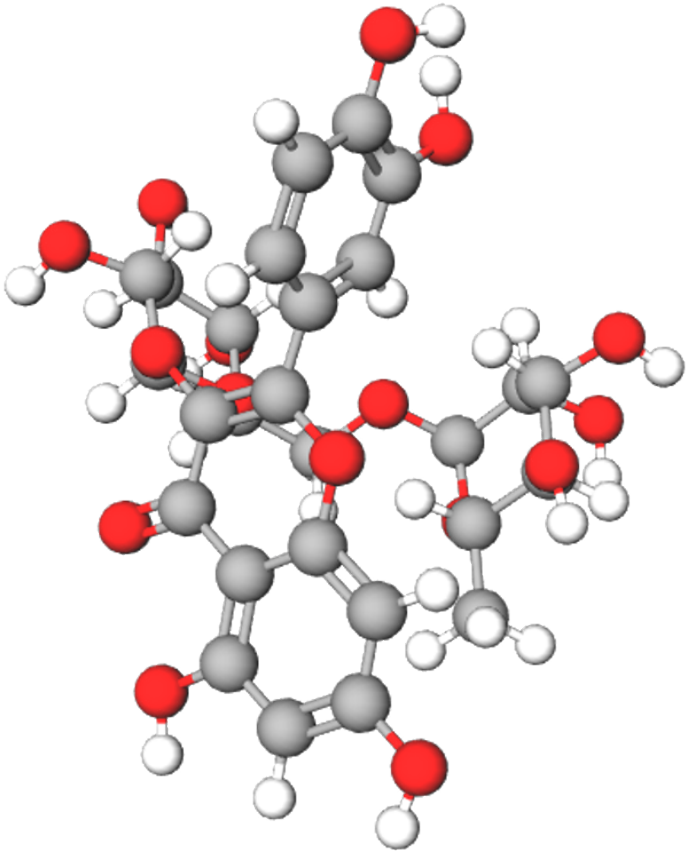

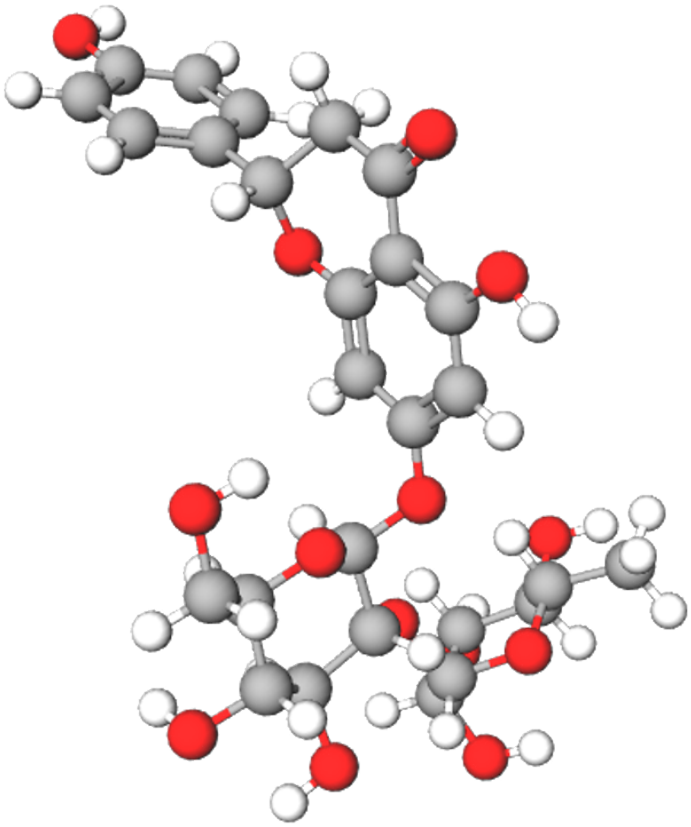

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Dissertação de Mestrado em Química Farmacêutica Industrial, orientada pela Professora Doutora Maria Manuel Cruz Silva e pela Professora Doutora Maria Teresa Teixeira Cruz Rosete e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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Abbreviations

^{13}C NMR- nuclear magnetic resonance of carbon 13

^{1}H NMR- nuclear magnetic resonance of proton

AChE- acetylcholinesterase

ATP- adenosine triphosphate

CALB- *Candida antarctica* lipase B

COX- cyclooxygenase

Cu- copper

CYP- cytochrome P-450

DMSO- Dimethyl sulfoxide

DNA- deoxyribonucleic acid

EC- enzyme commission

EGFR- epidermal growth factor receptor

FAD- flavin adenine dinucleotide

FMN- flavin mononucleotide

HDL- high-density lipoprotein

HIF1-α- hypoxia-inducible factor-1α

ICAM- intercellular adhesion molecule

IFN- interferon

IL- interleukin

iNOS- inducible nitric oxide synthase

JAK-2- Janus kinase 2

Log P- partition coefficient

LPS- lipopolysaccharide

LT- leukotriene

MAPK- mitogen activated protein kinase

NAD(P)- nicotinamide adenine dinucleotide phosphate oxidized

NAD(P)H- nicotinamide adenine dinucleotide phosphate reduced

NFAT- nuclear factor of activated T cells

NF-kB- nuclear factor-kappa B

NK- natural killer

NO- nitric oxide

Nrf2- nuclear factor erythroid 2-related factor

NSAIDs- non-steroidal anti-inflammatory drugs

PAGE- polyacrylamide gel electrophoresis

PG- prostaglandin

PPARs- peroxisome proliferator activated receptors

ppm- parts per million

PQQ- pyrroloquinoline quinone

PVDF- polyvinylidene difluoride

RNA- ribonucleic acid

ROS- reactive oxygen species

SAR- structure-activity relationships

SDS- sodium dodecyl sulphate

SEM- standard error of the mean

SNAP- S-nitroso-N- acetylpenicillamine

STAT- signal transducer and activator of transcription

TBS- tris-buffered saline

TLC- thin layer chromatography

TLR- toll-like receptor

TNF- tumour necrosis factor

UV- ultraviolet

VCAM- vascular cell adhesion molecule

VEGFR- vascular endothelial growth factor receptor

XO- xanthine oxidase

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Abstract

In the last years, epidemiological studies suggested that long term consumption of food rich in flavonoids is related to health benefits, such as prevention of diverse diseases including cancer and diabetes, along with cardiovascular and neurodegenerative diseases. Some of their protective effects have been associated to their anti-inflammatory activity. As a matter of fact, chronic inflammation is one of the leading causes of mortality in western world since it triggers multiple human pathologies, including diabetes, atherosclerosis, cancer and Alzheimer. Since the current anti-inflammatory drugs have several limitations and the abnormal production of pro-inflammatory mediators increases and sustains inflammation, compounds targeting their expression are good candidates for attenuating inflammatory diseases, for example chemicals targeting inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production. The low stability and solubility in lipophilic medium limit the practical application of flavonoids. Therefore, enzymatic synthesis of acylated derivatives of glycosylated flavonoids arises as an effective strategy to overcome this problem. Moreover, the use of chemical processes requires many protection/deprotection steps to obtain selective acylation, and the biocatalytic route, which is region- and stereoselective, is chosen as an effective method to perform such reactions and to improve their lipophilicity. Taking into account these assumptions, the present work aims to synthesize and characterize new acylated derivatives of the flavonoids rutin and naringin, catalysed by Novozym 435, with various vinyl esters (vinyl acetate, vinyl propionate, vinyl butyrate and vinyl cinnamate) as acyl donors and to disclose the potential anti-inflammatory effects of naringin derivatives using the *in vitro* model of inflammation lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. The results obtained suggest that the nature of the acyl donor can be a determining factor for the regioselectivity of the lipase and for the reaction yields. Moreover, the results show that the acylation of naringin enhanced its activity towards NO inhibition. Particularly, naringin 6"-butanoate (C4) was the most potent compound in inhibiting iNOS expression, followed by naringin 6"-propanoate (C3), leading to hypothesize that diacylated compounds or acylated compounds with long chain acyl donors can better interact with the cell membrane due to its increased lipophilicity, providing a mean of enhancing the internalization of naringin by cells, thus improving its anti-inflammatory effect.

Keywords: Rutin, naringin, biocatalysis, inflammation, glycosylated flavonoids, Novozym 435, acylated derivatives, iNOS, nitric oxide.

Resumo

Nos últimos anos, vários estudos epidemiológicos apontaram o consumo prolongado de alimentos ricos em flavonoides como um benefício para a saúde, no qual se inclui a prevenção de várias doenças, como o cancro e a diabetes, além das doenças cardiovasculares e neurodegenerativas. Alguns dos seus efeitos protetores têm sido associados à sua atividade anti-inflamatória. A inflamação crónica é, de facto, uma das principais causas de mortalidade no mundo ocidental, uma vez que desencadeia várias patologias humanas, incluindo a diabetes, aterosclerose, cancro e Alzheimer. Visto que os fármacos anti-inflamatórios atuais apresentam diversas limitações e que a produção anormal de mediadores pro-inflamatórios aumenta e sustenta a inflamação, compostos direcionados para a expressão destes últimos são bons candidatos para a atenuação de doenças inflamatórias, como por exemplo, substâncias químicas direcionadas à expressão da isoforma indutível da sintase do óxido nítrico (iNOS) e à produção de óxido nítrico (NO). A baixa estabilidade e solubilidade em meio lipofílico limitam a aplicação prática de flavonoides. Assim sendo, a síntese enzimática de derivados acilados de flavonoides glicosilados surge como uma estratégia eficaz para superar este problema. O uso de processos químicos requer muitas etapas de proteção/desproteção para obter acilação seletiva e, portanto, a via biocatalítica, que é regio e estereosseletiva, é escolhida como um método eficaz para a realização de tais reações e assim melhorar a sua lipofilicidade. Tendo em conta estas premissas, o presente trabalho visa sintetizar e caracterizar novos derivados acilados dos flavonoides rutina e naringina, utilizando a Novozym 435 como catalisador, com vários ésteres vinílicos (acetato de vinilo, propionato de vinilo, butirato de vinilo e cinamato de vinilo) como doadores de grupo acilo e também mostrar os potenciais efeitos anti-inflamatórios dos derivados de naringina, utilizando o modelo de inflamação *in vitro* de macrófagos RAW 264.7 estimulados com lipopolisacárido (LPS). Os resultados obtidos sugerem que a natureza do doador do grupo acilo pode ser um fator determinante para a regiosseletividade da lipase e para o rendimento da reação. Além disto, os resultados mostram que a acilação da naringina aumentou a sua atividade no que diz respeito à inibição do NO. Particularmente, a naringina 6"-butanoato (C4) foi o composto mais potente no que diz respeito à inibição da expressão de iNOS, seguido por naringina 6"-propanoato (C3), levando a supor que compostos diacilados ou compostos acilados com doadores de cadeia longa podem interagir melhor com a membrana celular devido ao aumento do seu carácter lipofílico, aumentando a internalização da naringina pelas células e melhorando assim o seu efeito anti-inflamatório.

Palavras-chave: Rutina, naringina, biocatálise, inflamação, flavonoides glicosilados, Novozym 435, derivados acilados, iNOS, óxido nítrico.

Chapter 1

Introduction

I. Introduction

1. Inflammation

Inflammation is a complex immunological process through which the body fights infection or injury. It is a crucial response for animals and in which there is an interaction with molecular mediators even the functions of immune cells, through a response that occurs at all biological levels (Allavena *et al.*, 2008).

The ancients characterized inflammation taking into account five cardinal signs, namely *rubor* (redness), *calor* (warmth), *tumor* (swelling), *dolor* (pain) and *functio laesa* (loss of function). The first four signs were named by Aulus Celsus in the ancient Rome in the first century AD (Medzhitov, 2010). The sensation of warmth is caused by the increase of blood influx through dilated vessels which causes the increase of redness (increased volume of erythrocytes). The swelling results from the amplified movement of fluid from dilated blood vessels and infiltration of cells into the damaged area. Pain is caused by the stimulation of sensory nerves due to the oedema and the effect of mediators, either from initial damage or from the inflammatory response itself. The loss of function is due to either the loss of mobility in a joint, caused by swelling and pain, or to the replacement with scar tissue.

Although, in ancient times inflammation was recognized as being part of the healing process, nowadays inflammation is considered an undesirable response and a keystone of pathology in which the changes observed are indicative of injury and disease. Today it is recognized that inflammation is far more complex, governed by different mechanisms of induction, regulation and resolution.

Inflammatory conditions range from acute inflammatory reactions in response to wounds and infections to chronic inflammatory states resulting in remodelling of the artery wall in atherosclerosis, the bronchial wall in asthma, neurodegenerative diseases and cancer. Inflammation begin as a protective response to challenges with various sources that include pathogens and foreign bodies, danger signals, radiation and toxic chemicals, autoimmune and chronic diseases, obesity, consumption of alcohol and tobacco.

1.1. Inflammatory responses

There are two inflammation stages, acute and chronic inflammation. Acute inflammation is an initial stage of inflammation to maintain homeostasis, through the activation of the immune system. This type of inflammation persists only for a short time in order to defend the host. If this inflammation lasts longer, it becomes harmful to the tissue and chronic inflammation sets in, which may predispose the host to various chronic inflammatory diseases.

In the case of sterile tissue injury in the absence of infection, cellular events of acute inflammation are temporally activated. The tissue damage is detected by leukocytes and by pain receptors (nociceptors) that causes pain sensation in the affected area (Basbaum *et al.*, 2009). It starts with an increase on protein exudation and then there is an accumulation of polymorphonuclear leukocytes in the inflamed tissue. This neutrophil infiltration is followed by recruitment of macrophages and mast cells into the tissues at the time of injury (Majno & Joris, 2004). Polymorphonuclear neutrophil extravasation requires adhesion to endothelial cells in the capillaries, which is mediated by selectins and integrins on neutrophils and by intracellular adhesion molecules and vascular cell adhesion on endothelial cells (Darveau, 2010). Selectins allow the passage of neutrophils along the capillaries and integrins allow firm attachment, conducting to transcellular diapedesis to the tissue. Then, neutrophils neutralize pathogens and promote the clearance of cellular and other debris by phagocytosis. The second stage is the programmed cell death of neutrophils via apoptosis, whose accumulation resulted before on a second wave of cellular infiltration of monocytes. There is a differentiation of monocytes into macrophages to remove the apoptotic neutrophils and the remains, which results on the beginning of tissue repair at the affected site (Serhan & Savill, 2005). Macrophages are then cleared either by egression to the lymphatic system or by apoptosis. The acute inflammation is usually finished once the triggering injury is eliminated, the infection is removed and the damaged tissue is repaired.

If the inflammatory trigger is not eliminated by the acute inflammatory response or persists for any other reason, that becomes harmful to the tissue and consequently develops into a chronic lesion. These lesions can be caused by chronic infections, unrepaired tissue damage, persistent allergens or foreign particles that cannot be digested. Chronic inflammation is usually located to the site with the inflammatory trigger and often results in different types of local tissue remodelling. For instance, persistent infections can lead to the formation of granulomas and the generation of tertiary lymphoid organs. In addition, inflammation induced

by allergens can result in respiratory epithelial tissue remodelling leading to asthma (Medzhitov, 2010).

A growing number of chronic inflammatory conditions has been described in situations that do not involve infection or tissue damage, in which many human inflammatory pathologies are included, such as obesity and type 2 diabetes, arthritis, cancers, atherosclerosis and neurodegenerative diseases. In these cases, it seems to be vicious cycles between inflammation and the pathological process. Accordingly, obesity can cause inflammation, whereas chronic inflammation can lead to obesity-associated diabetes and this process is a positive feedback, present in other chronic inflammatory diseases.

The study of inflammation in atherosclerosis has provided important new findings into the mechanisms underlying the recruitment of leukocytes (Libby, Ridker & Maseri, 2002) and in addition, studies have also showed the role of inflammation in Alzheimer's disease (Schott & Revesz, 2013). In this last illness the inflammatory components consist of microglia and astrocytes, the complement system, cytokines and chemokines (Heneka *et al.*, 2015). Cutaneous wound repair is a strongly regulated inflammatory process including blood coagulation, formation of new tissue and tissue remodelling, that are part of the innate host protection mechanism (Bielefeld, Amini-Nik & Alman, 2013). If thrombin, which is a protease that plays an important role in blood coagulation, is deregulated, that can lead to complications ranging from subtle subclinical to serious life-threatening coagulopathies (Popović *et al.*, 2012).

1.2. Immunity

The immune system has two lines of defence, innate immunity and adaptative immunity. Innate immunity is the body´s first line of defence and its function is the recruitment of immune cells to sites of infection through the production of cytokines (proteins involved in cell signalling). This production leads to the release of antibodies and other proteins that activates the complement system, a biochemical cascade to identify and make the foreign antigens more susceptible to phagocytosis. The innate immunity also promotes clearance of cell debris or antibody complexes to remove foreign substances (Turvey & Broide, 2010).

Innate immunity is triggered when a large sensor protein expressed in innate immunity cells, the inflammasome, recognizes diverse noxious signals and induces the release of pro-inflammatory cytokines (Rodríguez-Hernández *et al.*, 2013). In innate immune response,

macrophages and neutrophils, basophils, eosinophils, dendritic cells, mast cells and natural killer cells are involved. Macrophages and neutrophils have the function of engulf microbes. In addition, neutrophils contain granules to assist in the elimination of pathogenic microbes. Macrophages are also involved in antigen presentation to T cells and synthesize sensor proteins known as toll-like receptors (TLRs) that activate various responses, such as the activation of NK cells, nuclear factor-kappa B (NF-kB), and cytokines (Tian & Brasier, 2003). Dendritic cells act as important messengers between innate and adaptative immunity. Mast cells and basophils are involved in the initiation of acute inflammatory responses, such as allergy and asthma. Mast cells reside in connective tissue and basophils locate in the circulation. Eosinophils release several mediators to destroy parasites that are too large to be phagocytosed and they also control the inflammation associated to asthma and allergy. NK cells play an important role in the elimination of tumours and in the destruction of cells infected by viruses. This destruction is accomplished through the induction of apoptosis, a programmed cell death, by NK cells (Turvey & Broide, 2010).

Innate immunity has a limited durability and potency and when it is overloaded it can triggers the adaptative immunity through a process known as antigen presentation.

The adaptive immune response is activated after hours or days and works simultaneously with innate immunity to combat infection or injury. The mechanisms of adaptive immunity, also named as acquired immunity, are involved in highly specialized responses to specific antigens. The adaptive immune response has multiple functions, including the recognition of specific "non-self" antigens in the presence of "self" antigens, the production of specific immunologic effector pathways that eliminate specific pathogens or infected cells and the development of an immunologic memory to recognize and react to repeated exposures to a specific antigen (Bonilla & Oettgen, 2010).

Cells of the adaptive immune system include the T lymphocytes, generated in the bone marrow and thymus and usually accumulated in the spleen and lymph nodes, which are activated through the action of antigen presenting cells, and the B lymphocytes, produced by bone marrow, which create antibodies in response to the presence of antigens. Antibody-mediated immunity is mediated by B cells antibody production. The pathway of this immunity begins when the B cell's receptor recognizes and binds to antigen in its natural form, which then attracts the assistance of T helper cells. These cells release cytokines that help the B cell multiply and mature into antibodies. The secreted antibodies bind to antigens, indicating them for destruction through neutralization, classical complement activation, promotion of

phagocytosis and pathogen elimination. Then, the antigen-antibody complexes are cleared (Bonilla & Oettgen, 2010).

1.3. Mediators of inflammation

The cells and mediators present in inflammation depend on a wide range of factors, that include the stage of inflammation, the type of trigger, i.e. pathogen, autoimmune, danger signal, chemical or physical injury, the tissue involved and the type of inflammation (Punchard, Whelan & Adcock, 2004). The microenvironment in an inflammatory response is associated with cellular trans-differentiation, migration, proliferation, survival and extracellular matrix formation.

During the inflammatory response, mast cells and leukocytes are recruited to the site of injury, which leads to an increase in the oxygen uptake and consequently, to an increased release and accumulation of reactive oxygen species (ROS) (Hussain *et al.*, 2016). Bacterial pathogens are detected by sensors of the innate immune system, such as TLRs, that are expressed on sentinel cells, including tissue-resident macrophages, dendritic cells and mast cells. An outsider inflammatory mediator is the molecule called endotoxin, or lipopolysaccharide (LPS), which is present in the outer covering of some types of bacteria and binds to human and mouse TLR4 (Newton & Dixit, 2012). The activation of these pathogen-specific receptors induces the production of soluble mediators, such as metabolites of arachidonic acid, cytokines [tumour necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-10 (IL-10)] and chemokines [IL-8, CXC chemokine receptor 4 (CXCR4), CXC chemokine ligand 8 (CXCL8), CX3C chemokine ligand 1 (CX3CL1), CC chemokine ligand 2 (CCL2) and CCL3], which rapidly accelerate the inflammation progression by recruiting additional inflammatory cells and producing more reactive species. In contrast, the viral infection leads to the production of another class of cytokines, such as type 1 interferons (IFNs), with also the presence of cytotoxic lymphocytes. Parasitic infections and respiratory allergens induce the production of IL-4, IL-5, IL-13 and histamine (Medzhitov, 2010). In addition, during inflammation, many other mediators are present, such as nitric oxide (NO), adhesion molecules [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin] and lipid-derived eicosanoids [prostaglandin (PG) E2, PGI2, leukotriene (LT) B4, LTC4], that coordinate inflammatory response (Santangelo *et al.*, 2007) (***Figure 1***).

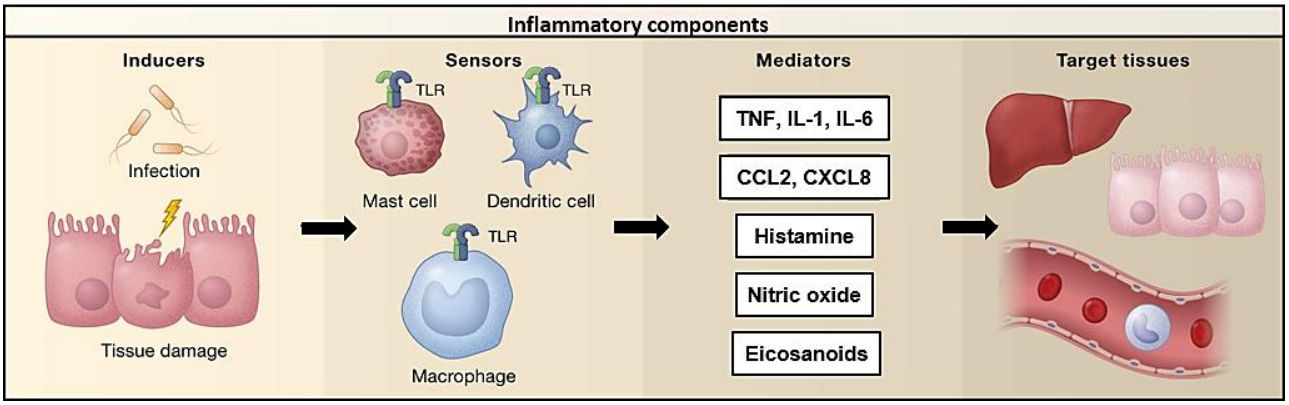

***Figure 1**- Inflammatory pathway components (Adapted from Medzhitov, 2010)*

The expression of these key mediators is regulated by changes in the activation of transcription factors, including NF-kB, activator protein (AP)-1, signal transducer and activator of transcription (STAT), peroxisome proliferator activated receptors (PPARs), nuclear factor erythroid 2-related factor (Nrf2), hypoxia-inducible factor-1α (HIF1-α) and nuclear factor of activated T cells (NFAT). NF-kB regulates the expression of cytokines and it is also activated by some cytokines, creating a feedback loop. During inflammation there is a decrease in oxygen and HIF1-α is activated to help immune cells survive longer. Furthermore, in lower oxygen levels conditions, HIF1-α induces transcription of genes that control processes such as angiogenesis and erythropoiesis. Depending on the type of inflammatory inducer, certain cytokines are triggered, activating a STAT protein, which either increases or decreases inflammation. The DNA-binding mode of these transcription factors is changed by signal transduction pathways, such as mitogen activated protein kinases (MAPKs), that regulate proliferation, differentiation, survival and death, phosphatidylinositol 3-kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (Akt) and ubiquitin-proteasome system.

Under normal physiological conditions, NO gives an anti-inflammatory effect, but in abnormal situations, it is considered a pro-inflammatory mediator due to its overproduction. When an inflammatory inducer triggers the production of cytokines, these induce the expression of inducible nitric oxide synthase (iNOS) that generates large amounts of NO over a period of days (Sharma, Al-Omran & Parvathy, 2007). Thus, the induction of iNOS and cyclooxygenase-2 (COX-2), that is selectively induced by pro-inflammatory cytokines, aberrant production of inflammatory mediators, as well as alterations in the expression of specific microRNAs, have been reported to play a key role in the inflammation. This continuous inflammatory/oxidative environment can damage healthy neighbouring epithelial

and stromal cells and over a long period of time may lead to multiple chronic diseases, such as diabetes, atherosclerosis, cancer and Alzheimer's disease.

