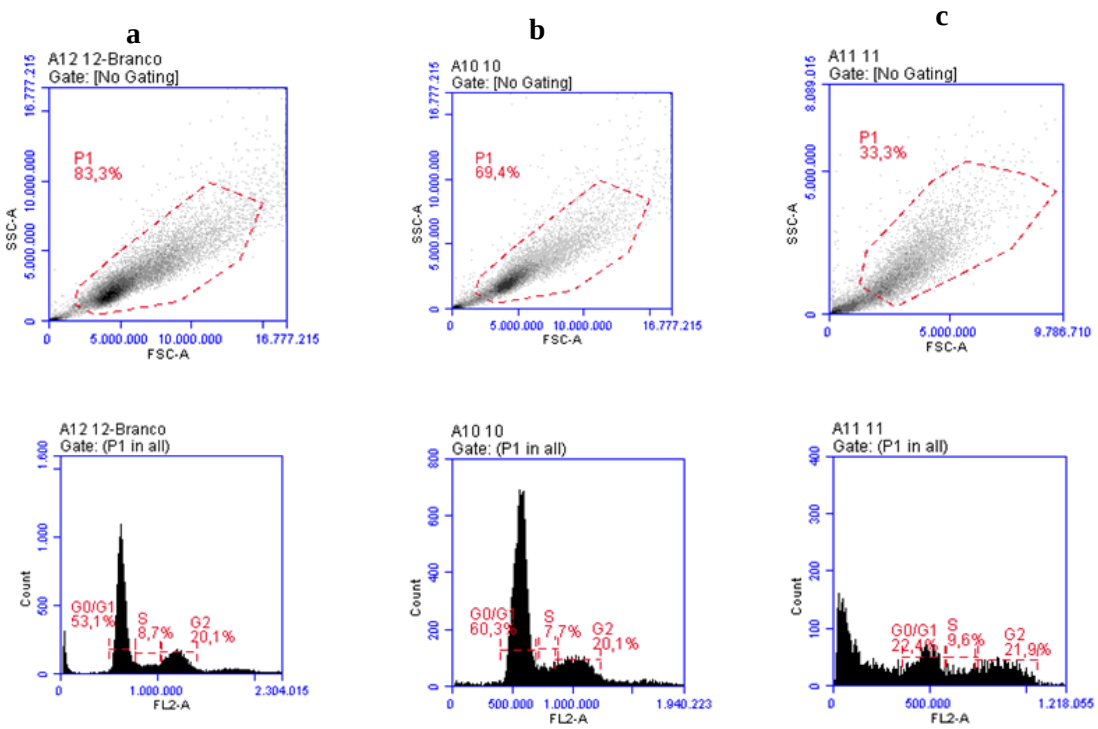


Figure 24: Cell cycle profile of the different samples: (a) control test, (b) *Z. officinale* and (c) *B. dioica*

4.2.6 Apoptosis

Flow cytometry is an important technique for detecting and quantifying the level of apoptosis in a population of cells at static points or over time. The apoptotic effect of *Z. officinale* and *B. dioica* was studied as seen in **Table 11** to better understand the mechanism of action of both samples.

The analysis of *Z. officinale* results shows that it has a lower percentage of living cells than the control test, 46.1% vs 63.6% but the percentage of apoptotic and dead cells was higher in *Z. officinale* than it was in living cells; 7.1% vs 6.6% for apoptotic cells and 39.3% vs 26.2% for dead cells respectively, suggesting that *Z. officinale* causes the death of the cells by apoptosis.

These results are conform with the study of Jennifer M. Rhode et al. [124]. These authors studied the effect of *Z. officinale* on the ovarian cancer cells and they found that it causes cell death in ovarian cancer cells via apoptosis properties through p53 pathway as well as autophagy induction.