1.4. Anti-inflammatory drugs

The knowledge about inflammation, including the cellular and molecular mechanisms behind the inflammatory response is crucial to disclose molecular targets able to be pharmacologically modulated and therefore to initiate drug development programs. There are two golden main approaches to manage the inflammation frame, the use of non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs or corticosteroids. More recently, other drugs have been used to control inflammation such as recombinant antibodies designed to neutralize specific pro-inflammatory mediators or disease modifying drugs, able to inhibit specific pathophysiological events. Indeed, the standard treatment for rheumatoid arthritis includes NSAIDs, such as aspirin, for pain and inflammation relief, and glucocorticoids or even disease modifying anti-rheumatic drugs to reduce other symptoms of the disease (Gaffo, Saag & Curtis, 2006).

NSAIDs are among the most widely used medications worldwide and the first ones, including aspirin and paracetamol, were found more than 100 years ago. Their mechanism of action was identified as inhibition of prostanoid production. The development of animal models of pain and inflammation led to the discovery of new NSAIDs, such as ibuprofen, ketoprofen, diclofenac and naproxen, in an attempt of sharing the therapeutic action of aspirin but without causing the main adverse event, namely gastric ulceration. However, they all generally showed similar therapeutic effects and side effects, i.e. the erosion of the gastric mucosa (Brune & Hinz, 2004).

NSAIDs inhibit prostanoids [prostaglandins (PGs) and thromboxane (TxA2)] biosynthesis through their activity on the COX system. The two COX isoforms (COX-1 and COX-2) have different functions and the inhibition of these enzymes results in different effects. COX-1 produces PGs and TxA2 that regulate gastrointestinal, renal, vascular and other physiological functions and COX-2 regulates the production of PGs involved in inflammation and pain. The finding that cyclooxygenase present in inflammatory lesions (COX-2) was distinct from that found in the stomach (COX-1) led to the development of selective COX-2 inhibitors, known as coxibs, which were designed to inhibit COX-2 while sparing COX-1 at therapeutic doses, having low gastrointestinal side effects. However, an alarming turn of events

took place in 2004 when rofecoxib was withdrawn from the market due to evidences of increased risk of myocardial infarction and other cardiovascular events, which led to concerns over the safety of coxibs (Brune & Patrignani, 2015).

Glucocorticoids, also called corticosteroids or glucocorticosteroids, are steroidal drugs that remain at the forefront of anti-inflammatory and immunosuppressive therapies. They are widely used in the treatment of inflammation, including rheumatoid arthritis, psoriasis, eczema, multiple sclerosis, leukaemia and in preventing a rejection to transplanted organs. Contrasting the non-steroidal drugs, these drugs do not relieve pain but decrease inflammation by inhibiting the synthesis of arachidonic acid and leukocytes activity (Coutinho & Chapman, 2011).

Cortisol is the main glucocorticosteroid, produced by adrenal cortex, that provides many physiological functions, including gluconeogenesis. In 1949, Hench *et al.* (1949) discovered that high levels of cortisol of adrenal cortex extracts exhibited an anti-inflammatory effect on rheumatoid arthritis. This discovery led to the use of cortisol to treat multiple inflammatory conditions and to the development of potent synthetic agents, including dexamethasone and prednisone.

The anti-inflammatory effects of glucocorticoids are mainly due to a reduction in the production or release of several inflammatory mediators, such as prostaglandins, and this results in suppression of vasodilation and increased vascular permeability, responsible for the cardinal signs of inflammation. They also inhibit certain aspects of leukocyte function, such as leukocyte migration into inflamed sites and phagocytosis among macrophages, as well as the number and activity of specific subsets of T lymphocytes. The anti-inflammatory activity of glucocorticoids starts with their binding to specific cytoplasmic receptors of targeted cells. The receptor-glucocorticoid complex undergoes translocation into the nucleus, where it binds to DNA and represses the transcription of many genes, as well as the activation of the pro-inflammatory transcription factor NF-kB. Multiple cellular functions are thereby modified and there is an inhibition in the production of pro-inflammatory cytokines and chemokines, cell adhesion molecules and important enzymes involved in the initiation and maintenance of host inflammatory response (Becker, 2013).

However, cortisol and synthetic glucocorticoids produce their therapeutic action at supra-physiological dosages, which result in a sobering list of adverse effects, including the

suppression of the hypothalamic-pituitary-adrenal axis that leads to adrenal atrophy, as well as the production of many other physiological responses (Becker, 2013) (***Figure 2***).

Some of these untoward effects can be avoided by administrating glucocorticoids topically, which led to the development of glucocorticoid containing creams. Nevertheless, applying this approach to the treatment of rheumatoid arthritis or other pro-inflammatory related pathologies is not feasible, so the patients should take intraarticular glucocorticoid formulations (Ericson-Neilsen & Kaye, 2014).

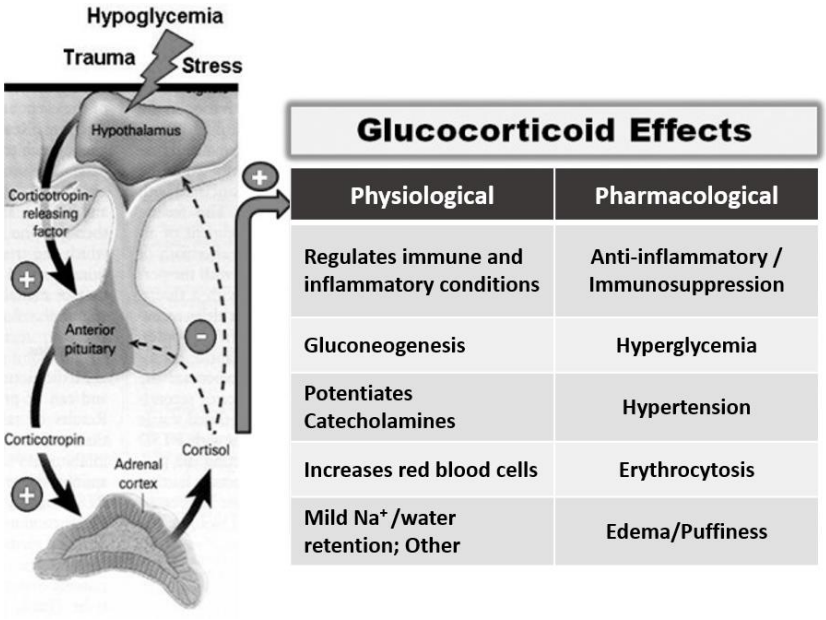

Figure 2*- Glucocorticoid effects (Adapted from Becker, 2013)*

Indeed, the current anti-inflammatory drugs have several limitations concerning effectiveness, safety and cost and it becomes imperative the search for new and safe anti-inflammatory agents, targeting the signal transduction pathways and mediators involved in inflammation.

2. Polyphenolic compounds

Polyphenolic compounds are secondary plant metabolites and are generally involved in defence against ultraviolet radiation or environment aggressions (Beckman, 2000). These natural substances are largely found in fruits, vegetables, cereals and beverages. Polyphenols may contribute to the bitterness, acidity, flavour, odour and oxidative stability of human diet.

To date, several thousands of polyphenolic compounds have been characterized. Their structures comprise several hydroxyl groups on aromatic rings. The variations around the basic chemical skeleton essentially relates to the degrees of oxidation, hydroxylation, methylation, glycosylation and the possible connections to other molecules, and its structure can range from simple molecules, such as phenolic acids, to highly polymerized compounds, like condensed tannins.

Polyphenolic compounds have many industrial applications, such as the use as natural colorants and preservatives for foods and in the production of paints, paper and cosmetic.

In the last years, epidemiological studies strongly suggested that long term consumption of diets rich in plant polyphenols is related to health benefits, such as protection against development of cancers, cardiovascular diseases, diabetes and neurodegenerative diseases (Arts & Hollman, 2005; Miranda *et al.*, 2016).

Polyphenols are the subject of extensive scientific research due to their beneficial effects on human health, namely their antioxidant action (Scalbert, Johnson e Saltmarsh, 2005; Zhang e Tsao, 2016). Plant polyphenols can scavenge free radicals and inactivate other pro-oxidants, due to their chemical structure.

Furthermore, polyphenols have long been assumed to provide health benefits in inflammation-associated chronic diseases (Francisco *et al.*, 2014; Nichols & Katiyar, 2010), including prevention of oxidative stress that activates a variety of inflammatory mediators (Hussain *et al.*, 2016).

2.1. Classes of polyphenolic compounds

More than 8000 polyphenols have been identified that arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid. They are classified into several classes based on the number of phenol rings that they contain and on the structural elements of these rings to one another. The main groups contain flavonoids, phenolic acids, tannins, stilbenes and lignans (D'Archivio *et al.*, 2007).

3. Flavonoids

Flavonoids are the most studied group of polyphenols and they have a C6-C3-C6 basic structure, consisting of two aromatic rings, A and B, bound together by three carbon atoms that form an oxygenated heterocycle, the C ring (***Figure 3***). The ring A derives from the acetate/malonate pathway and the ring B derives from phenylalanine through the shikimate pathway (Merken & Beecher, 2000).

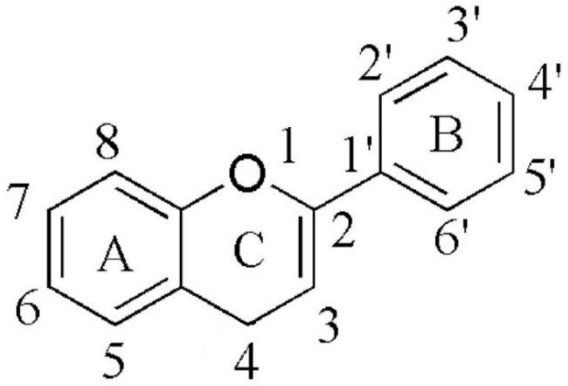

Figure 3*- Basic structure of flavonoids (Adapted from Wang, Li & Bi, 2018)*

Flavonoids can be classified into different subclasses, providing an extremely diverse variety of derivatives (Isoda *et al.*, 2014). They can be divided into six subclasses, i.e., flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones (***Figure 4***). Flavanones have the C ring saturated, while flavones have a double bond between positions 2 and 3. Isoflavones have the aromatic ring B attached to position 3 of the C ring and the flavonols have a hydroxyl group in position 3 of the C ring and a double bond between positions 2 and 3 of the C ring. These groups have in common a ketone in position 4 of the C ring. Additionally, flavanols have a hydroxyl group in position 3 of the C ring and anthocyanins are flavylium cations.

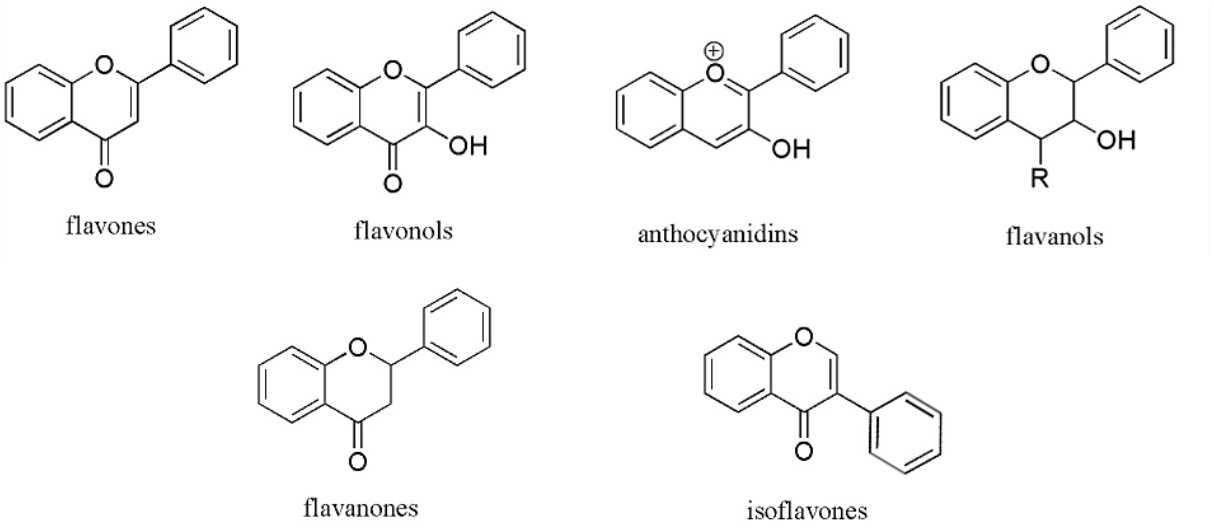

***Figure 4**- Chemical structures and subclasses of flavonoids (Adapted from Wang, Li & Bi, 2018)*

Inside each class of flavonoids, substitutions to rings A and B result into different compounds. The substitutions can be oxygenation, alkylation, glycosylation and acylation (Balasundram, Sundram & Samman, 2006).

3.1. Flavonoid glycosides

Among all polyphenols, flavonoids have received an increased attention due to their substantial biological benefits. Epidemiological studies have showed that the intake of natural flavonoids plays an important role in preventing modern diseases such as cancer (Xu *et al.*, 2016), diabetes (Grosso *et al.*, 2017; Xu *et al.*, 2018), inflammation (García-Lafuente *et al.*, 2009; Garcia-Larsen *et al.*, 2018) and obesity (Bertoia *et al.*, 2016; Vernarelli & Lambert, 2017).

The dietary flavonoids in nature exist as their glycosides, for instance glucoside, galactoside, rhamnoside, arabinoside and rutinoside (Oualid & M. S. Silva, 2012; Veitch & Grayer, 2011) and the most abundant are flavone *O*/*C*-glycosides and flavonol *O*-glycosides.

The biotransformation of flavonoid aglycone by glycosylation is considered of great interest due to the building-up of novel compounds with high solubility and metabolic stability, compared to their aglycones (Xiao, Muzashvili & Georgiev, 2014). Moreover, it is believed that flavonoid glycosylation, which is a common metabolic fate for majority of flavonoids, provides different benefits between *in vitro* and *in vivo* (Xiao, 2017).

In general, the first stage of metabolism of flavonoid glycosides is considered to be hydrolysis to its aglycone and after that they are metabolized by phase II enzymes in small intestine and then in the liver. If the flavonoid glycoside is not absorbed in the small intestine, it can be metabolized by microflora in the large intestine (***Figure 5***) (Xiao, 2017).

The absorption of flavonoid glycosides mainly depends on their permeability and they exhibit high hydrophilicity to diffuse across the cellular membrane. Therefore, in order to produce effects *in vivo*, the flavonoid glycosides need to be hydrolysed to their aglycones (Walle *et al.*, 2005), since these are more hydrophobic and can easily penetrate the cellular membrane through passive diffusion (Xiao, 2017).

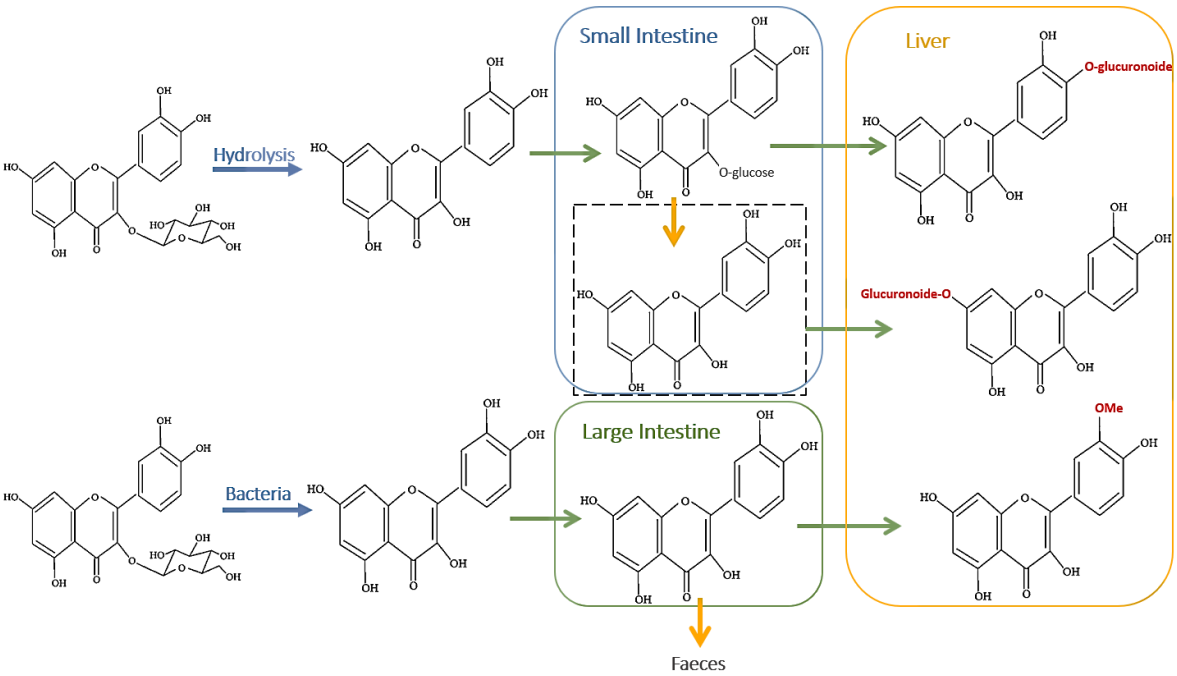

Figure 5- *Metabolism and absorption of flavonoid glycosides (adapted from Xiao, 2017)*

The antioxidant activity of dietary flavonoids depends on the number and location of the hydroxyl groups and this activity may also be influenced by their glycosides (Jeevitha *et al.*, 2016; Praveena *et al.*, 2014). When the antioxidant activity of luteolin is compared with its glycoside orientin, it is clearly observed that orientin exhibits a stronger antioxidant capacity than luteolin, due to the sugar substitution that decreases the negative charge on the oxygen atom at C- 3', increasing the ability of donating electrons (Praveena *et al.*, 2014).

There are some differences in the bioactivity of flavonoid glycosides between *in vitro* and *in vivo* studies. In cellular levels, the flavonoid glycosides display lower activity towards nitric oxide inhibition, iNOS expression and NF-kB activation, compared to their flavonoid aglycones (Choi *et al.*, 2012; Tokuyama-Nakai *et al.*, 2018). However, by oral treatment, flavonoid glycosides can display similar or even higher anti-inflammatory activity relatively to their aglycones (Xiao, 2017).

3.2. Rutin

Rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside, ***Figure 6***) is a flavonol found in several plant sources, such as passion flower, buckwheat, tea and apple. It is known as vitamin P and quercetin-3-*O*-rutinoside (Ghiasi, Taheri & Tafazzoli, 2010) and it is an important constituent of food and plant-based beverages (Gulpinar *et al.*, 2012). Rutin is a glycoside containing the aglycone quercetin along with disaccharide rutinose. The name 'rutin' comes from the plant *Ruta graveolens*. Rutin has shown to have multiple pharmacological activities, such as antioxidant (Azevedo *et al.*, 2013), cardioprotective (Wang *et al.*, 2018), anticarcinogenic (Dixit, 2014), neuroprotective (Javed *et al.*, 2012) and cytoprotective (Ugusman *et al.*, 2012).

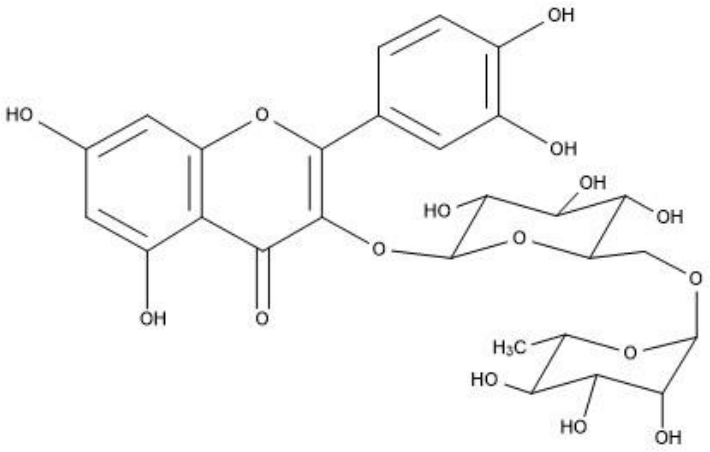

Figure 6- *Rutin*

Concerning the biosynthesis of rutin, it is important to emphasize that flavonoids constitute a diverse group of aromatic compounds derived from phenylalanine and malonyl-coenzyme. Chalcones derive from the condensation of three molecules of malonyl-CoA and a single molecule of 4-coumaryl-CoA, and they are converted to the flavanone naringenin by

chalcone isomerases, that forms the heterocyclic C ring. Flavanone 3-hydroxylase catalyses the hydroxylation of naringenin to dihydrokaempferol and it is further converted to flavonols (kaempferol and quercetin) through the flavonoid 3'-hydroxylase and flavonol synthase. These intermediates are modified by glycosyltransferases and rhamnosyl transferases to form diverse flavonoids, like rutin (Verhoeyen *et al.*, 2002) (***Figure 7***).

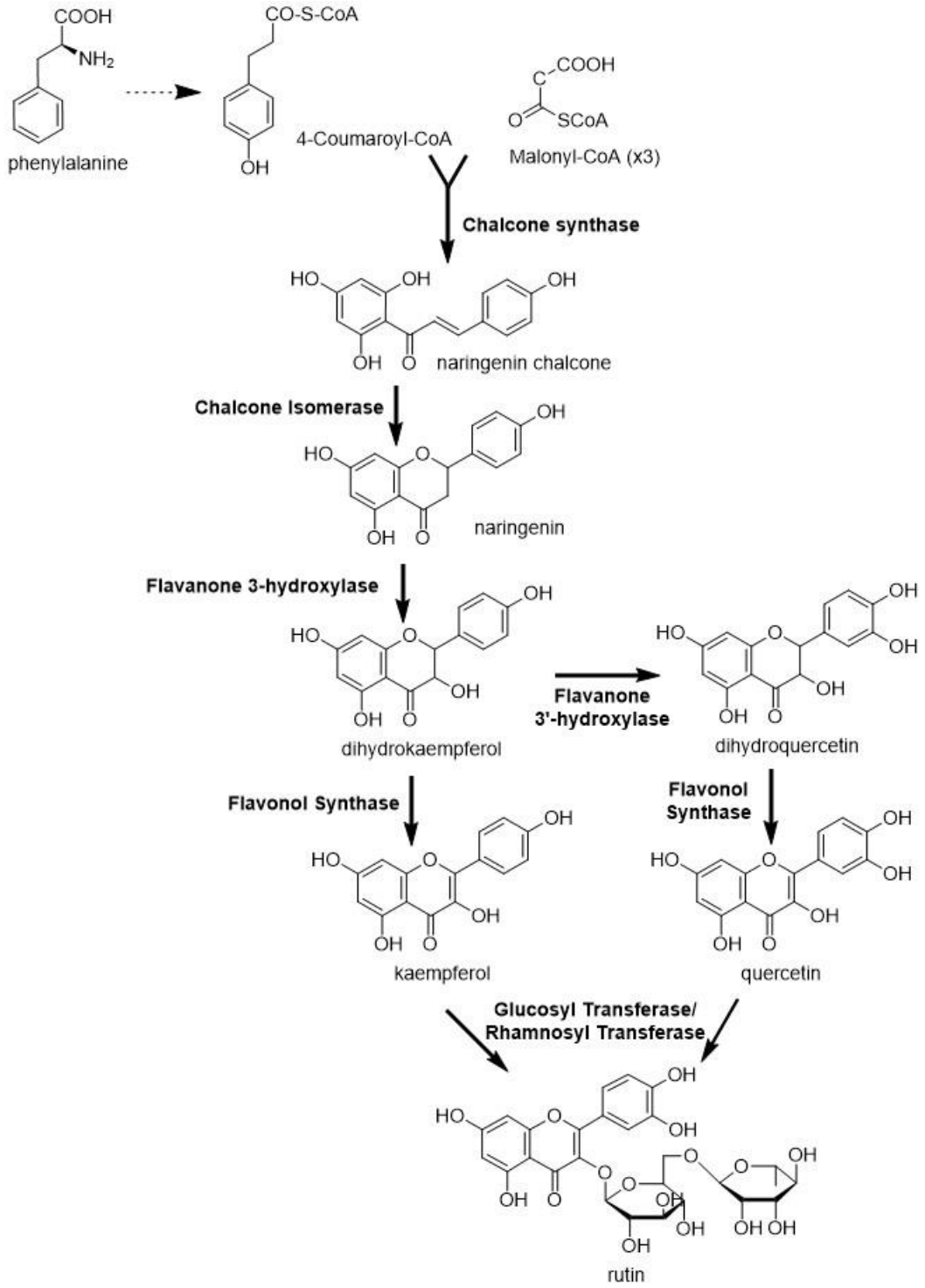

Figure 7*- Rutin biosynthetic pathway (Adapted from Verhoeyen et al., 2002)*

3.2.1. Pharmacokinetics and structure-activity relationships of rutin

The absorption of rutin depends mainly on its permeability and the main disadvantage associated to this flavonoid glycoside is its low bioavailability, caused by its limited membrane permeability and low aqueous solubility. Although it may show detectable bioactivity in multiple *in vitro* systems, the *in vivo* biological effects of rutin are delayed by its poor bioavailability (Gullón *et al.*, 2017).

The sugar moiety of the flavonoids may influence its retention time but also its absorption rates in the intestine. Rutin is absorbed more slowly than quercetin due to the digestion of microflora in the intestine (Carbonaro & Grant, 2005; Reinboth *et al.*, 2010). It is hydrolysed to quercetin by some bacterial populations with rhamnosidase activity and its hydrophilic moiety is removed, suggesting that glycosides containing a rhamnose moiety possibly will not be absorbed in the small intestine (Morand *et al.*, 2000). Therefore, rutin acts as a prodrug that deliver the bioactive quercetin to the damaged site, since it is absorbed as quercetin from the small intestine and is present in the form of the conjugate metabolites of quercetin (Crespy *et al.*, 2002; Scalbert *et al.*, 2002).

In a metabolic study, volunteers ingested rutin and the level of metabolite excretion was shown to be dependent on the colonic microflora of these volunteers. Rutin was converted into 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxytoluene and quercetin (Ganeshpurkar & Saluja, 2017; Jaganath *et al.*, 2006). Moreover, rutin is bound to plasma proteins and then conjugated to glucuronide, sulphate and methyl moieties (Schwedhelm *et al.*, 2003).

Regarding the structure-activity relationships (SAR) of rutin, it is known that the catechol group in the B ring, the double bond between C2 and C3 at the C ring and the ketone group in C4 at the C ring are essential for the inhibitory effect on angiotensin converting enzyme activity (Guerrero *et al.*, 2012). Comparing rutin with its aglycone quercetin, the glycosylation reduces the affinities for acetylcholinesterase by 1 to 5 times, depending on the forms of glycosides (Xie *et al.*, 2014) and greatly reduces the vascular relaxation effect in porcine coronary artery (Xu *et al.*, 2007). Moreover, the presence of a phenolic group in rutin contribute to scavenging free radicals from radiation, by adding hydrogen donation, and to inhibiting of oxidative stress (Patil, Mallaiah & Patil, 2013).

In a docking study, it was shown the molecular basis of interaction of rutin with immunomodulatory cytokines and NO. The interaction of rutin with TNF-α showed hydrogen

bonding with the backbone at Chain A and the sugar moiety also showed hydrogen bonding with Chain B. In addition, flavonol moiety showed π-amide interaction with Chain B and π-π interaction with Chain A. Regarding the docking with IL-1β, the polyphenolic flavonol nucleus of rutin, as well as the sugar moieties, exhibited hydrogen bond interaction with Arg-4, Phe-46, Gln-48 and Lys-103. The sugar unit affords quite strong interaction with three hydrogen bonds with Arg-4. The presence of such high number of hydrogen bonds makes rutin a potential inhibitor of IL-1β. Besides, the interaction of rutin with IL-6 revealed multiple hydrogen bonds with helix A and D. The interactions of rutin with NO, include hydrogen bridges with Ser-339, Trp-683, Val-685, Asp-601 and Hem-801, as well as π-π stacking with Trp-683, π-σ interaction with Met-341 and π-alkyl interaction with Met-341. This study also showed that, with rutin, the inhibitory constant for TNF-α was 25.62 μM, for IL-6 was 12.35 μM and for IL-1β was 61.42 μM, which partly explains the molecular mechanism of rutin to prevent inflammation (Ganeshpurkar & Saluja, 2018).

Rutin is known for its antioxidant capacity and this activity is enhanced by the polyhydroxylated substitutions, the 2,3-double bond combined with a 4-oxo group in the C ring and the 3-hydroxyl group (Wolfe & Liu, 2008).

3.2.2. Anti-inflammatory activity of rutin

Several mechanisms of action have been proposed to explain the anti-inflammatory activity of rutin, such as antioxidant and radical scavenging activities, the reduction of cellular activities of inflammation related cells (macrophages, lymphocytes), the modulation of pro-inflammatory enzyme activities, including COX and the nitric oxide producing enzyme, iNOS, and the modulation of other pro-inflammatory molecules, such as cytokines, and pro-inflammatory gene expression (Bispo da Silva *et al.*, 2017; Choi *et al.*, 2014; Imam *et al.*, 2017; Moutinho *et al.*, 2018; Nafees *et al.*, 2015; Song *et al.*, 2018).

In an inflammatory environment, macrophages produce an array of pro-inflammatory cytokines, including IL-1β and IL-6 and TNF-α, regulated by the activation of NF-kB and Nrf2, among other transcription factors. The release of these cytokines induces the expression of COX-2, which overexpression has been reported to play a role in inflammatory diseases. There is a correlation between TNF-α and both IL-1β and IL-6 (Vahabi *et al.*, 2011) and the expression of TNF-α and IL-1 causes the production of NO by iNOS, which is a pro-

inflammatory mediator due to its overproduction in chronic inflammatory conditions. It is known that rutin can inhibit these specific mediators in order to reduce inflammatory conditions.

Indeed, rutin (75 and 150 mg/kg per day, orally) demonstrated nephroprotective activity in a mouse model exposed to carbon tetrachloride by suppression of IL-1β and TNF-α and decrease of oxidative stress (Ma, Liu & Yang, 2018). Moreover, rutin have been exhibited neuroprotective effects *in vitro* and *in vivo*, for instance, in LPS-stimulated microglia (Bispo da Silva et al., 2017), spinal cord injury (Song et al., 2018), Alzheimer's disease (Xu et al., 2014) and head trauma-induced cognitive deficits (Kumar, Rinwa & Dhar, 2014). Microglial cells were stimulated with LPS and then treated with rutin (50 μM). Rutin displayed a non-toxic effect and increased microglial proliferation in a dose dependent manner. It also reduced the mRNA levels of TNF, IL-1β, IL-6 and iNOS and decreased the production of IL-6, TNF and nitric oxide. In a rat model of spinal cord injury, it was done an intraperitoneally injection with rutin (30 mg/kg) for 3 days. After the treatment, the locomotor functions increased and TNF-α, IL-1β and IL-6 levels decreased.

In addition, rutin showed protective effects against severe sepsis *in vivo*, with LPS-induced endotoxemia, due to reduction of pro-inflammatory cytokines (Soromou *et al.*, 2018) and also to suppression of NF-kB, TLR4 and COX-2 (Khajevand-Khazaei *et al.*, 2018). Rutin exhibited hepatoprotective effects as well, associated with upregulation of antioxidant enzymes activities and attenuation of inflammation and hepatotoxicity (AlDrak *et al.*, 2017; Nafees *et al.*, 2015). Rats with hepatotoxicity were subjected to oral treatment of rutin (50 and 100 mg/kg b.w.), which down regulated the expression of iNOS and COX-2, as well as the levels of pro-inflammatory cytokines, targeting the NF-kB and MAPK pathway (Nafees *et al.*, 2015). Rutin also has immunomodulatory properties in certain autoimmune diseases. For instance, treatment with rutin may be therapeutic for neutrophil-mediated inflammatory/autoimmune illnesses by decreasing the NO and cytokines production (Abd-Nikfarjam *et al.*, 2017), as well as for rheumatoid arthritis, by suppressing the inflammatory cells infiltration, cytokines production and/or NF-kB and iNOS activation (Gul *et al.*, 2018; Sun, Wei & Bi, 2017).

To evaluate the anti-inflammatory effect of rutin on tumour, transgenic mice with human papillomavirus type 16 were supplemented with rutin (413 mg/kg, daily for 24 weeks). After the treatment, the expression of COX-2, which is a key player in cancer related inflammation, was reduced in the dermis and epidermis and there was also a reduction on

leucocytic infiltration, contributing for a decrease in the tumour-associated inflammation (Moutinho *et al.*, 2018). Another transcription factor regulated by rutin is Nrf2, as shown in a study with fibroblasts exposed to UVA and UVB radiations. After the treatment with rutin (25 μM), there was a prevention of UV-induced inflammation and redox disparity at protein and transcriptional level, due to the normalization of Nrf2 expression (Gęgotek, Rybałtowska-Kawałko & Skrzydlewska, 2017).

Rutin also displays anti-asthmatic (Lv, Chen & Wang, 2017) and cardioprotective effects (Xianchu *et al.*, 2018) *in vivo*, due to suppression of inflammatory mediators, such as cytokines, and inflammatory cells infiltration.

3.3. Naringin

Naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside, ***Figure 8***) is a flavanone found in several fruits of the *Citrus* genus, mainly in grapefruit, and imparts a bitter taste to citrus juices (Gattuso *et al.*, 2007). It is a glycoside composed of the aglycone naringenin and the disaccharide neohesperidose attached to the hydroxyl group at C-7. The name 'naringin' probably comes from the Sanskrit term "naringi", which means orange (Sinclair, 1972). Naringin exhibits antioxidant, anti-inflammatory (El-desoky *et al.*, 2018), anti-apoptotic (Chtourou *et al.*, 2016), hepatoprotective (Pari & Amudha, 2011) and anti-carcinogenic (Li *et al.*, 2013) properties.

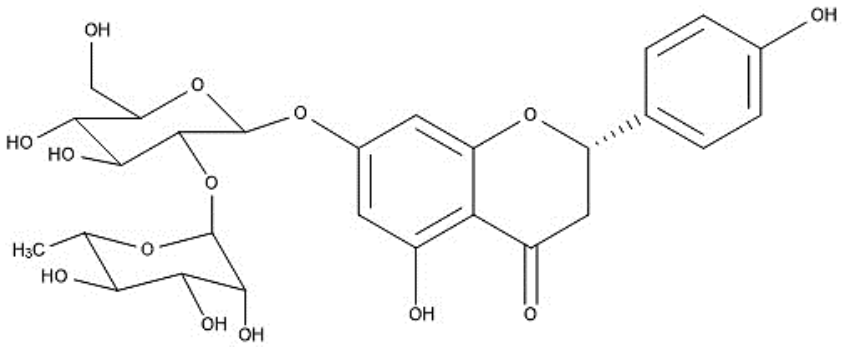

Figure 8- *Naringin*

The most abundant glycoside flavanones in citrus peel are naringin, hesperidin and neohesperidin that exhibit various health benefits such as suppression of carcinogenesis (Nair S *et al.*, 2017) and potent antihyperglycemic activity (Mahmoud *et al.*, 2015). Although aglycone

flavonoids show higher antioxidant capacity and radical scavenger efficiency than their respective glycosides (Cavia-Saiz *et al.*, 2010), the concentration of naringenin, in these fruits is lower than that of naringin.

Naringin is a promising candidate for treating a variety of diseases due to its extensive availability, low cost, a wide range of pharmacological actions and long history of use. Commercial grapefruit (*Citrus paradisi*) juice is the richest source of naringin (43.5 mg per 100 mL) (Gattuso *et al.*, 2007) and it has been described to increase the bioavailability of certain drugs either by inhibition of cytochrome P-450 (CYP) 3A4 in the small intestine or by inhibition of the P-glycoprotein, resulting in an additional increase in the fraction of drug absorbed (Dahan & Altman, 2004).

The flavonoid biosynthesis pathway shows that the aglycone naringenin is the precursor of hesperetin, naringin, neohesperidin and hesperidin. Naringin is formed by the action of 1,2-rhamnosyltransferase on the 7-*O*-glucoside form of naringenin (Molina-Calle, Priego-Capote & Luque De Castro, 2015) (***Figure 9***).

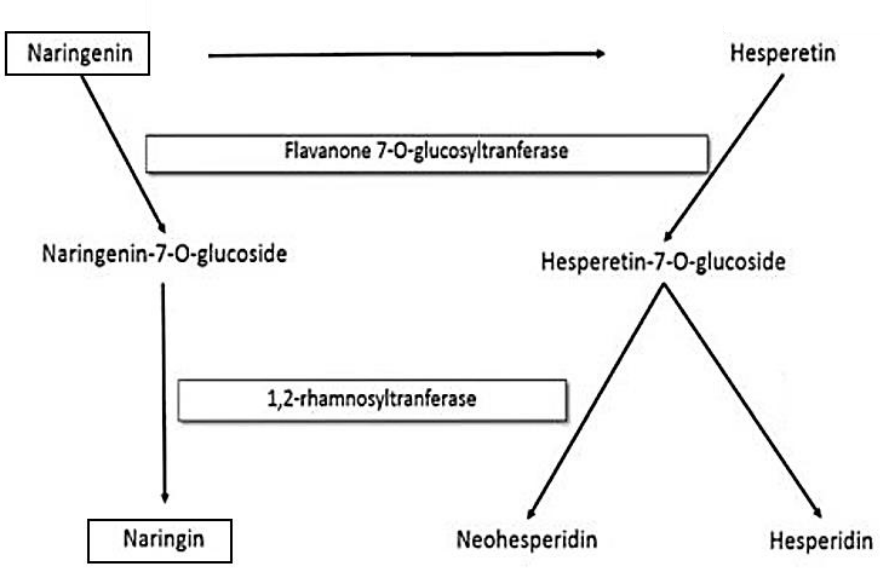

Figure 9*- Biosynthetic pathway of naringin (Adapted from Molina-Calle, Priego-Capote & Luque De Castro, 2015)*

3.3.1. Pharmacokinetics and structure-activity relationships of naringin

As observed with rutin, the oral absorption of naringin by gastrointestinal tract in its original form is reduced and the bioavailability of this flavanone is related to their glycosidic moiety that is known to increase its hydrophilic character (Felgines *et al.*, 2000). Naringin is hydrolysed to naringenin in the intestinal microflora by rhamnosidases and glucosidases and it is also transformed into many types of phenolic acids due to ring fission (Kim *et al.*, 1998).

Following oral administration of naringin to rats, it was revealed that naringin was present in stomach, small intestine, liver and trachea, whereas its metabolite naringenin showed up in liver, stomach, small intestine, kidney, lung and trachea (Zou *et al.*, 2012). After intraduodenally administration of naringin, the portal and lymphatic absorptions were about 95 and 5.0%, respectively, suggesting that naringin is primarily absorbed by portal blood rather than mesenteric lymph and it would be eliminated through bile excretion (Tsai & Tsai, 2012).

In humans, naringin undergoes extensive phase II metabolism in the liver via hydroxylation, deglycosylation, glucuronidation, sulfation, methylation, oxidation and acetylation to yield an array of conjugated products including naringenin (Zeng *et al.*, 2017). After oral administration of naringin to rats (42 mg/Kg) and dogs (12.4 mg/Kg), 4-hydroxyphenylpropionic acid was identified as the major metabolite of naringin (Liu *et al.*, 2012). In a tissue distribution study of naringin, it was observed that naringin was fast and widely distributed to all tissues except brain in rats due to its reduced blood brain barrier permeability (Zou *et al.*, 2012).

In a study to evaluate the antiangiogenic activity *in vitro* and *in vivo* of naringin the results demonstrated that it displayed no observable effect in this activity, suggesting that glycosylation at the C-7 position is not favourable to the antiangiogenic activity (Lam *et al.*, 2012). Moreover, the inhibitory activity of degranulation *in vitro* is also lost when a sugar moiety (regardless of the type and number of sugars) is coupled to the ring A of the flavanone (Noshita *et al.*, 2018). The presence of a single bond on flavanones makes easy the rotation but it becomes difficult to maintain a planar structure, which is necessary for intercalation with DNA, being the interaction with DNA made by groove binding (Tu *et al.*, 2015).

In a docking study, it was shown that naringin exhibits a stronger binding affinity towards acetylcholinesterase (AChE) than other flavanone glycosides. The interaction between naringin and AChE involves several hydrogen bonds. The hydroxyl group of B ring forms two hydrogen bonds, the oxygen atom and oxo group of C ring is also hydrogen bound.

In addition, the hydroxyl group attached to the A ring and the hydroxyl groups of attached sugar moieties form hydrogen bonds. Besides, there is a stacking interaction between A ring and Tyr341. Furthermore, it was done an *in vitro* inhibition study for naringin towards AChE and the IC_{50} was 446 μM (Remya *et al.*, 2014). This is in accordance with findings on an experimental model of Alzheimer's disease, whose chronic treatment with naringin dose dependently decreased the brain AChE activity, ameliorating the inflammatory surge, which is characteristic of this disease (Sachdeva, Kuhad & Chopra, 2014).

Another docking study showed that naringin and its metabolite naringenin could bind to multiple human protein targets, including JAK-2, ZAP-70 kinase, Angio-II-Type 1, TGFBR1, Kaep1, EGFR, VEGFR-2 and FGFR1 kinase, which are responsible for the protection of liver from chronic liver diseases (Shine *et al.*, 2018). This is in accordance with a study on ankylosing spondylitis that shows the protective effect of naringin, through suppression of inflammation and oxidative stress, and downregulation of JAK-2/STAT3 (Liu *et al.*, 2016).

3.3.2. Anti-inflammatory activity of naringin

Citrus fruits that are rich in flavanones, including hesperidin, naringin and neohesperidin, have been used for their anti-inflammatory properties (Benavente-García & Castillo, 2008).

The mechanisms of action proposed to explain the anti-inflammatory activity of naringin include antioxidant activity, reduction of cellular activities of inflammatory cells, inhibition of the synthesis and biological activities of different pro-inflammatory mediators, such as cytokines, chemokines, adhesion molecules and NO, as well as pro-inflammatory enzymes (COX-2 and iNOS) and modulation of pro-inflammatory transcription factors and pro-inflammatory gene expression. Naringin may specifically modulates the cytokines IL-1β, TNF-α, IL-6, chemokine IL-8 and CX3CL1, the transcription factors NF-kB, Nrf2, AP-1 and STAT, as well as lipid-derived eicosanoids (Caglayan *et al.*, 2018; Hsueh *et al.*, 2016; Okuyama *et al.*, 2018; Wang *et al.*, 2017; Xu, Zhang & Sun, 2017; Zhang *et al.*, 2018).

Indeed, naringin has exhibited neuroprotective effects through suppression of inflammation both *in vivo* and *in vitro*, for instance, in intracerebral haemorrhage (Singh *et al.*, 2017), cerebral ischemia/reperfusion (Okuyama *et al.*, 2018), doxorubicin-induced neurotoxicity and cognitive deficits (Ramalingayya *et al.*, 2018), glycoprotein 120-stimulated

microglia (Chen *et al.*, 2017) and in LPS-stimulated PC12 cells (Wang *et al.*, 2017). The PC12 cells, used as an analogue neuron model, were stimulated with LPS to induce cell death and with prior treatment with naringin (0-2,000 ng/ml). Naringin was able to rectify the antioxidant protein contents of Nrf2, as well as downregulate inflammatory gene and protein expression, such as IL-1β, IL-6, TNF-α, COX-2, TLR4, MAPK phosphorylation, AP-1 and NF-kB, exhibiting an anti-inflammatory effect in neuronal like cells. To evaluate the protective effect of naringin on intracerebral haemorrhage, it was used an experimental model of intracerebral haemorrhage, administrated with three doses of naringin (10.20 and 40 mg/kg) orally. The study showed that naringin alleviated dose dependently poststroke depression and neurological and cognitive deficits. The treatment also restored TNF-α and antioxidant levels, demonstrating the protective effect of naringin against ICH-induced inflammatory stress.

Additionally, naringin have been displayed hepatoprotective effects along with its anti-inflammatory activity (Caglayan *et al.*, 2018; El-desoky *et al.*, 2018; El-Mihi *et al.*, 2017). In rats exposed to hepatic fibrosis, naringin was administrated orally (40 mg/kg/day). After the administration of naringin, the mRNA level of cytokines, such as IL-1β, IL-6 and interferon gamma, decreased. Thus, naringin alleviated liver fibrosis through inhibition of PI3K/Akt pathway along with alleviation of oxidative stress and inflammation (El-Mihi *et al.*, 2017).

Besides, naringin also demonstrated to have nephroprotective effects *in vivo* (Caglayan *et al.*, 2018; Kandemir *et al.*, 2017). In both studies, rats were orally pre-treated with naringin (50 and 100 mg/kg b.w.) for 7 days and then they were subjected to renal toxicity induced by chemotherapeutic drugs. The treatment with naringin regulated inflammatory responses by decreasing the levels of TNF-α, NF-kB, IL-1β and IL-6 and activities of iNOS and COX-2. The results of these studies indicate that naringin acts as a potent protector against nephrotoxicity induced by chemotherapy.

To evaluate the potential of naringin against osteoarthritis, rats were orally pre-treated with naringin (5 and 10 mg/kg) for four weeks and then exposed to monosodium iodoacetate-induced osteoarthritis. The results showed that naringin decreased the levels of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α and pro-inflammatory mediator PGE2, protecting the tissue from the damage in the osteoarthritis model. Naringin also reduced the levels of specific pro-inflammatory mediators in LPS-stimulated RAW 264.7 cells. After the treatment with naringin (0.5 and 10 μg/mL), there was a reduction in the production of PGE2, NO, IL-6 and TNF-α by cells in a dose dependent manner (Xu, Zhang & Sun, 2017).

Naringin also have many other protective effects on which its molecular mechanism goes through the reduction of inflammation, including in high glucose conditions (Chen *et al.*, 2018; Liu *et al.*, 2017), asthma (Guihua *et al.*, 2016), periodontitis (Chang *et al.*, 2017), random skin flaps necrosis (Cheng *et al.*, 2017), colitis and colorectal carcinogenesis (Zhang *et al.*, 2018) and sepsis-induced injury of intestinal barrier (Li *et al.*, 2018). In all these studies, naringin reduced the levels of pro-inflammatory cytokines.

4. Biocatalysis

Biocatalysis uses mostly enzymes to catalyse chemical reactions to produce chemical compounds. The term biocatalysis refers to the chemical process through which natural biological catalysts perform faster reactions between organic components.

Enzymes are biological catalysts and, therefore, they are able to speed up chemical reactions in nature. The field of biocatalysis has received significant attention lately due to the potential applications in the pharmaceutical industry.

Given the increasing importance of single enantiomers and molecules with multiple chiral centres, methodologies involving enzymes overtaken the traditional synthetic chemistry with respect to the production of optically active small molecules, by their inherent chemo-, regio-, and enantiospecificity (Patel, 2000). It is still important to note that many enzymes are able to catalyse reactions for which there are no chemical alternatives.

In fact, the use of enzymes as catalysts is increasingly considered a promising and ecological alternative, in developing manufacture processes, with respect to its high selectivity and efficiency under mild conditions without generation of toxic products (Otrokhov *et al.*, 2013) and without the need for functional-group activation and protection and deprotection, which are characteristics of chemical methods. Therefore, biocatalysis has been an industrially attractive technology, mainly for the enantioselective synthesis of active pharmaceutical intermediates.

Over the last two decades the major obstacle to the widespread the use of biocatalysis was the limited number of available enzymes, that has changed thanks to advances in recombinant DNA technology (Reetz, 2013). That has opened unlimited access to a diversity of enzymes as tools to organic chemistry, that exhibit targeted process parameters, such as activity, specificity and stability. Moreover, their storage and stability can be optimized by immobilization, which is a mean of recycling as free-flowing solids and allows their economically viable recovery.

4.1. Green chemistry and biocatalysis

Green chemistry is defined as the use of chemical methods that use renewable raw materials efficiently, reduce or eliminate the use of hazardous solvents and avoiding the formation of products that are harmful (Anastas e Warner, 1998).

Biocatalysis is promising because it is suitable for green chemistry, due to their numerous economic and environmental benefits. The reactions are performed under mild conditions of temperature, pressure and pH and the enzymes are derived from readily available renewable sources and they are biodegradable, predominantly non-hazardous and nontoxic. Due to its high selectivity, it generates less secondary products, which could be hazardous to the humans and environment (Garcia-Junceda, 2008) (Sheldon, 2017). For all these reasons, biocatalysis lead to more step economic synthesis and purer products from processes that are more efficient and create less waste than conventional chemistry. Since the enzymatic processes are mostly performed under approximately the same conditions, the integration of multiple transformations into more economical and environmental attractive cascade processes is quite easy (Sheldon & Woodley, 2018).

Biocatalysis obeys to eleven of the twelve principles of green chemistry, since the principle 4 relates to the product and not the process (***Table 1***). Biocatalysis is definitely a green and sustainable process and it is not only environmentally beneficial, but also very profitable.

Table 1- Green chemistry principles and Biocatalysis (Adapted from Sheldon & Woodley, 2018)

	GREEN CHEMISTRY PRINCIPLE	BIOCATALYSIS
1	Waste prevention	Selectivity reduces waste
2	Atom economy	More atom- and step- economical
3	Less hazardous syntheses	Generally low toxicity of catalysts
4	Design for safer products	-
5	Safer solvents and auxiliaries	Often performed in water
6	Energy efficiency	Mild conditions/energy efficient
7	Renewable feedstocks	Enzymes are renewable
8	Reduced derivatization	Avoids protection and deprotection steps
9	Catalysis	Enzymes are the catalysts
10	Design for degradation	Enzymes are biodegradable
11	Real-time analysis	Applicability to biocatalytic processes
12	Inherently safer processes	Mild and safe reaction conditions

4.2. Classes of enzymes

Enzymes are powerful natural catalysts that have an enormous capacity to speed up biochemical reactions. They are classified by the Enzyme Commission (EC) of *Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (NC-IUBMB), attributing a four-part EC number to classify enzymes depending on the overall chemical transformation. The first part of the EC number corresponds to six different classes according to the type of reaction that the enzyme catalyses. These classes are further divided into subclasses and subgroups according to the chemical bond cleaved or formed, the reaction centre, the transferred chemical group, the nature of substrate and the cofactor used for catalysis (Martínez Cuesta *et al.*, 2015).