The comparison of *B. dioica* to the control test shows that there is no significant difference; for live cells, *B. dioica* has 66.4 %, while the Blanc has 63.6 %.For apoptotic cells, *B. dioica* has 6.8 %, while the control test has 6.6 %and for dead cells, *B. dioica* has 22.8 % vs 26.2 % for *B. dioica* and the control test, respectively.

The apoptotic effect of *B. dioica* not evaluated yet by other published studies.

Table 11: Apoptotic effect of *Z. officinale* , *B. dioica*, and control test

Samples	**control test**	***Z. officinale***	***B. dioica***
Live cells (%)	63.6	46.1	66.4
Apoptoticcells (%)	6.6	7.1	6.8
Deadcells (%)	26.2	39.3	22.8

Figure 25 shows that the apoptotic profiles of *B. dioica* (c) and control test (a) are most definitely the same, although there is a substantial variation between them and (b), showing that *Z. officinale* has an apoptotic impact as previously mentioned.

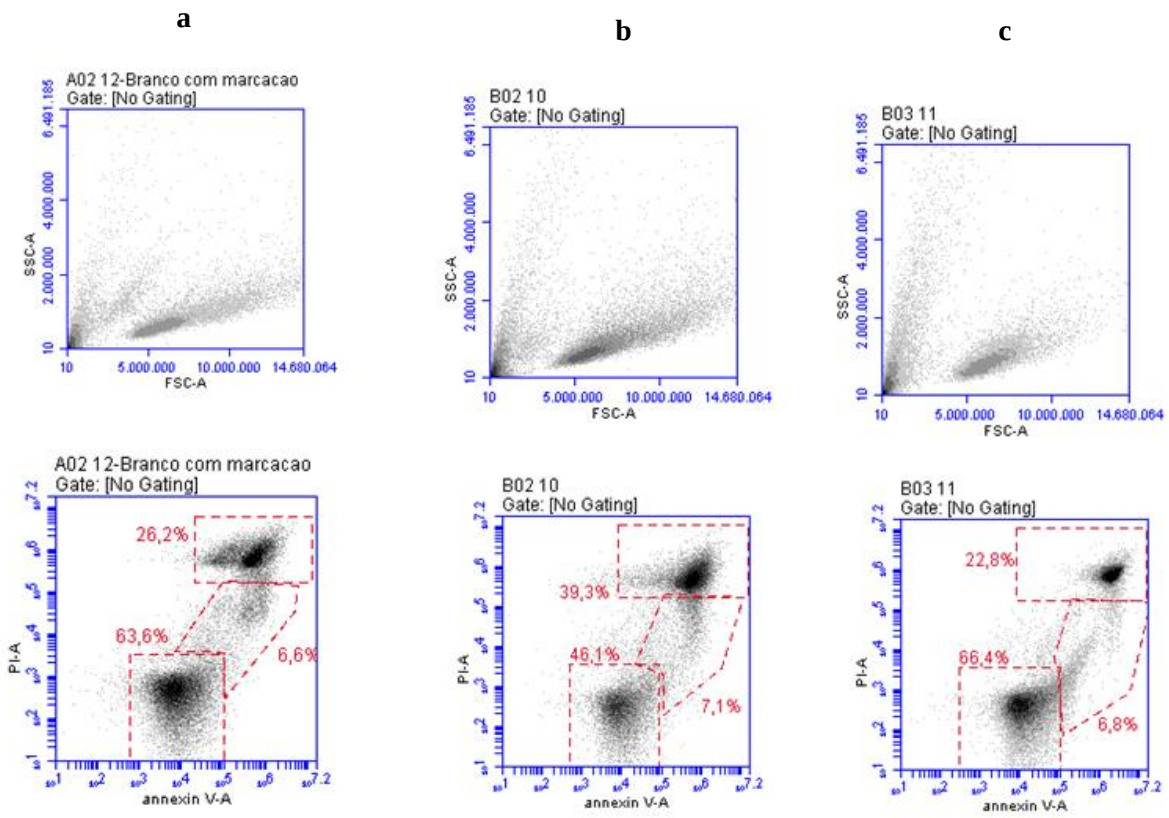


Figure 25: Apoptotic effect of (a) control test, (b) *Z. officinale*, (c) *B. dioica.*

5. Conclusions

Inflammation is a protective mechanism intended to remove bacteria or irritants to protect living tissues from infection and damage, and to potentiate tissue repair.