Oxidoreductases (EC 1) catalyse oxidation/reduction reactions by exchanging electrons between donor and acceptor. The reactions include electron transfer, proton abstraction, hydrogen removal, hydride transfer, oxygen insertion, among others. The classification is based on the order donor/acceptor oxidoreductase. The oxidases, peroxidases, oxygenases/hydrolases and dehydrogenases/reductases belong to this class. To achieve their function, oxidoreductases take multiple redox-active centres, including amino acid residues, metal ions or complexes and coenzymes, like FAD, FMN or PQQ, which can modulate their selectivity, potency, reactivity and stability. Thus, the potential industrial use of oxidoreductases is compromised due to the dependency of these enzymes on the external source with reduction or oxidation equivalents via cofactors like NAD(P)H and NAD(P) and/or enzyme bound coenzymes. In fact, most of the oxidoreductases need expensive cofactors, however several NAD(H) regeneration systems have been already developed to overcome this issue. For their application, oxidoreductases can also be classified according to their mode of catalysis and/or cofactor-dependence (Xu, 2005).

Transferases (EC 2) catalyse the transfer of a functional group from a donor molecule, often a coenzyme, to an adequate acceptor. The groups transferred include methyl, acyl, glycosyl, alkyl, phosphoryl and amino groups by means of a nucleophilic substitution reaction. Despite their potential for synthesizing interesting polymeric materials, transferases are not widely used in industrial processes. They are very sensitive, which avoids their isolation on a larger scale, however there is a classic example of industrial application of transferases, that is the use of glycosyltransferases for the synthesis of oligosaccharides (Johannes, Simurdiak & Zhao, 2006).

Hydrolases (EC 3) catalyse the hydrolytic cleavage of chemical bonds in the presence of water, including C-O, C-C, C-N and P-O, among others. Among hydrolases are amidases, proteases, esterases and lipases. Their most well-known applications are the hydrolysis of polysaccharides, proteins, nitriles and esterification of fatty acids and they have been used to produce intermediates for pharmaceuticals and pesticides. Besides, hydrolases are also used in reactions to digest proteins, carbohydrates and lipids in detergent formulations and in food industry. These hydrolytic enzymes are the biocatalysts most commonly used in organic synthesis due to their attractive properties for biocatalytic and industrial applications, for instance, the lack of cofactor dependency, easiness of use, acceptation of unnatural molecules as substrates, possession of high stereoselectivity and the capacity to perform reversed reactions under water-free conditions (Méndez-Sánchez, López-Iglesias & Gotor-Fernández, 2016).

Lyases (EC 4) catalyse the non-hydrolytic cleavage of C-O, C-C, C-N bonds, among others, by means other than hydrolysis or oxidation. This catalysis is either done by elimination to produce double bonds or by adding groups to double bonds. The most common lyases are decarboxylases, aldolases, hydratases, dehydratases, ammonia lyases and polysaccharide lyases. In industrial biocatalysis these enzymes are usually used in the synthetic mode through their reverse reaction, which is the addition of a molecule to an unsatured substrate.

Isomerases (EC 5) catalyse geometric or structural changes within one single molecule and they can perform highly selective and often complex molecular changes. Depending on the type of isomerism, these enzymes can be divided into epimerases, cis-trans isomerases, mutases, racemases and tautomerases. This enzyme class only represents a small number of enzymes, but one of them is of particular commercial interest, that is glucose isomerase (known as xylose isomerase with specificity for glucose), that catalyses the conversion of glucose to fructose, necessary to the production of natural sweeteners (Liese *et al.*, 2006).

Ligases (EC 6) catalyse a bond formation between two molecules with concomitant hydrolysis of a pyrophosphate in ATP or a similar triphosphate. The bonds formed include C-O, C-N, C-C and C-S. Ligases have not yet been applied in industrial biocatalysis due to their requirement for ATP or other cofactors.

4.3. Lipases

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyse the hydrolysis of ester bonds in triglycerides with subsequent release of fatty acids, diacylglycerols, monoacylglycerols and glycerol. These enzymes are widely distributed and they are obtained by extraction from animals, plants and natural or recombinant microorganisms. Lipases, which are serine hydrolases, catalyse esterification and transesterification reactions under low water conditions. Besides, they also catalyse alcoholysis, acidolysis and aminolysis, as well as hydrolysis reactions of organic carbonates (***Figure 10***). Their capacity to catalyse such reactions with high efficiency and stability, makes them significant biocatalysts, also due to their chemo-, regio- and enantioselectivity, mild reaction conditions, ability to use wide range of substrates, as well as not requiring cofactors (Stergiou *et al.*, 2013).

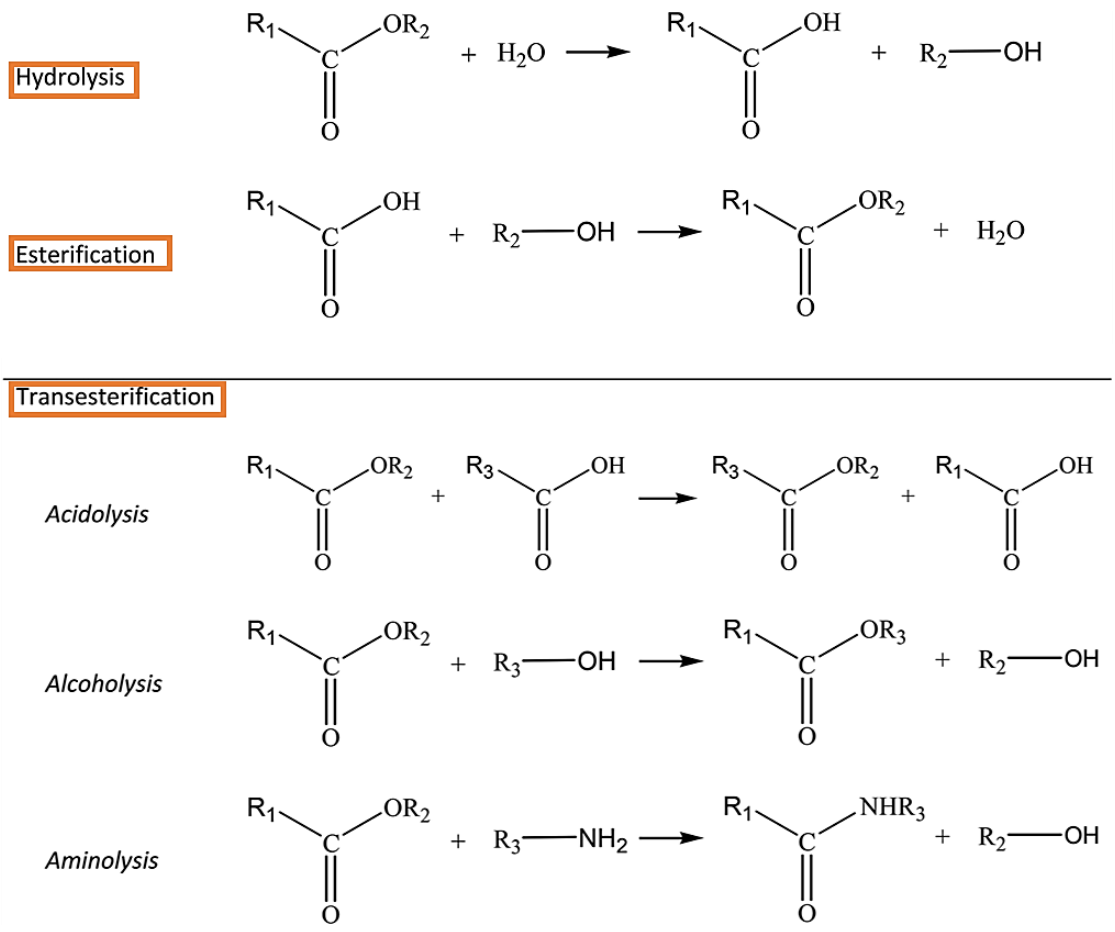

Figure 10- *The main reactions catalysed by lipases (Adapted from Aouf et al., 2014)*

Lipases are versatile enzymes and the most important biocatalysts frequently used to perform new reactions both in non-aqueous, aqueous media and in the synthesis of various pharmaceutical drugs. In this modern era, lipases remain the choice of enzymes for industry since their regio- and enantioselectivity have been employed for the resolution of chiral drugs, production of biofuels, modification of fats, synthesis of cosmetics, as flavour enhancers and to speed-up waste degradation (Angajala, Pavan & Subashini, 2016).

Lipases belong to α/β hydrolase fold super-family and perform catalysis via a catalytic triad composed by three residues (serine, histidine and aspartate or glutamate). An essential catalytic feature of lipases is the presence of an amphiphilic α helix peptidic loop covering the active site of the enzyme in solution, the lid domain. Despite their many advantageous features, it is important to clarify that any lipase-catalysed process is influenced by the lipase stability, selectivity, mass transfer and other factors (Aouf *et al.*, 2014; Stergiou *et al.*, 2013).

Various lipases display interfacial activation, that usually occurs at the lipid water interface, which affects enzyme activity. The interfacial activation involves the movement of the amphipathic flexible region, the lid. This unit lid upon interaction with a hydrophobic interface undergoes conformational changes, making the active site accessible to the substrate. Many factors can affect the way a lipase is activated and the degree of activation exhibited, and the nature of the interface is one of them (Aouf *et al.*, 2014).

However, *Candida antarctica* lipase B (CALB), one of the most used biocatalysts, is presented as an atypical lipase that has never shown any significant interfacial activation. This lipase is highly active towards a wide range of esters, thiols and amides but less active towards large triglycerides. Indeed, the phenomenon of interfacial activation of CALB is a more complex behaviour. This enzyme has two α-helices surrounding the active site (α5 and α10), which have been shown to be the most mobile parts of the structure and could work as a lipase lid by a relative motion between them, making an important role for CALB activity. A study of docking and molecular simulations has showed that CALB displays an increased catalysis for large, bulky substrates when adsorbed to a hydrophobic interface, accompanied with a high mobility for a small lid (helix α5) close to the active site. Thus, the interfacial activation of CALB towards large, bulky substrates requires a highly open conformation of helix α5 and a hydrophobic environment, which favours this conformation (Zisis *et al.*, 2015). Another study to evaluate the open and closed states of CALB indicated that Asp145 and Lys290 play a role in the conformation alteration. Asp145 and Lys290 are present in the two α-helices and are responsible to complete the closure of the catalytic cavity of CALB.

Accordingly, the interfacial activation of CALB is related to both the amino acid composition of the lid and to the reaction of this region to changes in the dielectric value or ionic strength of the media, resulting in a connection of factors towards this phenomenon (Stauch, Fisher & Cianci, 2015).

Lipase selectivity in a reaction system can be divided into three main groups, which are regioselectivity, substrate selectivity and enantioselectivity.

1) ***Regioselectivity*** (positional selectivity) is the preference to steer the reaction in one direction of chemical bond formation or breaking over all other directions. This property of lipases is very important to industry, since the production of isomeric mixtures display optimal function only under specific configuration (Sarmah *et al.*, 2018). It can be divided into the following types:

a) *Non-selective lipases*: they catalyse the complete hydrolysis of triglycerides into free fatty acids and glycerol, as well as mono- and diglycerides as intermediates, in a random way (***Figure 11***). The intermediates are hydrolysed more rapidly than the triglycerides in order to not accumulate in the reaction. These lipases are robust and can act on several substrates. In this reaction, the products are like those produced by chemical catalysis, but with less thermo-degradation due to ambient operating conditions (Kapoor & Gupta, 2012; Ribeiro *et al.*, 2011).

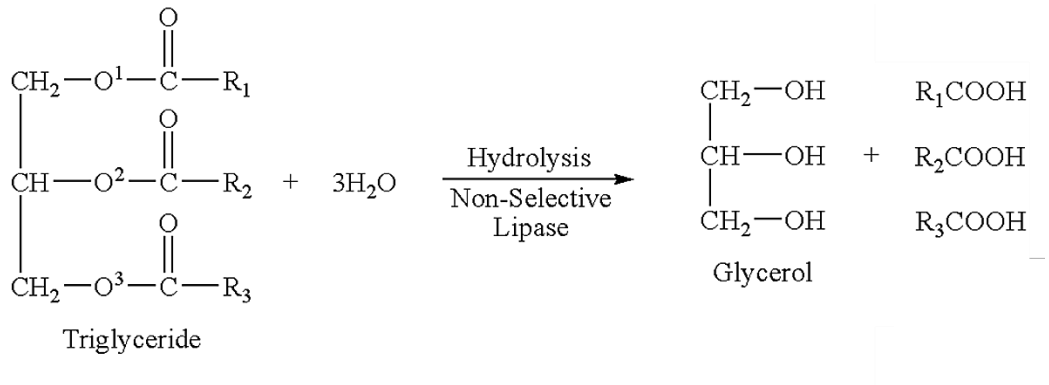

Figure 11*- Reaction catalysed by a non-selective lipase*

b) *1,3-Selective lipases*: these lipases only hydrolyse triacylglycerols at the C1 and C3 glycerol bonds and cannot hydrolyse ester bonds at secondary positions. They produce fatty acids, 2-monoacylglycerols and 1,2-diacylglycerols or 2,3-diacylglycerols (***Figure 12***), being the last two chemically unstable. Due to this instability, they undergo acyl migration to produce 1,3-diacylglycerol and 1- or 3- monoacylglycerols. Generally, the hydrolysis of

triglycerides by these specific lipases to diglycerides is must faster than those into monoglycerides (Kapoor & Gupta, 2012; Sarmah *et al.*, 2018).

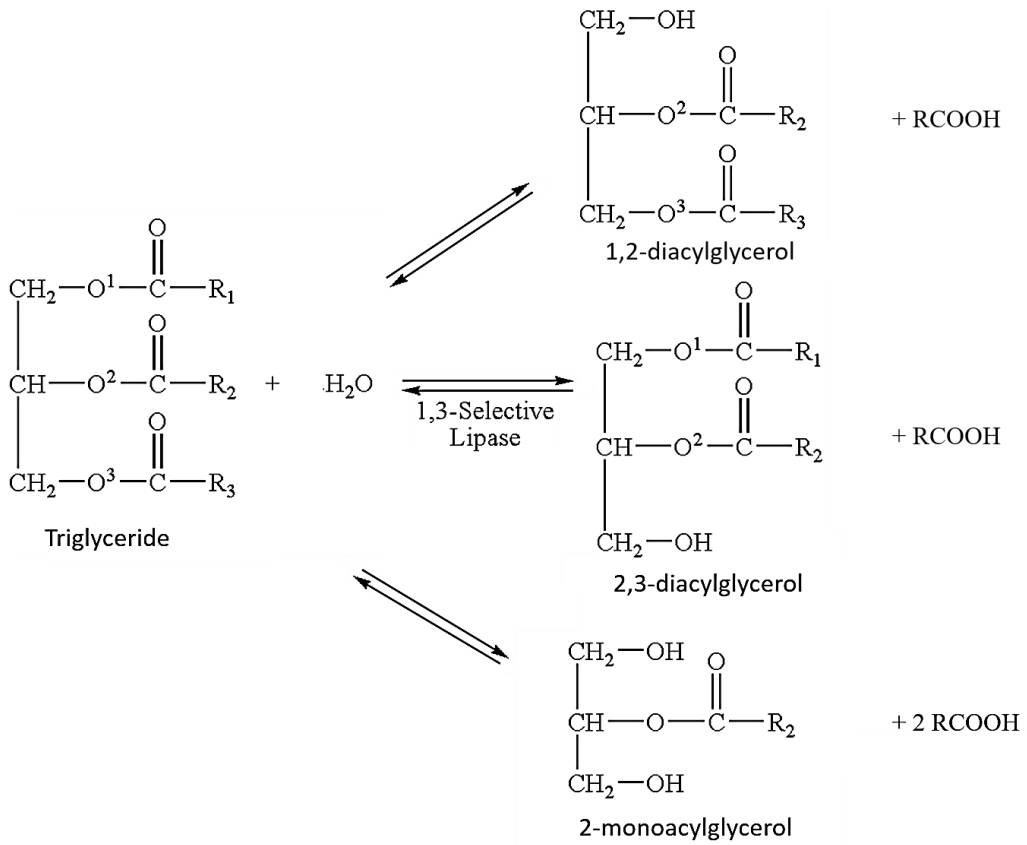

***Figure 12**- Reactions catalysed by 1,3-triacylglycerol selective lipases*

c) *Fatty acids selective lipases*: these lipases show fatty acid selectivity and they are specific for a particular type of fatty acid or for a specific group of fatty acids. They hydrolyse esters which have long-chain fatty acids with double bonds in *cis* position between C9 and C10 (Ribeiro *et al.*, 2011).

Moreover, regioselectivity is often observed in the monoacylation of a single hydroxyl group in a polyhydroxylated substrate, such as paclitaxel (Khmelnitsky *et al.*, 1997), steroidal diols (Silva, Riva & Sá E Melo, 2005) and glycosylated flavonoids (Araújo, De *et al.*, 2017b; Vavříková *et al.*, 2016).

2) Substrate selectivity is related to the lipase preference towards the substrate in reactions, where they can act on a specific substrate in a mixture of raw materials, facilitating the desired product synthesis. An example is the use of lipase in the production of biodiesel (Ribeiro *et al.*, 2011). Lipases show specificity to fatty acid chain length (short, medium, long),

to the degree of unsaturation or substitution of the fatty acid and to the nature of the nucleophilic substrate. Some lipases distinguish between certain fatty acids or groups of fatty acids and also display fatty acid chain length specificity. Most of the lipases select esters of medium (C4) to long chain (C16) saturated fatty acids, with a few exceptions like lipases from *Penicillium roqueforti* that can only hydrolyse esters of short chain (Mase, Matsumiya & Matsuura, 1995). Generally, the substrates that can be acted upon by these lipases include not only fatty acids but also alcohol parts. Lipases show higher specificity for primary alcohols and lower specificity for tertiary alcohols and their esters (Kuo & Parkin, 1993).

3) Enantioselectivity is the ability to differentiate enantiomers in a racemic mixture. These lipases selectively hydrolyse one of the enantiomers of a chiral molecule over the other, in a chemical reaction implying a racemic mixture (mixture of both enantiomers). Among the reactions catalysed by these specific lipases are included kinetic resolution of secondary alcohols to pharmaceutical drugs (Gotor-Fernández, Brieva & Gotor, 2006) (***Figure 13***), hydrolysis of menthol benzoate to cosmetic and food products (Vorlová *et al.*, 2002) and hydrolysis of glycidic acid methyl ester to medical care products (Singh & Banerjee, 2005).

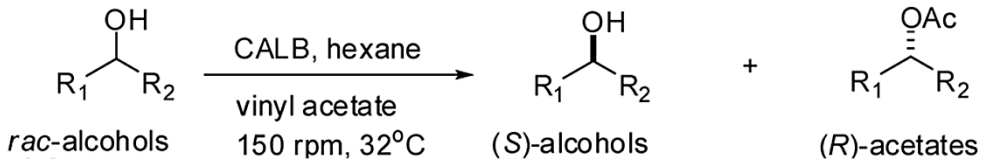

Figure 13- *Kinetic resolution of secondary alcohols by CALB (Adapted from Ferreira et al., 2012)*

In addition, lipase catalysis of esterification reactions has been applied to the kinetic resolution of racemic mixtures to the production of pharmaceuticals, including ibuprofen. Even though the S-enantiomer is known to be much more active than its R-enantiomer, ibuprofen is still sold as a racemate, also due to the higher cost of the first enantiomer compared with racemic profen. The most used lipases for the enzymatic resolution of ibuprofen through enantiomeric esterification are those of fungal sources such as *Candida rugosa*, *Rhizomucor miehei* that catalyse the S-enantiomer and the CALB that catalyses the esterification of the R-enantiomer (José, Toledo & Briand, 2016).

The enantioselectivity of lipases can vary according to the interaction of the active site and the structure of the substrate. Besides, stereospecificity was also shown to be dependent on physicochemical factors including temperature and solvent (Casas-Godoy *et al.*, 2012).

4.3.1. Catalytic mechanism of lipases

The catalytic mechanism of lipases is believed to be similar to that of serine proteases due to the similarity of their catalytic triads (serine, histidine and aspartate or glutamate) (Kapoor & Gupta, 2012; Stergiou *et al.*, 2013).

The mechanism starts with acylation, that is the transfer between the aspartate, histidine and serine residues of lipase, resulting in the activation of the hydroxyl group of serine, present in the active site. Consequently, there is a nucleophilic attack of the carbonyl group of the susceptible substrate by the hydroxyl residue of serine, resulting in the formation of tetrahedral intermediate. An oxyanion hole stabilizes the charge distribution and the tetrahedral intermediate loses an alcohol molecule to give an acyl-enzyme intermediate (Aouf *et al.*, 2014; Casas-Godoy *et al.*, 2012).

Then the deacylation takes place, whereby the nucleophile (that is water molecule in the case of hydrolysis) attacks the acyl-enzyme to produce tetrahedral intermediate, which lastly, loses an acid molecule to give the enzyme in its native form, regenerating the catalytic site (Casas-Godoy *et al.*, 2012; Kapoor & Gupta, 2012).

4.4. Biocatalysis in organic solvents

Water is considered the solvent of life, however it is a poor solvent for most synthetic reactions and it can limit the applications of biocatalysts. Only a small number of industrially attractive substrates are sufficiently water soluble and unwanted side reactions such as hydrolysis, racemization, polymerization and decomposition are favoured over synthetic reactions in aqueous media. Besides, water represents a challenge for reaction engineering due to its high boiling point and high heat of vaporization, which makes the enzymatic catalysis an expensive process (Stepankova, Damborsky & Chaloupkova, 2015).

The use of organic solvents as reaction media for biocatalytic reactions is considered extremely useful since they are usually required to increase the solubility of hydrophobic compounds, to change the thermodynamic equilibrium to favour synthesis and to block water dependent side reactions. Another reason that makes enzymatic catalysis in organic solvents so appealing is the improved thermal stability that often some enzymes show and their selectivity, which can differ from that in water or in between solvents (Carrea & Riva, 2000).

Lipases are widely used in biocatalysis performed in organic solvents. The reactions in this type of media not only improve the solubility of substrates with different polarities but also allow the reaction in the reverse direction (opposite to hydrolysis), accompanied with easy recovery of the product. There are still many other advantages in the use of organic solvents, such as ease of lipase recovery and reusability without immobilization, catalysis of a wide variety of chemical reactions which are not possible in aqueous media and elimination of microbial contamination. Organic solvents are generally inert and can increase the activity, stereoselectivity and regiospecificity of the lipase (Kumar *et al.*, 2016).

Solvent polarity is considered to be the key parameter to predict lipase activity and the hydrophobic solvents are usually preferred for lipase catalysed reactions. Polar solvents affect negatively the activity of lipases due to a competition between solvent and protein for water. This competition compromises the protein activity by decreasing the hydration state. However, very hydrophobic solvents are not always the right ones for lipase catalysis that involves polar substrates such as phenolic compounds or sugars, due to their low solubility in these solvents. Thus, solvents with intermediate polarities are frequently used, including acetonitrile, *tert*-butanol, 2-methyl-2-butanol and acetone (Aouf *et al.*, 2014).

Many lipases can undergo denaturation, resulting in a reduced catalytic activity, due to altered temperature, shear stresses, exposure to interfaces and chemical denaturants, in the

presence of organic solvents. However, various engineering methods have been developed to stabilize lipases in the presence of organic media, including immobilization. Lipase immobilization has many advantages in terms of biocatalyst activity, thermostability, enzyme recovery and reuse. By taking advantage of the interfacial hydrophobicity, immobilization of lipases has been performed by adsorption on hydrophobic adsorbents, covalent attachment to solid materials and/or entrapment in organic or inorganic polymers or microemulsions. This technique makes the use of lipases in industry more attractive due to the ease of separation and removal from the reactant and product, improved stability, protection of the lipase against exterior influences like pH, solvents or temperature, rapid termination of reactions and continuous operation, improving process economy. An example of a widely used immobilized lipase is CALB, adsorbed on a macroporous polyacrylic resin (Novozym 435) which maintains the lid of the active site always in open conformation and gives support, making it a more robust biocatalyst (Sharma & Kanwar, 2014).

Lipase catalysis in organic media has been widely used for the resolution of alcohols, acids or lactones through transesterification. Indeed, the practical applications of lipases in organic media include ester synthesis, either by esterification or transesterification, which became dominant in this type of solvent (Dhake, Thakare & Bhanage, 2013).

In lipase catalysed esterification, the water content is an important factor regarding the ester yield. In this type of reaction, the water molecule, which is a stronger nucleophile than alcohols, is produced as a side product and has the potential to revert the forward reaction (reversible reaction), decreasing both the reaction rate and the ester yield. Therefore, the removal of water from the reaction media becomes a crucial factor, either by performing multi-step reactions or by removing it *in situ*. Various methods have been described such as dehydrating agents, molecular sieves and specialized membranes. However, the use of a completely anhydrous media has become controversial since enzymes need media containing certain amounts of water to retain their native structure (Stergiou *et al.*, 2013).

On the other hand, in lipase catalysed transesterification the crucial factor affecting, whether is a reversible or non-reversible reaction, is the choice of the acyl donor. The acyl donor more appropriate for each process depends on the nucleophile used, for example, activated esters are the most adequate reagents to perform the resolution of alcohols, to avoid the reversibility of the reaction and increase the ester yield. By far, the best activated and most used acyl donors on a small scale are enol esters such as vinyl acetate, which is the cheapest and most reactive acyl donor (Paravidino & Hanefeld, 2011). During the reaction,

vinyl alcohol is released as a side product which tautomerizes to form acetaldehyde. Hence, no nucleophile remains and the reaction becomes irreversible (***Figure 14***).

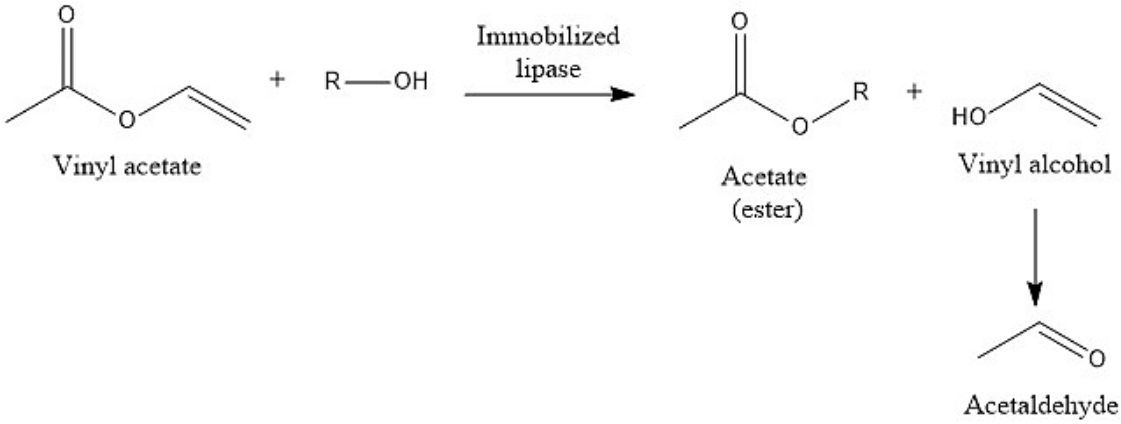

Figure 14*- Irreversible transesterification with a vinyl ester as the acyl donor catalysed by an immobilized lipase.*

Some vinyl esters are commercially available and are often used in large excess, thus making the use of a solvent unnecessary. However, the major obstacle to the scale-up of vinyl esters mediated acylation, namely with the use of vinyl acetate, is the generation of a stoichiometric amount of acetaldehyde, which is an irritating agent that has been classified as an inhalation carcinogen (Paravidino & Hanefeld, 2011).

The use of organic solvents as reaction media for enzymatic reactions is considered a useful approach for biocatalyst applications. Though, the advantages of using this type of solvents is limited by the risk of enzyme deactivation and the environmental hazardous nature of solvents. Significant progress has been made towards the substitution of organic solvents to more environmental-friendly alternatives, such as ionic liquids, deep eutectic solvents, supercritical fluids and fluorous solvents (Stepankova, Damborsky & Chaloupkova, 2015).

4.5. Modulation of the biological activity of flavonoids through structural modifications

Depending on the structure of flavonoids, the absorption and bioavailability of these compounds is still limited by their weak stability and low lipophilicity. Thus, structural modifications of these compounds is a mean to improve their pharmacological activities (Chebil *et al.*, 2007).

Acylation of natural flavonoids is known to improve their lipophilicity resulting in enhanced cellular uptake and modified biological activity upon dietary intake. Because of the numerous reactive hydroxyl groups of these compounds, the enzymatic catalysis, which is more regioselective, is described as the best route to perform such reactions. Lipase catalysed acylation of flavonoids can be achieved by direct esterification or transesterification (Araújo, De *et al.*, 2017a).

The regioselectivity and conversion rates of the enzymatic acylation of flavonoids are determined by the composition and water content of the reaction media, the nature and chain length of the acyl donor, the acyl donor and flavonoid molar ratio and the temperature of the reaction.

CALB is an excellent biocatalyst and it is widely used in enzymatic acylation reactions. CALB has the capacity of acylating flavonoid glycosides, however it has a little activity for tertiary alcohols and aglycone polyphenols and no visible activity for flavonoid aglycones. Indeed, a docking study showed that no product can be obtained from the catalytic acylation of quercetin by CALB (Christelle *et al.*, 2011) and there is only one study reporting the acylation of flavonoid aglycones by CALB, to date (Kyriakou *et al.*, 2012).

The flavonoid glycoside isoquercitrin (IQ) was acylated with mono and dicarboxylic acids by Novozym 435 (immobilized CALB), giving IQ acetate, IQ diacetate, IQ butyrate, IQ hexanoate, IQ octanoate, IQ dodecanoate, IQ palmitate, IQ hemiglutarate, IQ hemiadipate and IQ hemidodecanedioate. The conversion rates with dicarboxylic acids were limited by the acceptance of CALB and depended on the chain length. The selective acylation of primary hydroxyl groups by CALB, allowed to synthesize multiple derivatives with increased lipophilicity and conserved or even improved antioxidant properties. The ability of IQ esters with longer aliphatic chain to pass through a lipophilic membrane was much higher compared to IQ, however these esters were the weakest inhibitors of lipid peroxidation compared to other derivatives. The ability to inhibit lipid peroxidation was significantly improved for IQ

hemidodecanedioate, which means that the length of aliphatic chain was a crucial factor for the interaction with lipid bilayers (Vavříková *et al.*, 2016).

The hydrophilic/lipophilic nature of an antioxidant compound is an important factor governing the accessibility to the active sites of oxidative injuries *in vivo* and the glycosylation of flavonoids, which is an important modification, is known to increase their hydrophilic character. The glycosylated flavonoid quercetin-3-*O*-glucoside (Q3G) was acylated with six long chain fatty acid esters, namely stearic acid, oleic acid, linoleic acid, linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), by immobilized CALB. ALA, EPA and DHA acid esters of Q3G significantly attenuated the cytotoxicity induced by H_2O_2 in human primary hepatocytes and lung fibroblasts, whereas oleic acid and linoleic acid esters of Q3G displayed significant cytoprotection only in primary hepatocytes. Moreover, ALA and DHA esters of Q3G significantly decreased the production of lipid hydroperoxides under oxidative stress conditions, which points out fatty acid esters of Q3G as potential cytoprotective agents (Warnakulasuriya, Ziaullah & Rupasinghe, 2016).

The influence on inflammation and hyperlipidaemia of acylation of quercetin-3-*O*-glucoside, was evaluated *in vitro* and *in vivo*. Isoquercitrin was acylated with eicosapentaenoic acid in dried acetone by Novozym 435, giving an ester yield of 81%, approximately. The ester compound was more effective in reducing the production of TNF-α, PGE2, COX-2 levels and NF-kB in macrophages with induced inflammation by LPS, compared to the isoquercitrin. Moreover, in rats fed with a high-fat diet, the isoquercitrin ester increased the serum high-density lipoprotein (HDL)-cholesterol and decreased hepatic total cholesterol concentration, as well as reduced C-reactive protein, IL-6 and IFN-ɣ, exhibiting anti-inflammatory and hypolipidemic properties (Sekhon-Loodu *et al.*, 2015).

Another glycosylated flavonoid, malvidin 3-glucoside was acylated with saturated fatty acids with different chain lengths (from C4 to C16) using CALB as biocatalyst. The hydrophobicity index and partition coefficient (Log P) increased with the fatty acid chain length, which is an indication of the lipophilicity increase from short chain esters to long chain esters of malvidin 3-glucoside. This study showed that a proper acylation could enhance physical and chemical properties of anthocyanins as well as improve their antioxidant capacity, that reaches a maximum with the acylation of malvidin 3-glucoside with 8 carbons (Cruz *et al.*, 2017).

Flavonoids selectively acylated with aliphatic acids by lipases may not only alter their physicochemical properties but also improve their biological properties. This was shown with

the acylation of rutin, naringin and hesperidin with various monocarboxylic acids (hexanoic, octanoic, decanoic, lauric and oleic acids) by CALB. The highest conversion yields were obtained for hesperidin. The acylation of rutin and naringin with lauric (12C) and oleic (18C) acids had low conversion yields, since the highest CALB acylation yields of naringin and rutin are obtained using short chain acyl donors (Katsoura *et al.*, 2006). Three of the acylated derivatives, namely naringin octanoate, naringin decanoate and hesperidin decanoate, exhibited a considerable increase in the inhibitory effect on xanthine oxidase (XO) activity, one of the main enzymatic sources of reactive oxygen species. This acylation process enables the use of flavonoid fatty acid esters in oil-based systems and may lead to the development of potential new drugs for XO inhibition (Araújo, De *et al.*, 2017b).

Rutin acylation can take place at both the 3"-OH position of glucose and the 4"'-OH position of rhamnose. On the other hand, naringin acylation takes place at the 6"-OH position (Araújo, De *et al.*, 2017a) (***Figure 15***).

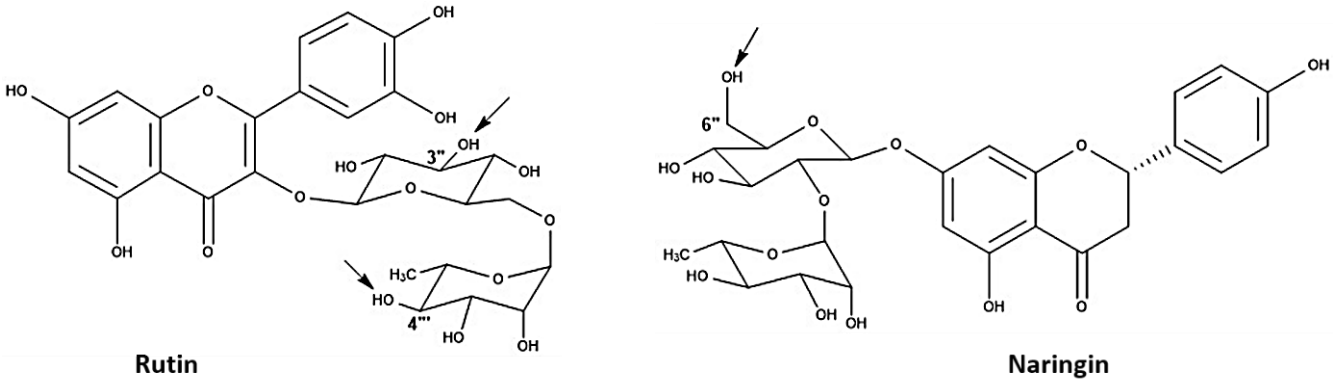

Figure 15*- Rutin and naringin enzymatic acylation position sites*

Chapter II

Aims

II. Aims

The structural modification of glycosylated flavonoids constitutes a challenge to chemical synthesis. Due to the numerous reactive hydroxyl groups of these compounds, acylation through chemical processes requires many protection/deprotection steps. Therefore, the enzymatic catalysis, which is regioselective, is described as the best route to perform such reactions. In addition, structural modifications of these compounds through acylation are a mean to improve their pharmacological activities due to their enhanced cellular uptake and modified biological activity.

The presented study set out to semi-synthesize and characterize new acylated derivatives, with relevant biological activity, of the glycosylated flavonoids rutin and naringin, catalysed by Novozym 435, with various vinyl esters (vinyl acetate, vinyl propionate, vinyl butyrate and vinyl cinnamate).

Furthermore, this work also intends to disclose the potential anti-inflammatory effect of naringin acylated derivatives using the *in vitro* model of inflammation lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Specifically, the anti-inflammatory effect of these compounds set sights on the cell viability, the inhibition of the production of nitric oxide, the evaluation of antioxidant activity and on the expression of inducible nitric oxide synthase in the presence of the acylated derivatives and naringin.

Moreover, structure-activity relationships of naringin derivatives will be also highlighted and discussed.

Chapter III

Materials and Methods

III. Materials and Methods

1. Equipment

The nuclear magnetic resonance spectra of protons (^{1}H NMR) were performed using a Varian Unity 400 spectrometer, at 400 MHz, and the nuclear magnetic resonance spectra of carbon (^{13}C NMR) were performed using a Bruker Avance III spectrometer, at 100 MHz. The chemical shifts are presented on the σ (ppm) scale and they are relative to deuterated methanol and deuterated chloroform as solvents.

The melting points were measured on a Büchi-540 apparatus. The enzymatic reactions were performed in a New Brunswick Scientific, C24 Incubator Shaker, orbital shaker, at 175 rpm.

The ELISA automatic microplate reader was purchased from SLT, Austria. The protein detection was performed using the imaging system Typhoon™ FLA 9000, from GE Healthcare. The quantification of bands densitometry was performed using Image Quant 6.0 software, from Molecular Dynamics, Amersham Biosciences.

2. Materials

Rutin and naringin were purchased from Tokyo Chemical Industry (TCI). Chloroform, ethyl acetate, ethanol and methanol were obtained from CARLO EBRA Reagents and butanone was from BDH Laboratory supplies. Acetone, dichloromethane, silica gel 60 (for column chromatography) and thin layer chromatography (TLC) 60 F_{254} were obtained from Merck Co. Vinyl esters (vinyl acetate, vinyl propionate, vinyl butyrate and vinyl cinnamate) were purchased from Sigma-Aldrich Co. The immobilized lipase B from *Candida antarctica* (Novozym 435) was also obtained from Sigma-Aldrich Co. All the solvents and reagents were used as received.

The enzymatic reactions were monitored by TLC eluted either with chloroform/acetone/methanol (60:35:5) (v/v/v) or chloroform/methanol/water (5:2:0.3) (v/v/v).

The mouse leukemic monocyte macrophage cell line, RAW 264.7, was purchased from *American Type Culture Collection* (ATCC® TIB-71™). The Dulbecco's Modified Eagle Medium (DMEM) was purchased from Sigma-Aldrich Química, Madrid, Spain. The lipopolysaccharide

(LPS; from *E. coli*-serotype 026: B6) was obtained from Sigma Chemical Co., St. Louis, USA. The polyvinylidene difluoride (PVDF) membranes were purchased from Amersham Biosciences, Uppsala, Sweden. The primary antibody for iNOS (1:1000) was purchased from R&D Minneapolis, MN, USA, and the anti-β-tubulin antibody (1:20000) was obtained from Sigma-Aldrich, St. Louis, USA. The anti-mouse secondary antibodies were obtained from GE Healthcare.

3. Enzymatic synthesis and purification of rutin and naringin esters

Some enzymatic reactions of rutin and naringin acylation, that were previously done (Semedo, 2015), were repeated and optimized in order to increase the ester yield.

The reaction medium contained acetone or butanone, previously treated with 4 Å molecular sieves activated by being kept at 100 °C for 1h, 12h prior to the reaction. The glycosylated flavonoid, rutin or naringin, was dissolved in dried butanone or acetone, respectively. The acyl donor and Novozym 435 were added to the solution in 100 mL flasks. The mixtures, in sealed vials, were heated in the orbital shaker with constant stirring at 175 rpm. When most of the glycosylated flavonoid had been converted to the ester form, the reaction was stopped. The lipase was filtered off and the solvent was evaporated under vacuum at 55 °C. After that, the products were isolated by column chromatography on silica gel column. The extent of the reaction was monitored by TLC analysis. The solvents of the elutes were evaporated and the glycosylated flavonoid derivatives were dried in a desiccator under vacuum for 1 week.

3.1. Enzymatic acylation of rutin

- ***Rutin 3", 4'"-diacetate***

To a solution of rutin (300 mg; 0.49 mmol) in butanone (8 mL), it was added 2 mL of vinyl acetate and 300 mg of Novozym 435 and the mixture was shaken at 50 °C. After 24h the reaction was stopped and rutin 3", 4'"-diacetate (185 mg; 54.20 %) was isolated by column chromatography eluted with chloroform/methanol (5:2) (v/v).

Melting point: 189.2-192.7 °C

- ***Rutin 3", 4"'-dibutanoate***

To a solution of rutin (50 mg; 0.082 mmol) in butanone (2 mL), it was added 0.2 mL of vinyl butyrate and 20 mg of Novozym 435 and the mixture was shaken at 50 °C. After 4 days and 18h the reaction was stopped and rutin 3", 4"'-dibutanoate (46 mg; 74.82 %) was isolated by column chromatography eluted with ethyl acetate/ethanol/water (90:9.5:0.5) (v/v/v).

Melting point: 198.1-200.0 °C

3.2. Enzymatic acylation of naringin

- ***Naringin 6"-acetate***

To a solution of naringin (200 mg; 0.34 mmol) in acetone (15 mL), it was added 0.6 mL of vinyl acetate and 100 mg of Novozym 435 and the mixture was shaken at 40 °C. After 6h the reaction was stopped and naringin 6"-acetate (103 mg; 48 %) was isolated by column chromatography eluted with dichloromethane/acetone/methanol (60:35:5) (v/v/v).

Melting point: 72.1-74.5 °C

- ***Naringin 4"',6"-diacetate***

To a solution of naringin (200 mg; 0.34 mmol) in acetone (15 mL), it was added 3 mL of vinyl acetate and 200 mg of Novozym 435 and the mixture was shaken at 50 °C. After 2 days and 20h the reaction was stopped and naringin 4"',6"-diacetate (135 mg; 58.96 %) was isolated by column chromatography eluted with chloroform/acetone/methanol (70:15:10) (v/v/v).

Melting point: 198-199.5 °C

- ***Naringin 6"-propanoate***

To a solution of naringin (200 mg; 0.34 mmol) in acetone (10 mL), it was added 1.5 mL of vinyl propionate and 200 mg of Novozym 435 and the mixture was shaken at 50 °C. After 5h the reaction was stopped and naringin 6"-propanoate (132 mg; 60.19 %) was isolated by column chromatography eluted with dichloromethane/acetone/methanol (5:5:5) (v/v/v).

Melting point: 133.4-135.2 °C

- ***Naringin 6"-butanoate***

To a solution of naringin (200 mg; 0.34 mmol) in acetone (10 mL), it was added 1 mL of vinyl butyrate and 200 mg of Novozym 435 and the mixture was shaken at 50 °C. After 5h the reaction was stopped and naringin 6"-butanoate (165 mg; 73.63 %) was isolated by column chromatography eluted with dichloromethane/acetone/methanol (5:5:5) (v/v/v).

Melting point: 68.1-69.4 °C

- ***Naringin 4"',6"-dipropanoate***

To a solution of naringin (200 mg; 0.34 mmol) in acetone (3 mL), it was added 1 mL of vinyl propionate and 200 mg of Novozym 435 and the mixture was shaken at 50 °C. After 3 days it was added to the mixture more 40 mg of lipase and 0.2 mL of vinyl propionate. The reaction was stopped after a total of 5 days and naringin 4"',6"-dipropanoate (150 mg; 62.87 %) was isolated by column chromatography eluted with chloroform/acetone/methanol (60:35:5) (v/v/v).

Melting point: 92.0-94.3 °C

- ***Naringin 6"-cinnamate***

To a solution of naringin (200 mg; 0.34 mmol) in acetone (5 mL), it was added 1 mL of vinyl cinnamate and 200 mg of Novozym 435 and the mixture was shaken at 50 °C. After 6 days the reaction was stopped and naringin 6"-cinnamate (153 mg; 62.50 %) was isolated by column chromatography eluted with chloroform/acetone/methanol (60:35:5) (v/v/v).

Melting point: 158.5-159.6 °C

4. Assessment of the anti-inflammatory effect

4.1. Cell line culture

The mouse leukemic monocyte macrophage cell line, RAW 264.7 ATCC® TIB-71 ™, is a well-established model used to investigate how a compound might interfere with inflammation in macrophages (*in vitro*). The cells were cultured in DMEM supplemented with 1.5 g/L sodium bicarbonate, 3.5 g/L D-glucose, 10% non-inactivated fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Every 2 days, about 10-20% of the cells were transferred to another culture flask containing 10 mL of supplemented DMEM, again at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Morphological changes were monitored by microscope observation. Cells were used in the biological assays when they had 80-90% of confluence. Cell viability was confirmed by counting on a haemocytometer (Neubauer chamber) using trypan blue.

4.2. Measurement of nitric oxide (NO) production

The anti-inflammatory activity of the glycosylated polyphenol naringin and its derivatives was evaluated in the RAW 264.7 macrophages cell line. To achieve this goal, the NO production was determined by nitrite concentration in cell culture media, using a colorimetric reaction with Griess reagent (Green *et al.*, 1982). Thus, the macrophage cells (0.1×10^6 cells/ 300 µL) were plated in 96-well plates and stabilized for 12h, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After stabilization, cells were either maintained in control medium or pre-incubated with different concentrations of the naringin esters and naringin (***Table 2***) for 1h. Ethanol and DMSO were used as vehicles and were added to control plates. All compounds were dissolved in ethanol except for naringin 6"-cinnamate, that was dissolved in DMSO. Later, cells were activated with 50 ng/mL LPS for 24h.

***Table 2**- Tested concentrations of naringin and its derivatives.*

Compounds	Tested concentrations (µM)
Naringin	400; 200; 100; 50; 25
Naringin 6''-acetate	400; 200; 100; 50; 25
Naringin 4''',6''-diacetate	250; 200; 100; 50; 25
Naringin 6''-butanoate	400; 200; 100; 50; 25
Naringin 6''-propanoate	400; 200; 100; 50; 25
Naringin 6''-cinnamate	400; 200; 100; 50; 25

After 24h of LPS incubation, 170 µL of culture supernatants were collected and mixed with equal volume of Griess reagent [prepared in a ratio (1:1) of reagent A and reagent B, stable at 4 °C, in which reagent A is prepared with 1% (w/v) sulfanilamide containing 5% (w/v) H_3PO_4 and reagent B with 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride] in a 96-well plate, in dark at room temperature. After 30 min, it was measured the absorbance at 550 nm with an ELISA automatic microplate reader. The nitrite concentration was calculated using a standard curve of sodium nitrite and results are expressed as percentage of LPS relatively to the controls with the respective vehicles.

4.3. Cell viability by resazurin assay

The viability of RAW 264.7 cells exposed to the compounds was measured using resazurin assay (O'Brien *et al.*, 2000), which is based on the reduction of the non-fluorescent resazurin to the fluorescent resorufin.

After the removal of 170 µL of culture supernatants for Griess assay, the 96-well plate culture medium was aspirated. Then, a solution of resazurin (50 µM) was added to the cells. After 1h, the quantification of resorufin was performed using a microplate reader at 570 nm, with a reference wavelength of 620 nm. Data are presented as percentage of viability relatively to their respective vehicles.

4.4. Determination of scavenging activity

To evaluate the NO scavenging activity of naringin esters, it was used S-nitroso-N-acetylpenicillamine (SNAP) as NO donor. For that, 300 µL of culture medium with the compounds were mixed with 300 µM SNAP, in a 96-well plate. The solution was maintained in a humidifier incubator at 37 °C for 3h. After the treatments, the nitrite quantification was performed by Griess assay, as described above (III.4.2.).

4.5. iNOS expression

4.5.1. Total protein extracts

RAW 264.7 cells (0.6×10^6 cells/2000 µL) were plated in 12-well plates and stabilized for 12h, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

After stabilization, cells were either maintained in culture medium (control) or pre-incubated, during 1 h, with naringin (400 µM), naringin 6"-acetate (400 µM), naringin 4"',6"-diacetate (250 µM), naringin 6"-butanoate (400 µM), naringin 6"-propanoate (200 µM and 50 µM) and naringin 6"-cinnamate (100 µM)- concentrations that inhibited NO production and were noncytotoxic to macrophages. It was also done controls for the vehicles ethanol and DMSO.

Then, the cells were incubated alone with the compounds or with 1 µL/mL LPS for 24h, with or without compounds, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

After the treatments, RAW 264.7 cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 2 mM ethylenediamine tetraacetic acid), supplemented with 1 mM dithiothreitol (DTT), proteases (1:7 RIPA) and phosphatases (1:10 RIPA) inhibitor cocktails. The cells were scraped and the suspension was collected into cooled microtubes. After sonication, the cells were centrifuged at 12000*g* for 10 minutes at 4 °C to remove nuclei and the insoluble cell debris. The supernatant was collected and stored at -80 °C until use.

Protein concentration was measured by the bicinchoninic acid protein assay (Smith *et al.*, 1985), which is based on the reduction of Cu^{+2} to Cu^{+1} by proteins, in an alkaline solution, that results in a purple colour formation by bicinchoninic acid. The cells were diluted in a 96-well plate and it was added 200 µL of reagent containing 4% copper (II) sulphate and

bicinchoninic acid (ratio of 1:1). The solutions were incubated at 37 °C for 30 minutes. It was measured the absorbance at 570 nm using a microplate reader. The protein concentration was calculated using a standard curve of bovine serum albumin (BSA).

Cell lysates were denatured in sample buffer (0.25 mM Tris pH 6.8, 4% (w/v) sodium dodecyl sulphate, 200 mM DTT, 20% glycerol and bromophenol blue), at 95 °C for 5 min.

4.5.2. Western blot analysis

Western blot analysis was carried out to evaluate the levels of iNOS. After denaturation, 30 µg of proteins were separated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS-PAGE) in 10% (v/v) polyacrylamide gel, for 1h at 160 V, and transferred to PVDF membranes, as previously reported by the team (Liberal *et al.*, 2014). The PVDF membranes were preactivated in methanol for 5 minutes, followed by water for 5 minutes, under stirring. After being kept in transfer buffer, the membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T) under agitation, for 1h, in order to prevent non-specific antibody binding. The membranes were then incubated, overnight at 4 °C, with the primary antibody for iNOS (1:1000). The membranes were washed with TBS-T and incubated with the anti-mouse secondary antibodies (1:20000), diluted in TBS-T with 1% skimmed milk, for 1h.