Inflammatory processes have a direct relationship with serious diseases such as atherosclerosis and Alzheimer's disease.

Different types of synthetic substances are used in the treatment of inflammation but unfortunately, they have side effects; that's why there is a crucial need to find natural alternatives with higher efficiency and lower side effects. Some natural substances have already been used, such as Omega-3 EFAs (fish oil), white willow bark, green tea among others, thus, there is a crucial need to develop new strategies to evaluate the activity of these natural substances.

The research study presented focused on evaluating the chemical characterization and bioactive properties of *B. dioica* and *Z. officinale*, two plants noted for their potential anti-inflammatory properties.

Both samples have revealed the presence of organic acids; however, *B. dioica* has a higher overall organic acids quantity than *Z. officinale*. *B. dioica* contained oxalic, citric, and fumaric acids, with citric acid being the most abundant. *Z. officinale* contained oxalic, malic, citric, and fumaric acids, with oxalic acid being the most abundant. All these organic acids are known for their potential importance in a lot of physiological process.

Four phenolic compounds were found in the analysis of *Z. officinale* composition, nevertheless it was not possible to identify the compounds present in *B. dioica* until this moment, being this analysis ongoing.

The anti-inflammatory results indicate that both samples and their combination have interesting anti-inflammatory activity, yet *B. dioica* has the most impressive findings because its EC_{50} value is lower than that of dexamethasone, which is used to treat a variety of anti-inflammatory diseases, so this discovery may have a major impact on the pharmacology field.

The cytotoxic activity assay revealed that both samples were less active against normal cell lines than cancer cell lines; this property is critical because it allows targeting cancer cell lines while avoiding harming normal cell lines simply by lowering the samples concentration. *Z. officinale* had a strong cytotoxic effect, and it was particularly effective against NCI-H460. *B. dioica* exhibited an intriguing cytotoxic activity that was

much higher than that of ellipticine (a drug used to treat a variety of tumors) and it was more potent against MCF-7 and NCI-H460.

The *i*NOS activity results show that *B. dioica* has a higher *i*Nos activity than *Z. officinale*, indicating that it reduces nitric oxide synthesis more effectively.

The CAA data revealed that *B. dioica* has a higher percentage of oxidation inhibition and antioxidant activity (lowest GI_{50}) than *Z. officinale*. However, their activity was lower than quercetin, a positive control and known for its potential antioxidant activity.

Only *B. dioica* interferes with the cell cycle by suppressing the G0/G1 process, according to cell cycle experiment.

The apoptosis analysis reveals that only *Z. officinale* causes cell death by apoptosis.

The findings are fascinating and have the potential to benefit a variety of fields, including pharmacology, industry, chemistry, food, and so on.

Z. officinale has been used as a potential plant in multiple fields, but *B. dioica* is a new finding in this study that needs to be more exploited.

After this work's examination, four fundamental perspectives will be established: *i)* complete the chemical characterization of both samples, *ii)* examinate the anti-inflammatory activity using other methods and optimize them, *iii)* Repeat all these tests *in vivo* to corroborate the *in vitro* data and to ensure their safety,*iv)*manufacturing of a topical cream containing *B. dioica* and *Z. officinale* extracts that may be applied directly to the site of inflammation.

Overall, both samples revealed their promising anti-inflammatory potential. Is also relevant to state that these studies were the first reports on the chemical characterization and bioactive properties of *B. dioica*, since there are no reports on the literature describing these data.

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