Protein detection was performed using the enhanced chemifluorescent (ECF) substrate and the imaging system Typhoon™ FLA 9000. The quantification of bands densitometry was performed using the Image Quant 6.0 software. Anti-β-tubulin antibody (1:20000) was used for loading control and data were normalized by calculating the ratio between the intensities of the bands of iNOS and β-tubulin. Results are expressed as percentage of LPS relatively to their respective vehicles.

4.6. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) of, at least, three experiments. Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by *Dunnett's* post-hoc test and applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA). A p value <0.05 was considered to represent significant differences.

Chapter IV

Results and Discussion

IV. Results and Discussion

Acylation of glycosylated flavonoids has been described as an efficient strategy to not only modify their physicochemical properties but also improve their biological properties. These compounds have in their structure several hydroxyl groups and their acylation through chemical methods is not feasible since it requires many protection/deprotection steps. Therefore, in this work, the acylation of rutin and naringin was accomplished by enzymatic catalysis, which is regioselective and allows the isolation of pure derivatives in high yields.

The selection of lipase and solvents was made based on previous work done by Semedo (2015). The enzyme screening showed that Novozym 435 (immobilized CALB) was the best enzyme to acylate rutin. Indeed, this enzyme is an excellent biocatalyst in what concerns enantioselectivity and regioselectivity, and it is widely used in acylation of flavonoid glycosides (Araújo, De *et al.*, 2017b; Cruz *et al.*, 2017; Sekhon-Loodu *et al.*, 2015).

The selection of the solvent is a determining factor for the performance of reaction. Butanone was the selected solvent to perform enzymatic acylation of rutin and acetone was the chosen solvent to perform enzymatic acylation of naringin (Semedo, 2015).

1. Enzymatic synthesis of glycosylated flavonoid esters

The best reaction conditions were selected and reactions were performed on a larger scale. The isolation of the corresponding ester gave yields ranging from 48 to 84% of the starting material and the identification of the products was made by ^{1}H NMR and ^{13}C NMR analysis, along with the starting substrate for comparison.

1.1. Enzymatic acylation of rutin

Rutin has multiple hydroxyl groups (four phenolic and six secondary alcohols; ***Figure 16***) that can undergo acylation by CALB. It was previously described that enzymatic acylation of rutin occurs on the secondary hydroxyl groups of the sugar moiety, primarily on the 4'''-OH position of rhamnose. However, it is reported as well the enzymatic acylation on a second position, giving a diacylated compound (Araújo, De *et al.*, 2017a).

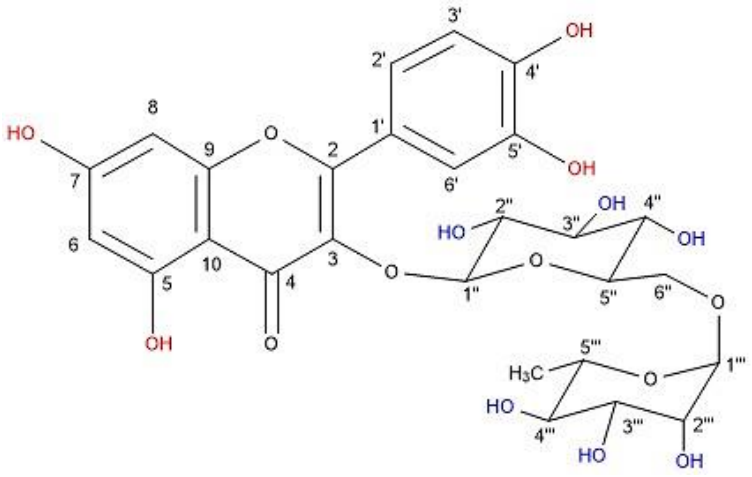

***Figure 16**- Hydroxyl groups of rutin*

Rutin reactions were carried out in butanone since it was the solvent that better favoured the conversion of the substrate.

When vinyl acetate was used as acylating agent, it was observed, by TLC, two different products, one resulting from monoacylation and the other from diacylation. The control of the reaction conditions allows to get one (resulting from monoacylation) or another (resulting from diacylation) product in a higher relative proportion. Therefore, it was possible to obtain the diacylated derivative by using an excess of vinyl acetate and a higher amount of enzyme.

The diacetate derivative was isolated by column chromatography, giving an ester in 54.20 % yield (***Figure 17***).

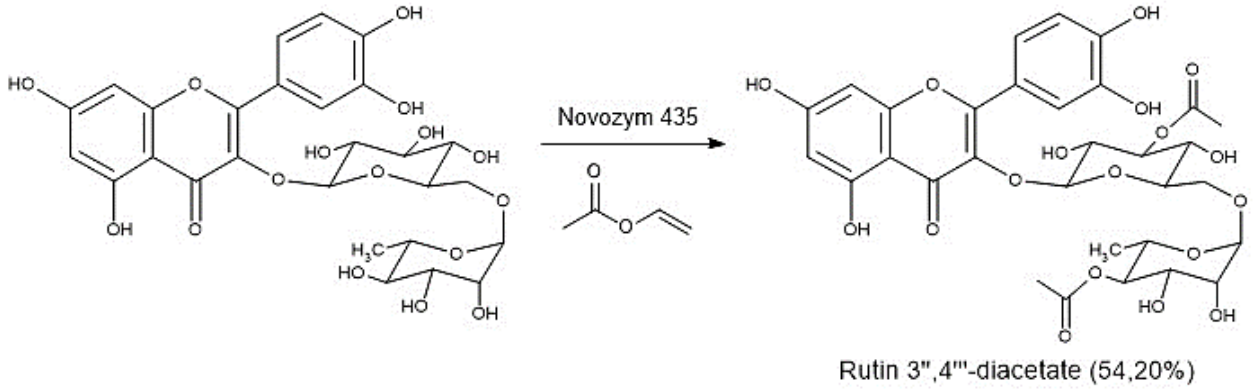

Figure 17- Lipase-catalysed diacylation of rutin with vinyl acetate

The data suggested by ^{1}H NMR and ^{13}C NMR spectra and DEPT 135, from a previous work (Semedo, 2015), confirm that the acylation positions are both the carbons of position 4 of rhamnose (C-4"') and position 3 of glucose (C-3"). Danieli *et al.* (1997) have reported these results by acetylation of rutin with CALB, that yielded rutin 3",4"'-*O*- diacetate as the only product.

When vinyl butyrate was used as acyl donor, it was obtained the diacylated compound as the only product, that was isolated by column chromatography, giving an ester yield of 74.82 % (***Figure 18***).

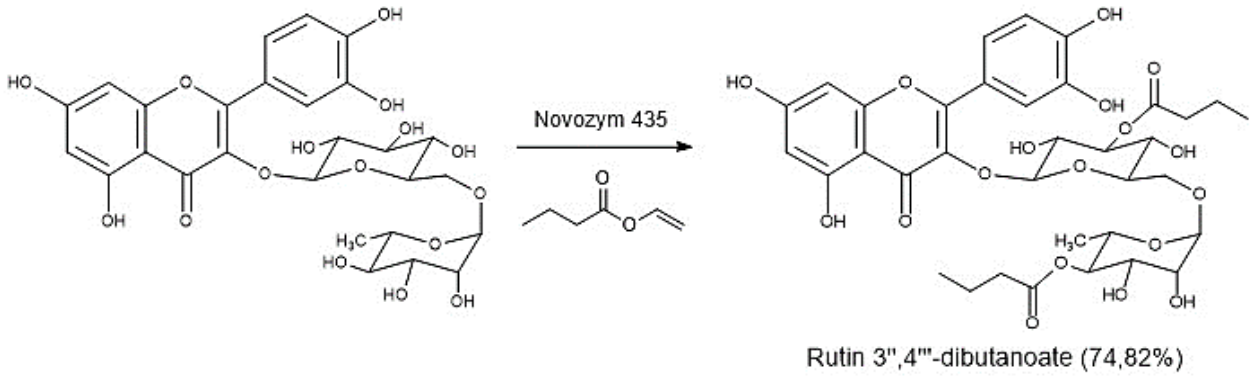

Figure 18- Lipase-catalysed diacylation of rutin with vinyl butyrate

The data proposed by ^{1}H NMR and ^{13}C NMR spectra and DEPT 135, from a previous work (Semedo, 2015), confirm that the acylation positions are both the carbons of position 4 of rhamnose (C-4''') and position 3 of glucose (C-3'').

1.2. Enzymatic acylation of naringin

Naringin has multiple hydroxyl groups (two phenolic, one primary and five secondary alcohols; ***Figure 19***) that can undergo acylation by CALB. It was previously described that enzymatic acylation of naringin occurs on the sugar moiety, mainly on the primary hydroxyl group (Araújo, De *et al.*, 2017a), since this portion of the molecule is more flexible, exhibiting then an easier access to the active site of CALB.

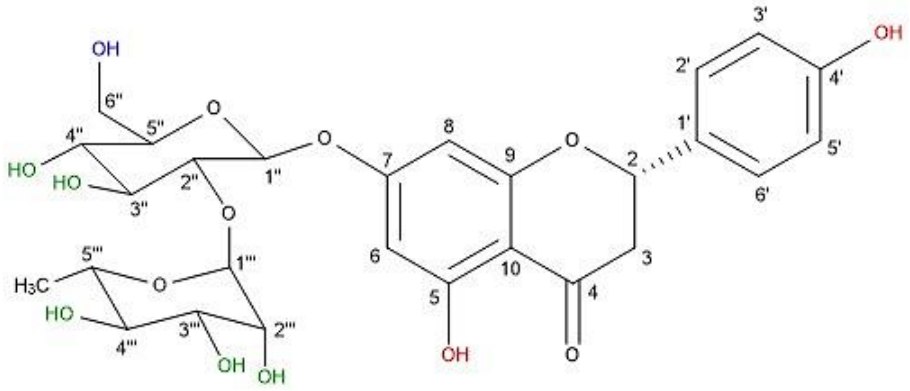

Figure 19- Hydroxyl groups of naringin

The ^{1}H NMR spectrum of naringin (***complementary figure A-1***) provided the characteristic signals of the flavonoid nucleus, such as δ_H 12.05 ppm, attributed to the hydrogen of the C-5 carbon hydroxyl group, involved in an intramolecular hydrogen bond with the C-4, and δ_H 9.60 ppm, relative to the hydroxyl present at C-4 '. Two doublets centered at δ_H 7.33 ppm (2H, d, J = 12 Hz, H-2' and H-6') and δ_H 6.80 ppm (2H, d, J = 8 Hz, H-3' and H-5'), the chemical shift region of aromatic hydrogens present on the C-ring. In addition, a multiplet was observed at δ_H 6.11 ppm referring to the H-6 and H-8 hydrogens of ring A. The doublet at δ_H 5.30 ppm (J = 4 Hz) and the singlet at δ_H 5.11 refer to the hydrogens attached to the C-1'' and C-1''' anomeric carbons, respectively. The double doublet centred at δ_H 5.52 ppm with J = 4 Hz refers to the H-2 of ring C. The doublet at δ_H 1.15 with J = 4 Hz is characteristic of the methyl group present at the 6''''-position.

The ^{13}C NMR spectrum of naringin (***complementary figure A-2***) provided the shifts of the 27 carbons: 197,22 ppm (C-4); 164,87 ppm (C-7); 162,94 ppm (C-5); 162,76 ppm (C-9); 157,83 (C-4'); 128,65 ppm (C-1'); 128,55 ppm (C-2'); 128,45 ppm (C-6'); 115,21 ppm (C-3' and C-5'); 103,31 ppm (C-10); 100,39 ppm (C-1"); 97,42 ppm (C-1'"); 96,31 ppm (C-6); 95,12 ppm (C-8), 78,63 ppm (C-2); 77,14 ppm (C-5"); 76,90 ppm (C-3"); 76,08 ppm (C-2"); 71,82 ppm (C-4'"); 70,47 ppm (C-3'"); 70,39 (C-2'"); 69,58 (C-4"); 68,29 ppm (C-5'"); 60,44 ppm (C-6"); 42,10 ppm (C-3); 18,04 ppm (C-6'").

Naringin acylation reactions were carried out using acetone as the solvent for vinyl acetate, vinyl propionate, vinyl butyrate and vinyl cinnamate.

When vinyl acetate and vinyl propionate were used as acyl donors, it was observed, by TLC, two different products, one resulting from monoacylation and the other resulting from diacylation. From the enzymatic acylation of naringin with vinyl acetate, the product resulting from monoacylation was isolated, giving an ester yield of 48 % (***Figure 20***), which can be explained by the difficult task to perform an efficient separation of the two resulting products.

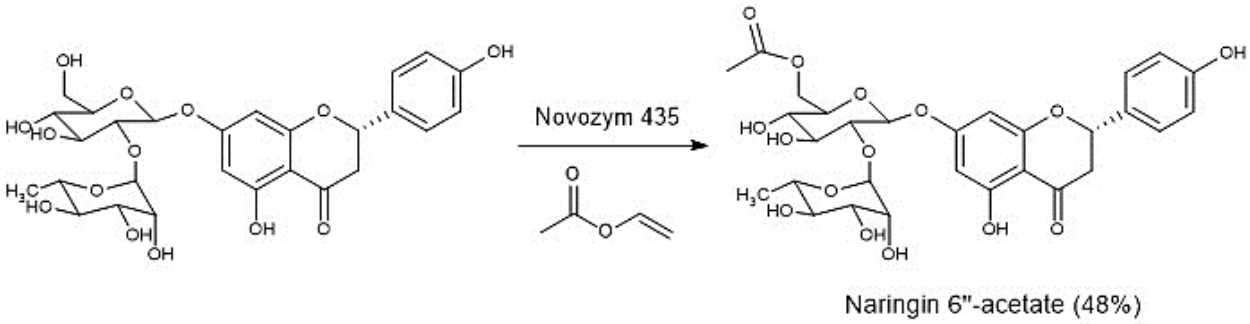

Figure 20- Enzymatic monoacylation of naringin with vinyl acetate

The ^{1}H NMR spectra of naringin 6"-acetate (***complementary figure A-3***) shows a singlet (3H) at δ_H 1.14 ppm characteristic of the methyl group of the acetate, while the ^{13}C NMR spectra (***complementary figure A-4***) shows an additional peak at 170,12 ppm corresponding to the acetyl group.

The data provided by ^{1}H NMR and ^{13}C NMR spectra is in agreement with previous results of the group (Semedo, 2015) and suggest the presence of an ester bonded at the C-6 oxygen of the glucose moiety of naringin, confirming the position of monoacylation by vinyl acetate, which agrees with literature.

From the enzymatic acylation of naringin with vinyl propionate, the product resulting from monoacylation was isolated, giving an ester yield of 60.19 % (***Figure 21***).

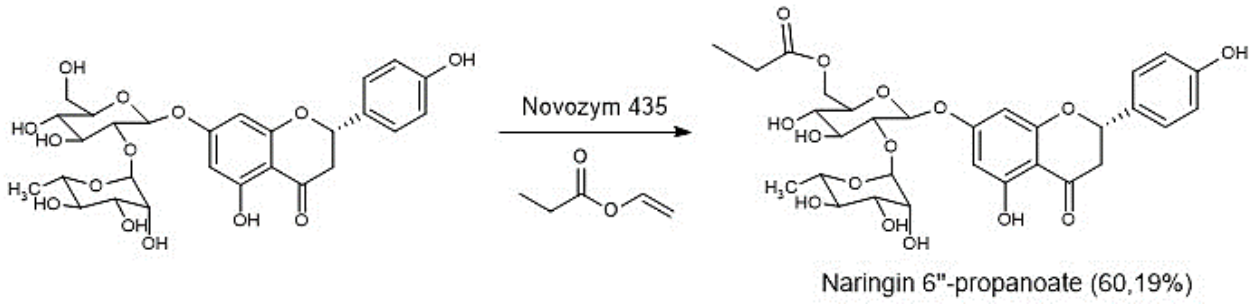

Figure 21- Enzymatic monoacylation of naringin with vinyl propionate

The data provided by ^{1}H NMR and ^{13}C NMR spectra (***complementary figure A-9 and A-10***) suggest the presence of an ester bonded to the hydroxyl group of C-6 of the glucose moiety of the naringin molecule, confirming the position of monoacylation by vinyl propionate, which agrees with the previous work (Semedo, 2015).

It was possible to obtain the diacylated derivatives by using an excess of vinyl acetate and a higher amount of enzyme. The diacetate derivative was isolated by column chromatography, giving an ester yield of 58.96 % (***Figure 22***).

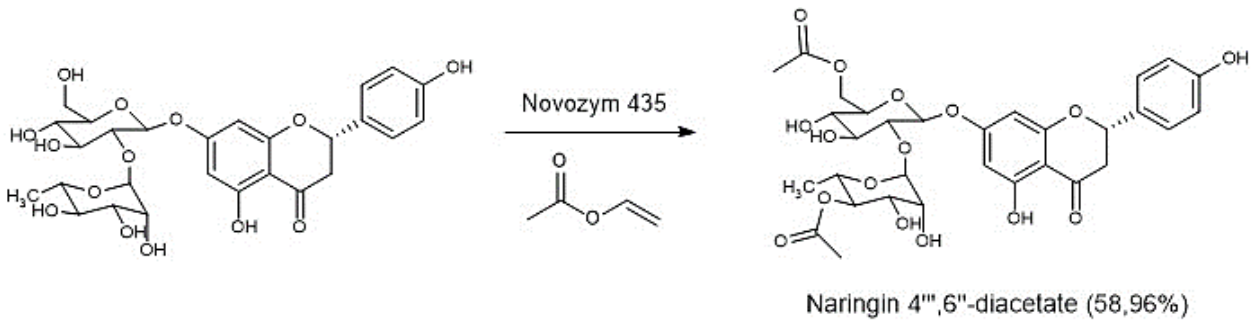

Figure 22- *Enzymatic diacylation of naringin with vinyl acetate*

The ^{1}H NMR spectra (***complementary figure 5***) of the product obtained shows two singlets at 2.00 and 2.04 ppm corresponding to the methyl hydrogens of the acetate groups. The ^{13}C NMR spectra (***complementary figure 6***) shows two additional signals at 172.62 and 172.73 ppm corresponding to the carbonyl groups of the acetates.

The diacylation by vinyl acetate took place at the C-4''' position besides the C-6'' position.

From the reaction with vinyl propionate, it was also possible to obtain the diacylated derivative by using an excess of vinyl propionate and a higher amount of enzyme. The diacetate derivative was isolated by column chromatography, giving an ester yield of 62.87 % (***Figure 23***).

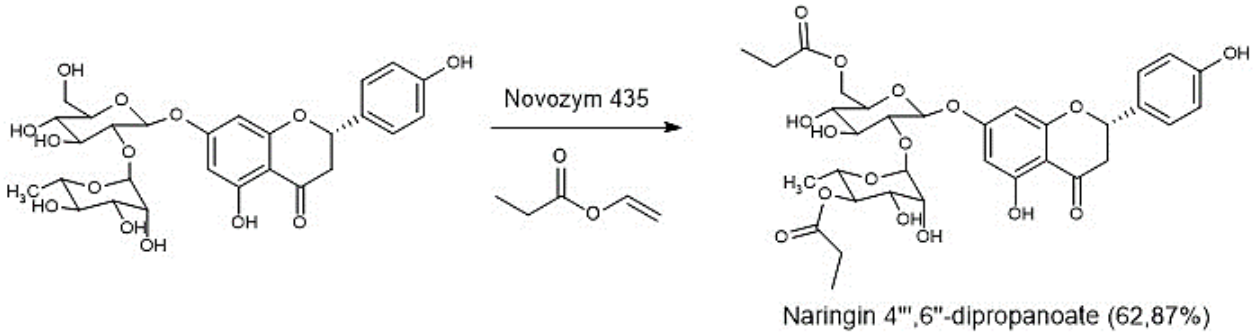

Figure 23- Enzymatic diacylation of naringin with vinyl propionate

The ^{1}H NMR spectra (***complementary figure A-11***) of the product obtained shows two triplets at 1.01 and two multiplets at 2.33. The ^{13}C NMR spectra (***complementary figure A-12***) shows two additional signals at 117.02 and 129.49 ppm corresponding to the carbonyl groups of the propionates. The diacylation by vinyl propionate took place at the C-4''' position besides the C-6'' position.

When vinyl butyrate was used as acylating agent, the product resulting from monoacylation was isolated by column chromatography, giving an ester yield of 73.63 % (***Figure 24***).

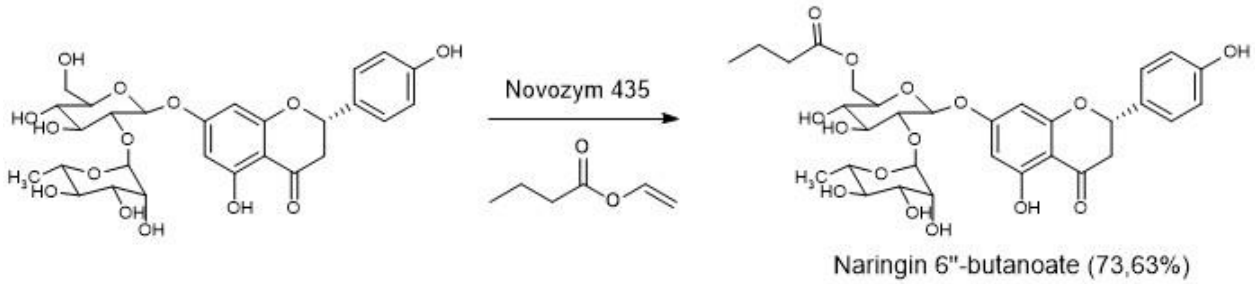

Figure 24- Enzymatic monoacylation of naringin with vinyl butyrate

The data provided by ^{1}H NMR and ^{13}C NMR spectra (***complementary figures A-7 and A-8***) is in agreement with the previous work (Semedo, 2015).

When vinyl cinnamate was used as acyl donor, it was obtained the monoacylated compound as the only product, that was isolated by column chromatography, giving an ester yield of 62.50 % (***Figure 25***).

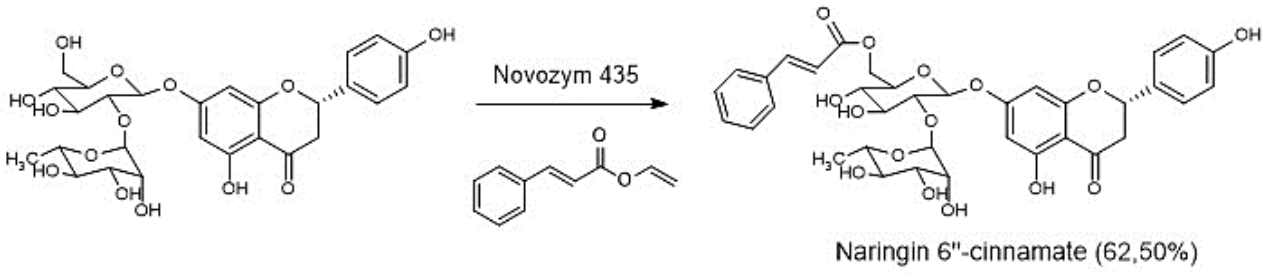

Figure 25- Enzymatic acylation of naringin with vinyl cinnamate

The data provided by ^{1}H NMR spectra indicates the presence of an additional aromatic moiety (7.3 – 7.8 ppm) and the ^{13}C NMR provides a carbonyl signal at 164.76 ppm.

1.3. Effect of the acyl donor in the acylation reaction fate

The binding site of CALB is elliptical and narrow and with the increase in the number of carbons of the acyl donor or the increase in the size of the molecule, the steric hindrance leads to low reaction competence.

In both rutin and naringin enzymatic acylation, it was possible to notice that when vinyl butyrate (C4) was used as acylating agent, the conversion yields was among the highest, that is 74.82 % for rutin acylation and 73.63 % for naringin acylation. These data are in accordance with previous reports (Katsoura *et al.*, 2006) which indicate that the highest CALB acylation yields of naringin and rutin are obtained using short chain acyl donors up to 4 carbons, being the highest conversion yield (65 %) with an acyl donor chain length of 4 carbons.

In the same study, it is also reported the acylation of naringin with vinyl butyrate, giving a diacylated compound as a second product along with the monoacylated compound as the main product. Indeed, this was confirmed in various acylation reactions performed in this study, in which it was observed, by TLC, the monoester in higher proportion along with the diester. The control of the reaction allows to get the diacylated compound, namely by using an excess of acyl donor.

It can be also assumed that the regioselectivity of the enzyme not only can be related to the amount of acylating agent but also to the nature of the acylating agent. Once again, the structure and length of the acyl donor can be determinant to the regioselectivity of the enzyme towards one or two hydroxyl groups. An example of this is that diacylation is possible with the use of short chain acyl donors (vinyl acetate, vinyl propionate, vinyl butyrate) and it is impossible with the use of bulky acyl donors like vinyl cinnamate, regardless the reaction time and the amount of vinyl cinnamate, as observed in this study.

2. Assessment of the anti-inflammatory effect

The evaluation of the anti-inflammatory effect was carried out for naringin and its derivatives, since the anti-inflammatory effect of rutin and its derivatives was previously evaluated (Barbosa, 2016). In that work, it was reported that rutin and its derivatives exhibited significant antioxidant activity for NO radical, which is similar to their inhibitory activity towards NO production in macrophages and independent of structural modifications, suggesting that the effect of rutin and its derivatives is related to the scavenger activity of these compounds and not to the modulation of the production of NO by inhibiting iNOS expression. This conclusion was confirmed by the evaluation of iNOS expression in macrophage cells treated with a derivative ester of rutin, demonstrating that the compound does not interfere with iNOS expression.

2.1. Solubilization of naringin and its derivatives

The solubilization conditions remain the same of the previous work (Barbosa, 2016), in which naringin and its derivatives were dissolved in ethanol, for an accurate comparison.

However, two new acylated compounds exhibited solubilization concerns. The naringin 6"-cinnamate was not able to be dissolved in ethanol, precipitating in this solvent. Therefore, DMSO was used as vehicle for this compound at a concentration that does not interfered with the anti-inflammatory activity. Indeed, DMSO is the most used solvent for *in vitro* biological assays, namely in studies with polyphenols (Vavříková *et al.*, 2016; Wang *et al.*, 2017; Warnakulasuriya, Ziaullah & Rupasinghe, 2016).

The derivative naringin 4"',6"-dipropanoate presented solubility problems in culture media, even when it was dissolved in ethanol or DMSO, probably due to the introduction of two ester groups that increases its lipophilicity and therefore decreases its solubility in water. For this reason, this derivative was not tested for its anti-inflammatory effect.

2.2. Effects of naringin and its derivatives on cell viability

Along with nitric oxide production evaluation, the effect of naringin and its esters derivatives was evaluated on the viability of RAW 264.7 cells at concentrations ranging from 25 to 400 μM (***Table 2***).

Naringin and most of its derivatives did not presented cytotoxic effects (***Figure 26***). Only naringin 6"-cinnamate decreased the cell viability at a concentration of 400 μM to 0%. Hattori *et al.* (2016) evaluated the anti-inflammatory activity of naringin alkyl esters on RAW 264.7 cells and the derivatives with more than 12 carbons displayed cytotoxicity. These results suggest that acyl donors with long or bulky chains like vinyl cinnamate weaken the integrity of the cells.

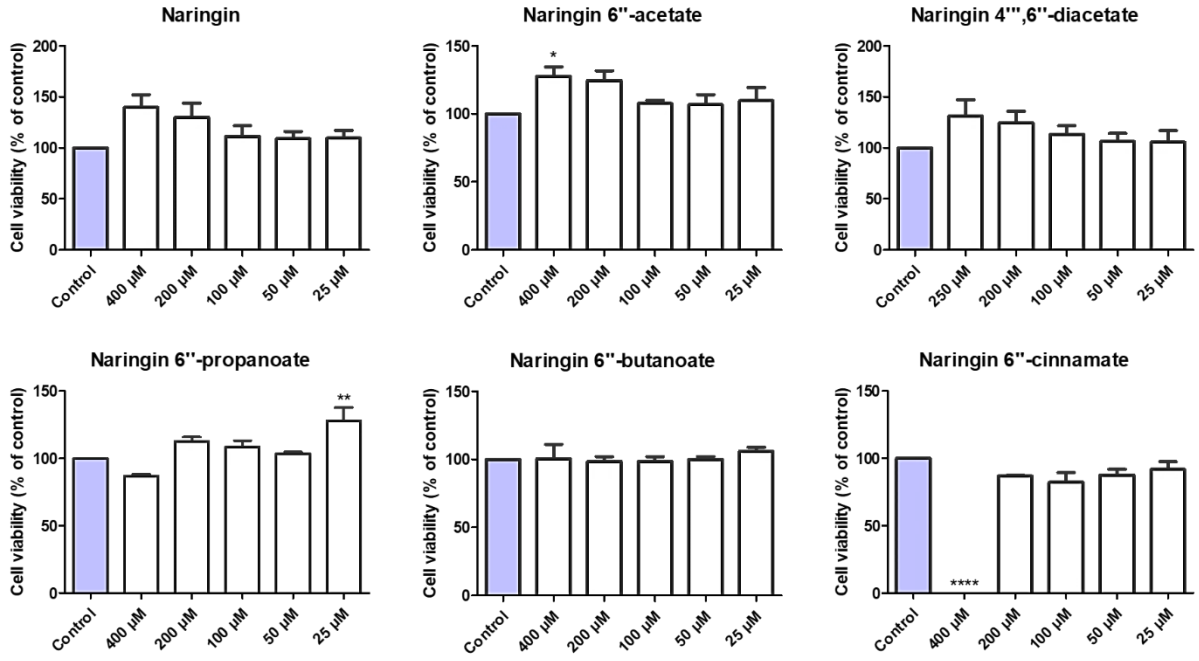

Figure 26- *Effect of naringin ester derivatives on cell viability.* Raw 264.7 cells were either maintained in control medium or pre-incubated with different concentrations of the naringin derivatives and naringin for 1h, and cell viability was measured 24 h after cells treatment using resazurin assay. Data are expressed as percentage of viability relatively to their vehicles and represent the mean ± SEM from 2 and 3 independent experiments. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$, significantly different from control, as determined by one-way ANOVA followed by *Dunnet's* post-hoc test.

2.3. Effects of naringin and its derivatives on nitric oxide production

In the presence of an inflammatory trigger, such as the *Toll-like* receptor agonist LPS, macrophages increase the expression of nitric oxide synthase (iNOS) and cytokines. The pro-inflammatory cytokines also induce the expression of iNOS that generates large amounts of NO over a period of days. iNOS activity is independent of the level of calcium in the cells, unlike the other two isoforms of nitric oxide synthase (neuronal NOS and endothelial NOS). The production of NO by iNOS lasts much longer and tends to produce much higher concentrations of NO in the cell (Sharma, Al-Omran & Parvathy, 2007).

The overproduction of NO leads to a sustained inflammatory environment that conducts to tissue damage and many inflammatory diseases. Therefore, compounds targeting the NO production are good candidates for attenuating inflammatory diseases (Hofseth, 2008).

Hence, the effect of naringin and its derivatives was evaluated on NO production by macrophages cultured in the presence of LPS.

The results confirmed that naringin inhibit NO production in the presence of LPS, mainly at the concentration of 400 μM, presenting a significant inhibition of 38% (62.03 ± 9.92 %; $p < 0.01$) (***Figure 27***), which is in accordance with other reported studies (Kanno *et al.*, 2006; Xu, Zhang & Sun, 2017).

The cultures pre-treated with the naringin derivatives presented a significant decrease on the nitrite production comparing to LPS treated cells, in almost a dose-dependent manner (***Figure 27***). At a concentration of 400 μM, naringin 6"-cinnamate displayed a big reduction on NO production (15.35 ± 1.31%), however this result is probably due to its strong cytotoxicity. The NO production generated by cells treated with naringin ester derivatives, at a concentration of 200 μM, that was not cytotoxic, was decreased for all the tested compounds when compared with naringin, which allows to conclude that the structural modifications through acylation improved the anti-inflammatory activity of naringin.

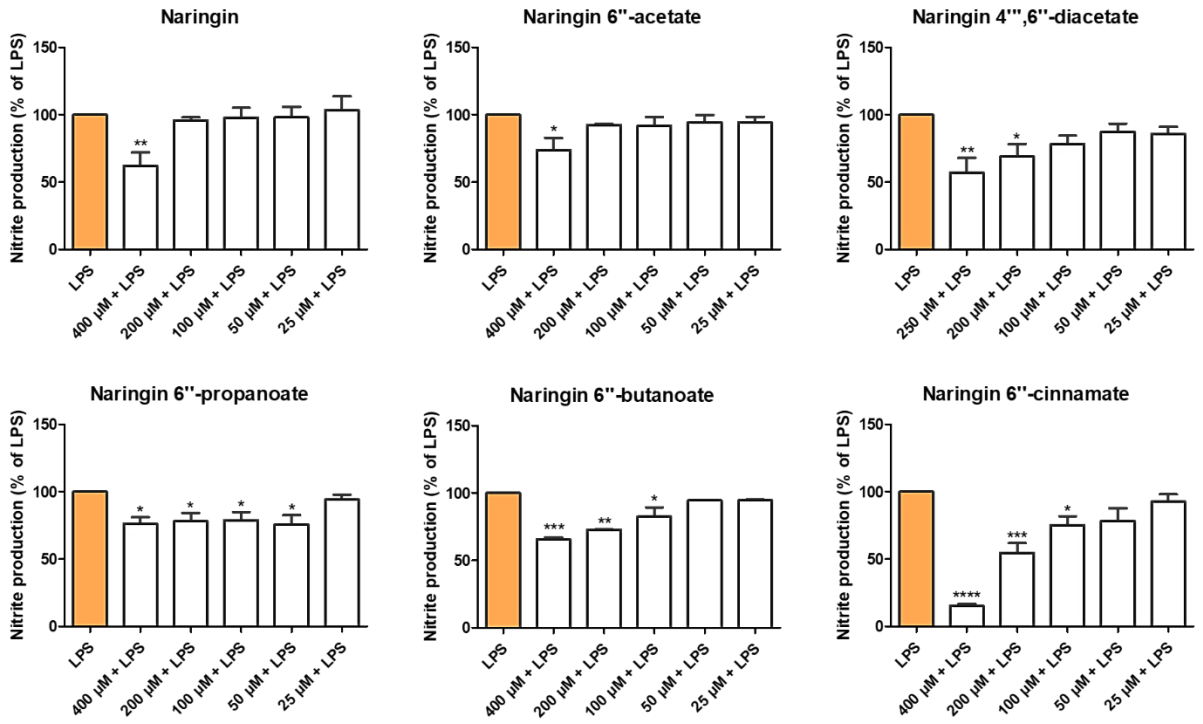

Figure 27- *Effect of naringin derivatives on NO production.* Cells were pre-treated with naringin derivatives and naringin for 1h, followed by the addition of LPS (50 ng/mL) for 24h. Cell culture supernatants were collected and nitrite concentration in the medium was measured by Griess reaction. Results are presented as percentage of LPS relatively to the respective vehicle and represent the mean ± SEM from 2 and 3 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, significantly different from LPS, as determined by one-way ANOVA followed by *Dunnet's* post-hoc test.

Besides, naringin 6''-cinnamate and naringin 4''',6''-diacetate were the most potent compounds, exhibiting an inhibition on NO production of 45% (54,61 ± 7,20%) and 31% (68,83 ± 9,45%), respectively, at a concentration of 200 µM (***Figure 27***). The third most potent compound was naringin 6''-butanoate, suggesting that diacylated compounds and with long or bulky chains can modulate intracellular molecular targets due to the increase of their lipophilicity, preventing nitrite accumulation. These results are in agreement with Hattori *et al.* (2016), who performed naringin acylation with vinyl esters and showed that the inhibition of NO production is related to the increase of chain length of naringin derivatives, being naringin C-12 derivative the most potent compound.

2.4. Effects of naringin and its derivatives on NO scavenging activity

To further clarify the mechanism whereby naringin and its ester derivatives lead to a decreased production of nitrites, the NO scavenging capacity of these compounds was investigated, using SNAP as NO donor.

All the naringin derivatives were evaluated for the NO scavenging capacity at various concentrations, except the naringin 6"-butanoate since it was previously evaluated (Barbosa, 2016) and it did not exhibit NO scavenging effect.

The NO scavenging effect of naringin and its other derivatives was also not very expressive (***Figure 28***), when compared with the decrease on NO production displayed by these compounds. The results suggest that the activity exhibited by these compounds is mainly related to an inhibition on NO production by macrophages.

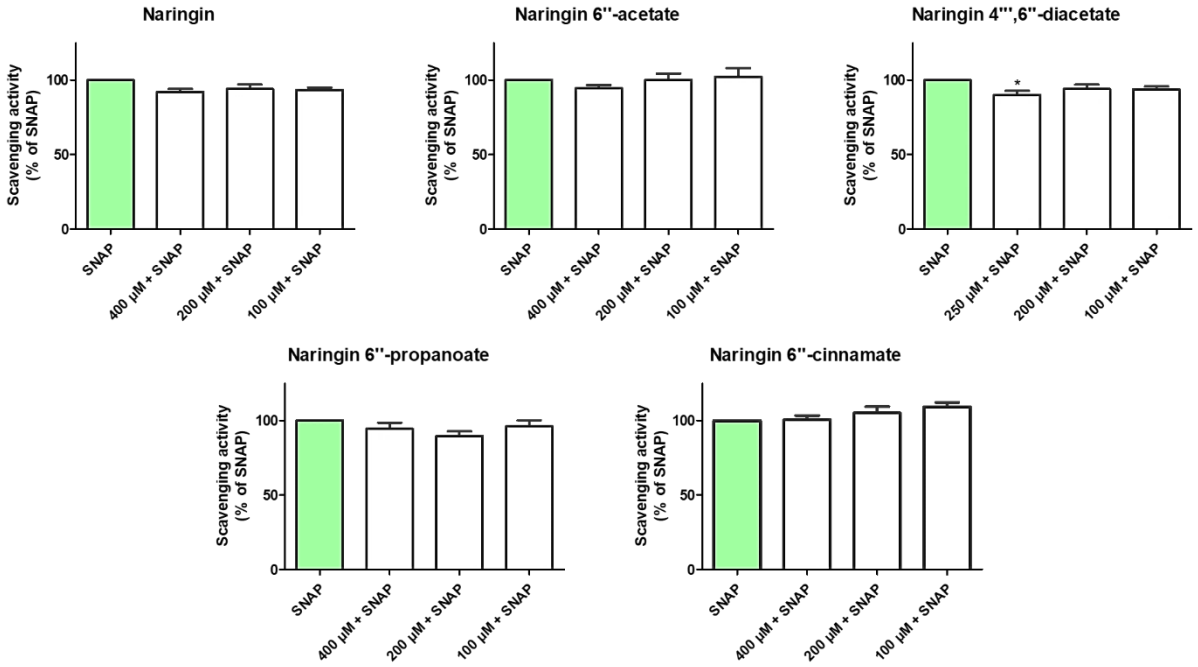

Figure 28- *Effect of naringin derivatives on NO scavenging activity.* To evaluate the scavenging properties of naringin derivatives and naringin, SNAP was used as NO donor. The mediums were collected after an incubation of 3h with SNAP and/or the naringin ester and/or naringin and nitrite concentration was measured by Griess reaction. Data are expressed as percentage of SNAP and represent the mean ± SEM from 3 independent experiments. *p < 0.05, significantly different from SNAP, as determined by one-way ANOVA followed by *Dunnet's* post-hoc test.

2.5. Effects of naringin and its ester derivatives on iNOS expression

To investigate if the inhibition of NO production in macrophages exposed to LPS and pre-treated with naringin and its derivatives could be also due to a down-regulation on iNOS protein expression, the western blot assay was performed in cell lysates. Overall, the concentrations with higher inhibitory effect on NO production and without cytotoxicity were selected to carry out this experiment (***Figure 29***). However, given that there was a significant decrease in cell viability when comparing LPS with 200 µM naringin 6''-cinnamate plus LPS (***complementary figure A-13***), it was selected the concentration of 100 µM naringin 6''-cinnamate. The expression of iNOS is presented in relation to tubulin expression, that is used as an internal standard because it remains intact throughout the experiment.

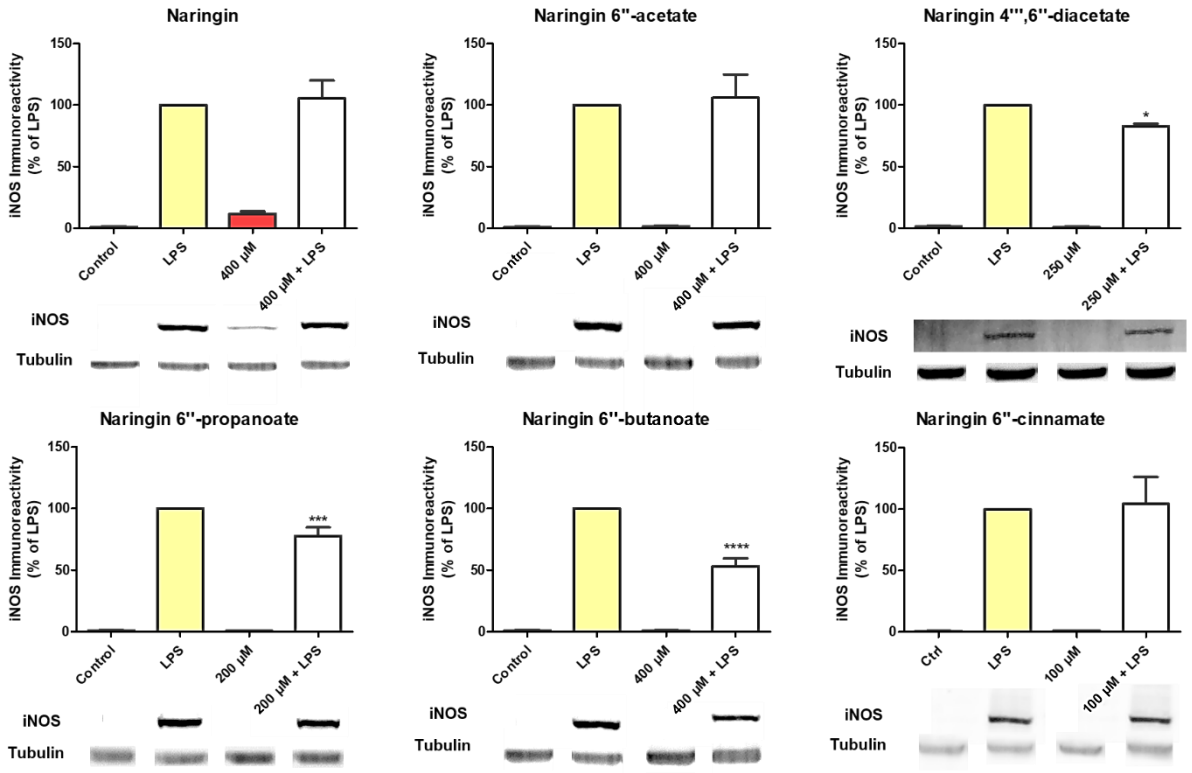

Figure 29- *Effect of naringin derivatives on iNOS expression*. Raw 264.7 cells were pre-incubated with naringin derivatives and naringin, and then were stimulated with LPS for 24h. Representative immunoblots are presented under the graphs. Results are presented as percentage of LPS relatively to their vehicles and represent the mean ± SEM from 3 independent experiments. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$, significantly different from LPS, as determined by one-way ANOVA followed by *Dunnet's* post-hoc test.

There are no differences on iNOS expression between cells treated with LPS alone and cells co-treated with naringin 6"-acetate and naringin 6"-cinnamate. However, naringin 4"',6"-diacetate, naringin 6"-propanoate and naringin 6"-butanoate showed a significant inhibition on iNOS expression of 17 (82.75 ± 2.36%), 22 (77.65 ± 6.98%) and 47% (53.04 ± 6.56%), respectively (***Figure 29***). These results are similar to those obtained for NO production and it is observed that the most effective compound has a longer acyl chain, which is also in accordance with another study (Hattori *et al.*, 2016). In contrast, the naringin 6"-cinnamate no longer has the strongest effect on iNOS expression inhibition. One hypothesis to explain is that naringin cinnamate may interfere with iNOS activity, thus decreasing NO production, but not with its expression. Although, it is possible to observe once again that the anti-inflammatory effect of naringin is potentiated by the introduction of ester groups, namely on iNOS expression.

Strikingly, the results of western blot assay also allowed to observe that in macrophages treated with naringin at concentration of 400 μM, without the activation by LPS, there was an increase on iNOS expression, when compared with the control, differing from what would be expected (Caglayan *et al.*, 2018; Kandemir *et al.*, 2017). Further experiments should be performed to decipher the molecular mechanisms behind this pro-inflammatory effect of naringin alone.

3. Structure-activity relationships of naringin and its derivatives

Naringin exhibits low ability to penetrate the cell membrane which compromises their potential anti-inflammatory activity. In contrast, its acylation allows the increase in the lipophilicity (Araújo, De *et al.*, 2017a), that enhances with the increase of carbons added. Accordingly, our results demonstrated that the acylation of naringin enhanced its anti-inflammatory activity. Naringin 6"-butanoate (C4) was the most potent derivative in inhibiting iNOS expression, followed by naringin 6"-propanoate (C3), leading to hypothesize that compounds diacylated or acylated with long chains have a more effective anti-inflammatory activity, what is in agreement with the study of Hattori *et al.* (2016).

In this study, the authors also investigated the incorporation of naringin C-12 ester in the cell membrane and the results showed that the naringin C-12 ester bond is cleaved by membrane esterases, allowing free naringin to translocate to the cytosol. Once inside the cell, naringin exerts an anti-inflammatory effect by suppressing iNOS expression via modulation of the NF-kB pathway. These results, along with the results of the present study, indicate that the addition of lipophilic ester groups to naringin provide a mean of enhancing the internalization of naringin by cells, increasing its anti-inflammatory properties.

Chapter V

Conclusions

V. Conclusions

Flavonoids are subject of extensive scientific research due to their beneficial effects on human health, namely in inflammation associated chronic diseases.

The acylation of natural flavonoids is known to improve their lipophilicity, resulting in enhanced cellular uptake and modified biological activity, and the enzymatic catalysis is described as the best way to carry out such reactions, due to the high regioselectivity.

Thus, in this work, the enzymatic syntheses of two diacylated rutin esters, as well as four naringin monoesters and two diacylated naringin esters were performed using Novozym 435 and various vinyl esters, in organic media, and their anti-inflammatory potential was evaluated.

The CALB preference for the hydroxyl group at the C-4 position of rhamnose was verified in the enzymatic acylation of rutin with vinyl acetate. Moreover, when is synthesized the diacylated derivative, obtained in the enzymatic acylation reactions of rutin with vinyl acetate and vinyl butyrate, the acylation occurs at the hydroxyl group of C-4 position of rhamnose and also at the hydroxyl group of C-3 position of glucose.

In the enzymatic acylation of naringin with vinyl acetate, vinyl propionate, vinyl butyrate and vinyl cinnamate, the lipase preference for the C-6 position of glucose was confirmed. In addition, in the enzymatic acylation reactions of naringin with vinyl acetate and vinyl propionate, a diacylated derivative was obtained, whose acylation positions were at C-6 of glucose and C-4 of rhamnose.

The results demonstrate the high regioselectivity of lipases towards specific hydroxyl groups of several that are possible to undergo acylation (phenols and secondary and primary alcohols) of complex substrates.

In addition, the results suggest that the nature of the acyl donor can be a determining factor for the regioselectivity of the lipase and for the reaction yield. For instance, the acylation with short chain acyl donors can give higher conversion yields and the acylation with bulky acyl donors, like vinyl cinnamate, cannot give diacylated derivatives, only monoesters.

Furthermore, the results point out the usefulness of enzymatic catalysis in the regioselective transformation of the flavonoids under study, in order to improve their biological properties, namely anti-inflammatory activity.

The anti-inflammatory effect exhibited by the acylated derivatives consists of reduction of NO production and modulation of iNOS expression in macrophages stimulated with LPS. As previously reported, rutin acylated derivatives showed a similar anti-inflammatory activity relatively to rutin, suggesting that this bioactivity is not dependent on its lipophilic profile.

In contrast, the enzymatic acylation of naringin enhanced its activity towards NO inhibition. The derivatives demonstrate an increase in the anti-inflammatory potential compared to naringin, which has low ability to penetrate the cell due to its low lipophilicity.

The derivatives naringin 6"-cinnamate and naringin 4"',6"-diacetate were the most potent compounds in the inhibition of NO production, followed by naringin 6"-butanoate. Since none of the derivatives showed relevant NO scavenging activity, it can be assumed that their inhibition of nitrite accumulation is due to the modulation of iNOS expression. Indeed, the western blot results demonstrated that some derivatives decreased the iNOS expression, namely naringin 4"',6"-diacetate, naringin 6"-propanoate and naringin 6"-butanoate. Naringin 6"-butanoate (C-4) was the most potent compound in inhibiting iNOS expression, followed by naringin 6"-propanoate (C-3), leading to hypothesize that diacylated compounds or acylated compounds with long chain acyl donors can better interact with the cell membrane due to its increased lipophilicity, having a more potent anti-inflammatory effect.

The results also allowed to observe that naringin has a pro-inflammatory effect at 400μM, without the activation by LPS, when compared with the control, differing from what would be expected. Further experiments should be performed to decipher the molecular mechanisms behind this pro-inflammatory effect of naringin alone.

Lipase catalysed acylation of naringin is an effective way to enhance its lipophilicity, providing a mean of enhancing the internalization of naringin by cells, thus improving its anti-inflammatory effect.

The structure-activity relationships reported here allow future enzymatic synthesis of enhanced biologically active compounds and the study of their anti-inflammatory activity *in vivo* will enable the discovery of potential therapeutics. Additionally, further studies should be performed to deeply explore the signalling pathways and the molecular mechanisms behind the anti-inflammatory effect of naringin derivatives, for instance the transcription factors NF-kB, AP-1, as well as their effects on additional pro-inflammatory mediators, such as cytokines and prostaglandins.

Chapter VI

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VI. References

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Chapter VII

Complementary information

VII. Complementary information

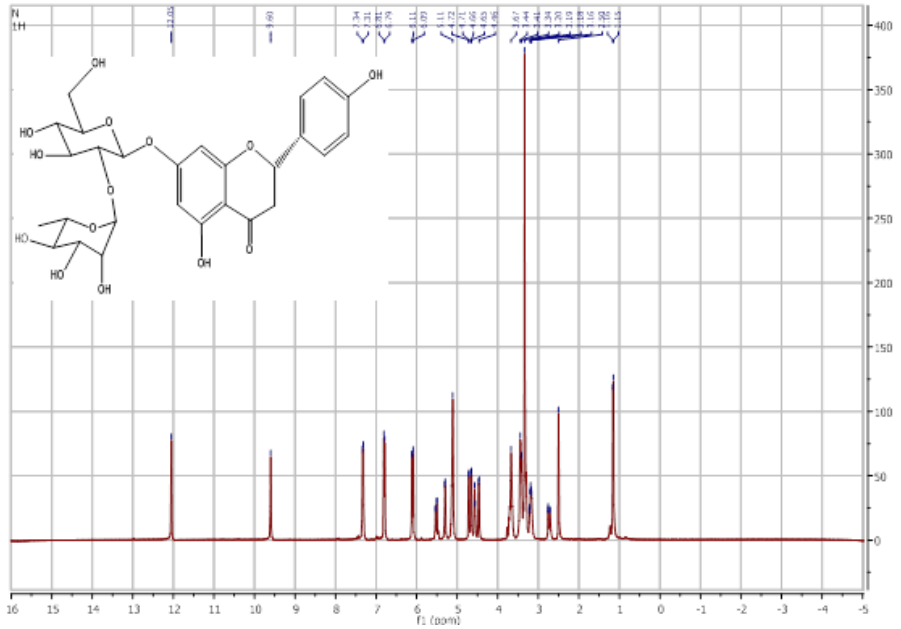
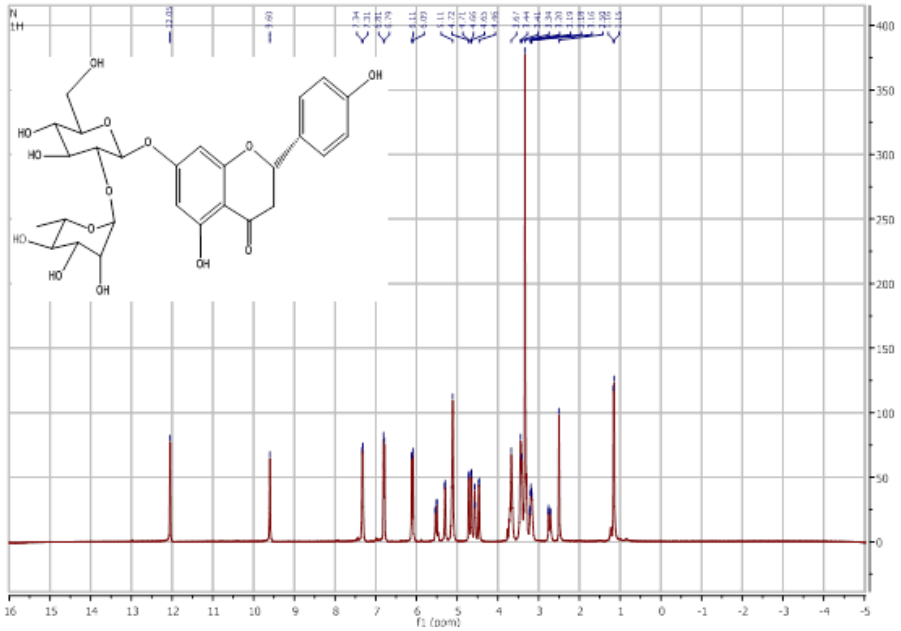


Figure A-1 - ^{1}H NMR spectra of naringin



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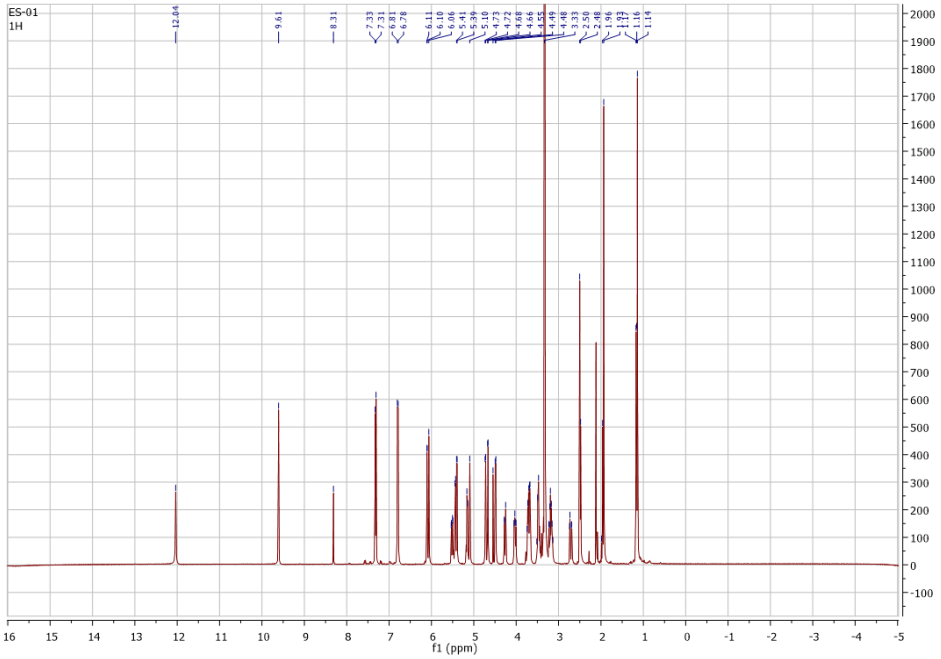

Figure A-3- ^{1}H NMR spectra of the resulting product of the enzymatic monoacylation of naringin with vinyl acetate

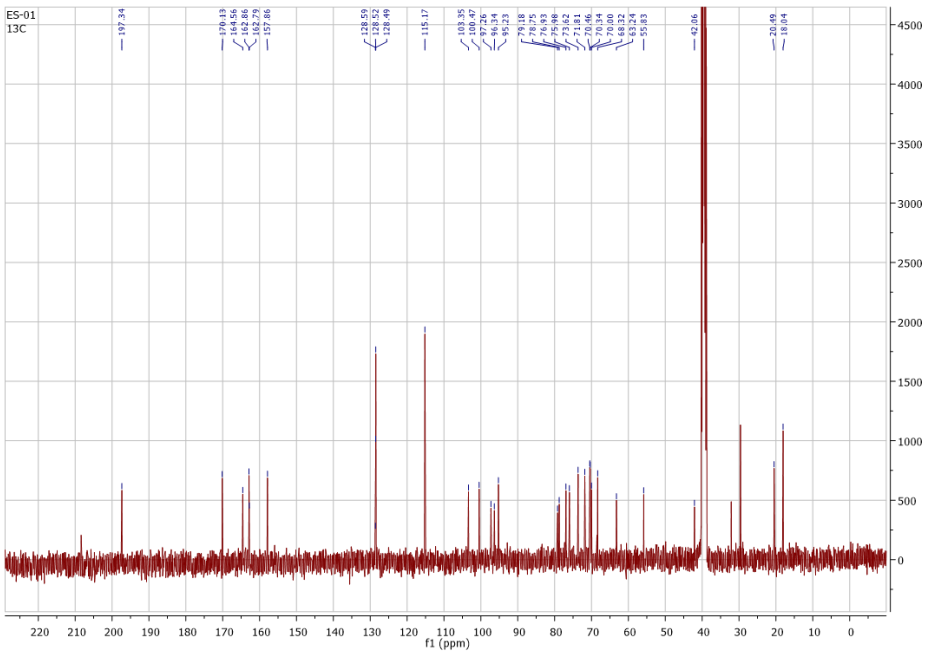

Figure A-4- ^{13}C NMR spectra of the resulting product of the enzymatic monoacylation of naringin with vinyl acetate

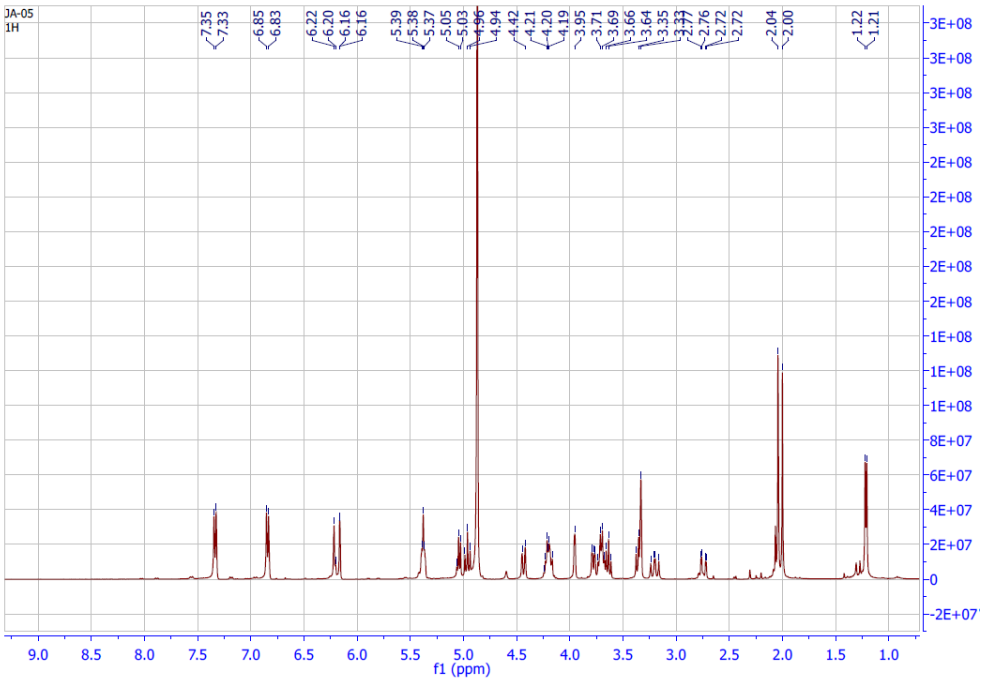

Figure A-5- ^{1}H NMR spectra of the resulting product of the enzymatic diacylation of naringin with vinyl acetate

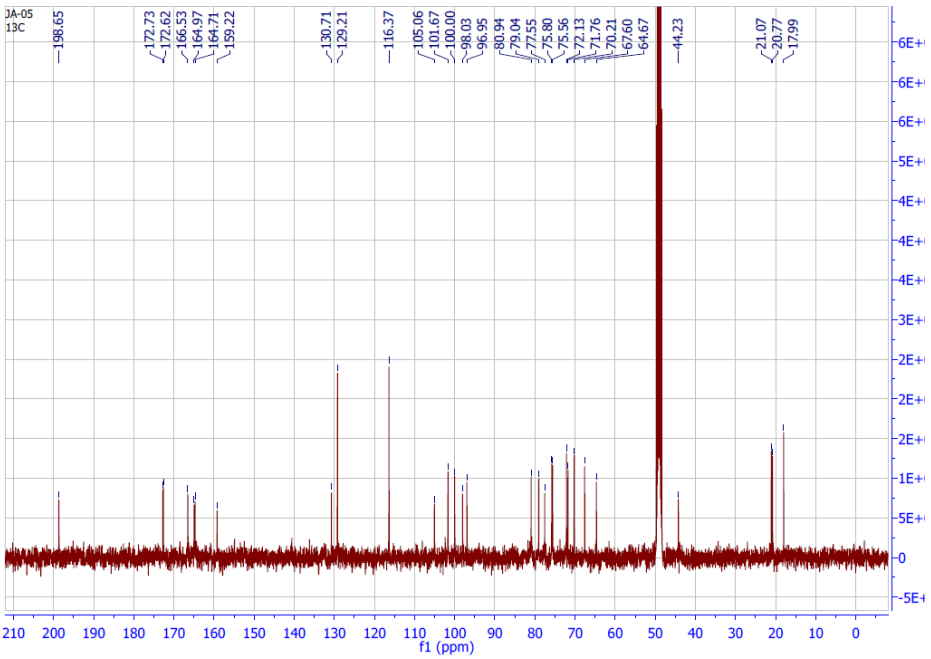

Figure A-6- ^{13}C NMR spectra of the resulting product of the enzymatic diacylation of naringin with vinyl acetate

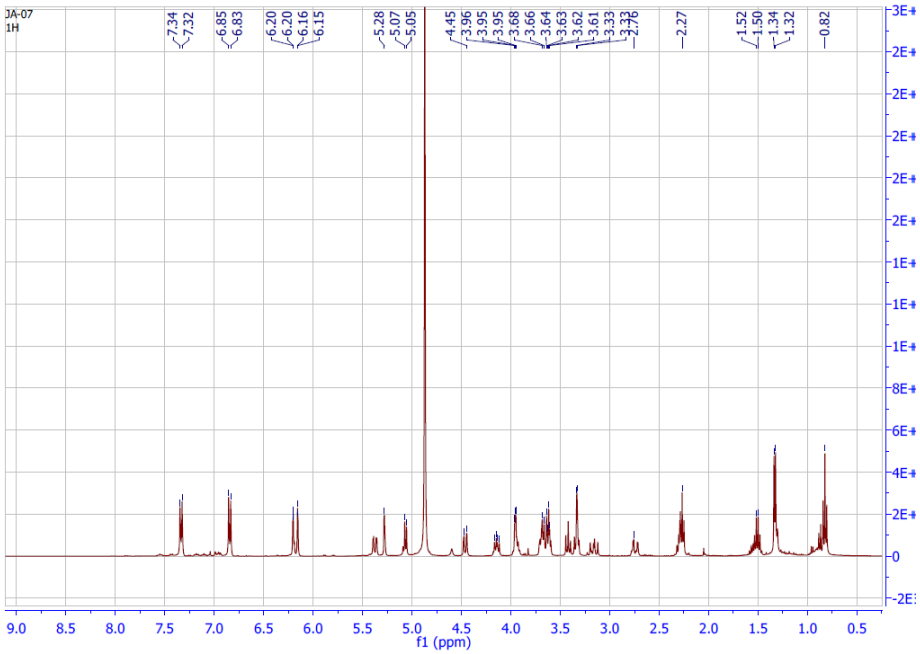

Figure A-7-^{1}H NMR spectra of the resulting product of the enzymatic monoacylation of naringin with vinyl butyrate

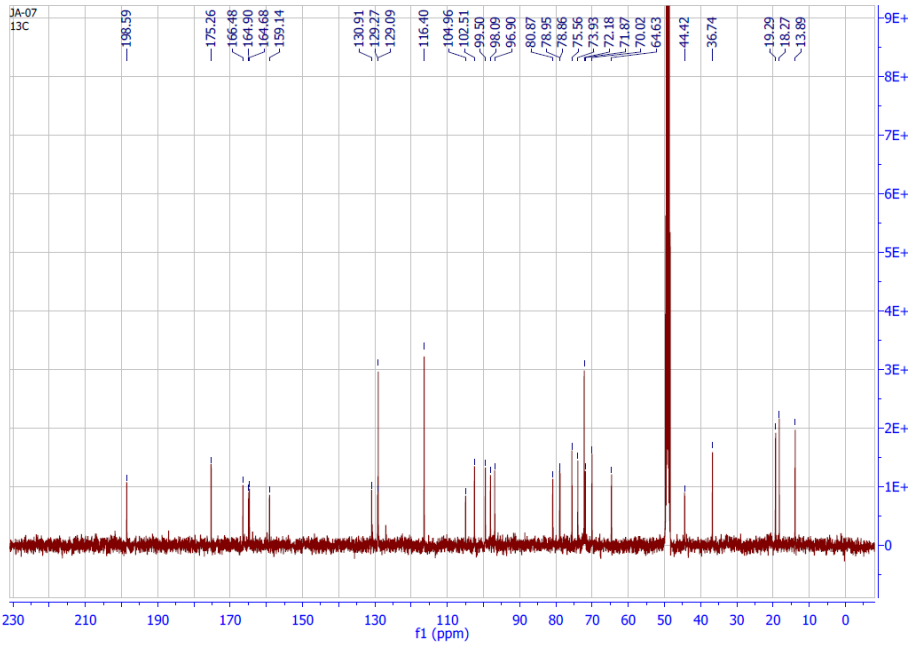

Figure A-8- ^{13}C NMR spectra of the resulting product of the enzymatic monoacylation of naringin with vinyl butyrate

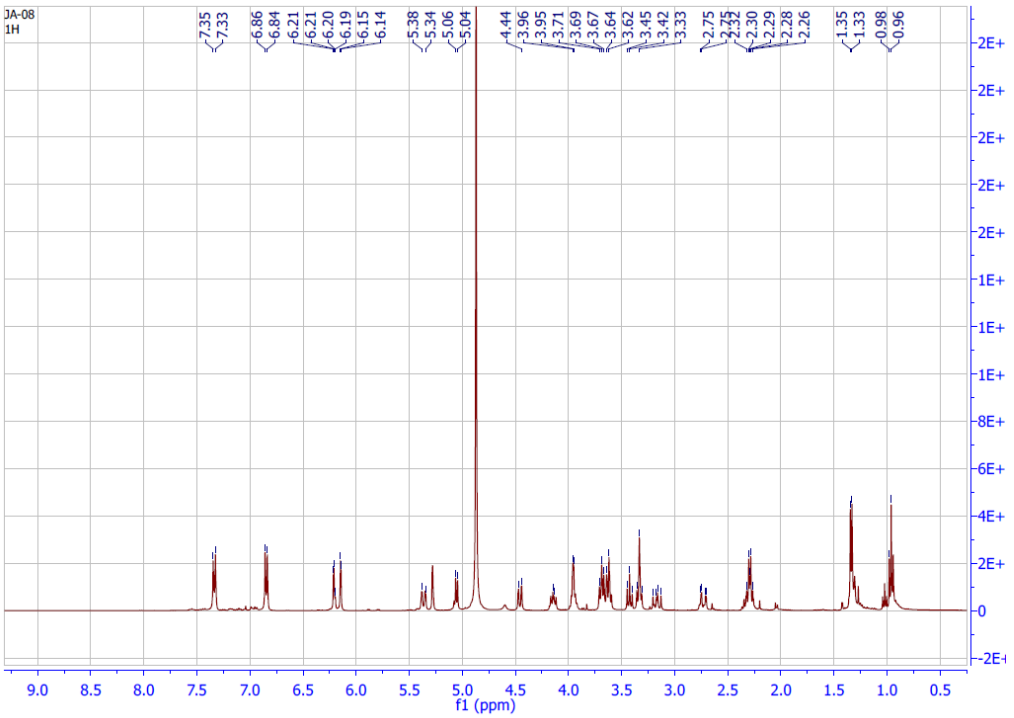

Figure A-9- ^{1}H NMR spectra of the resulting product of the enzymatic monoacylation of naringin with vinyl propionate

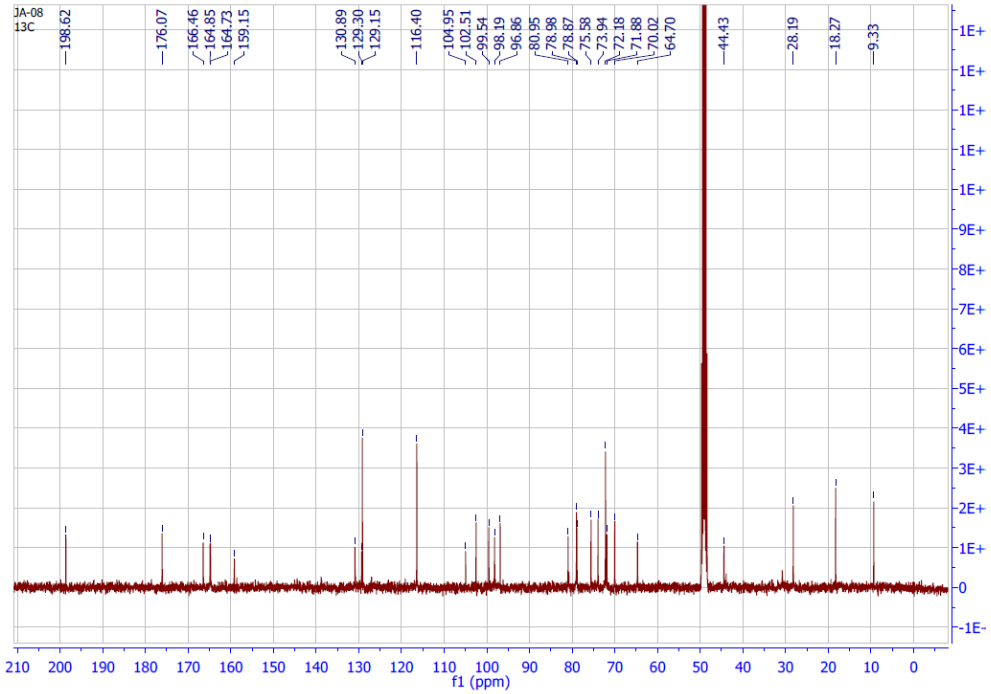

Figure A-10- ^{13}C NMR spectra of the resulting product of the enzymatic monoacylation of naringin with vinyl propionate

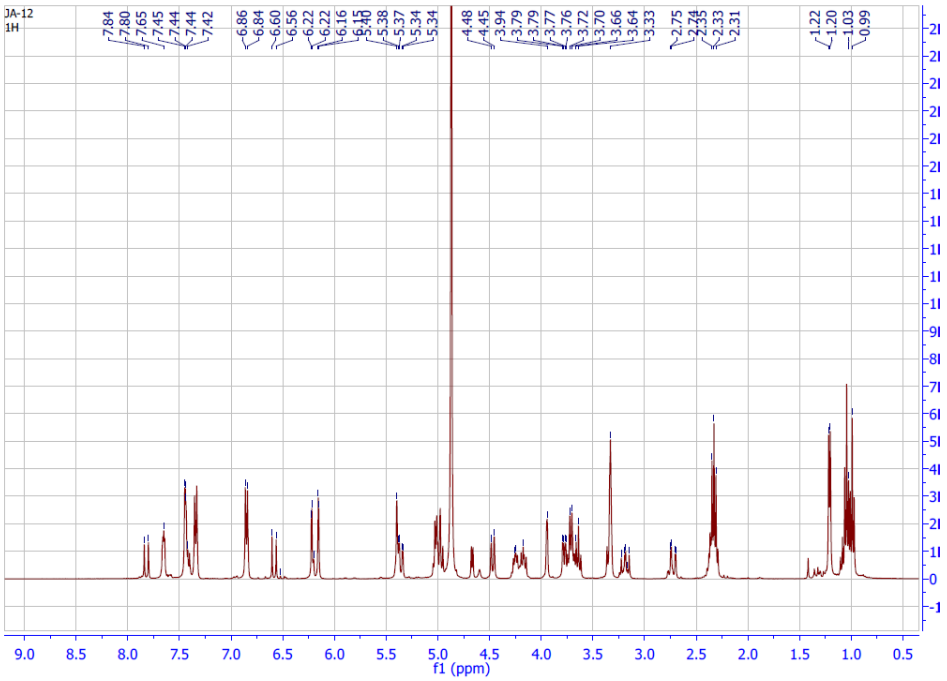

Figure A-11- *^{1}H NMR spectra of the resulting product of the enzymatic diacylation of naringin with vinyl propionate*

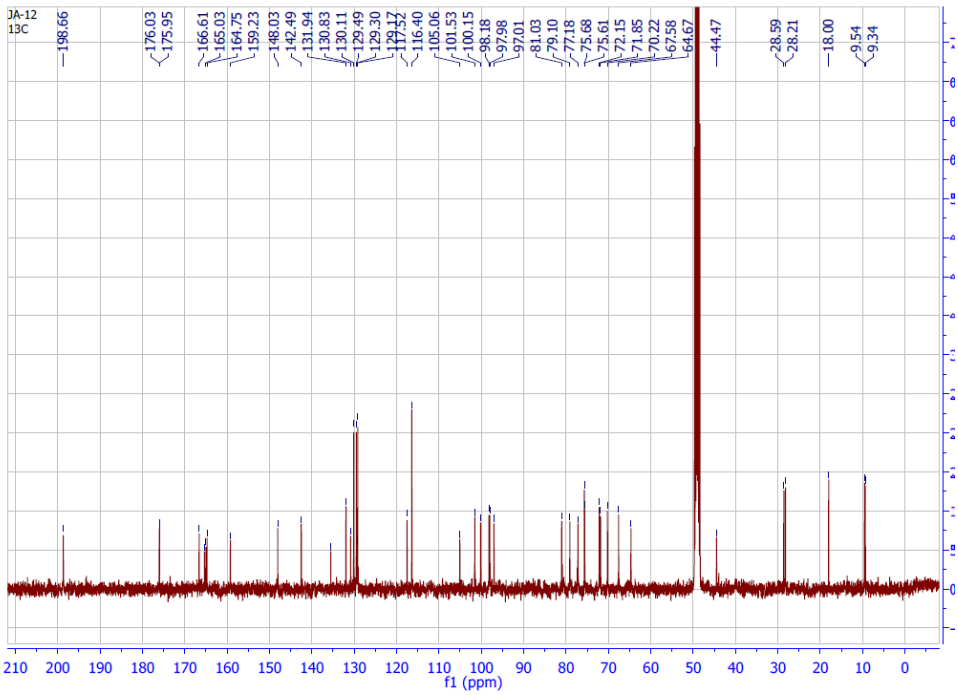

Figure A-12- *^{13}C NMR spectra of the resulting product of the enzymatic diacylation of naringin with vinyl propionate*

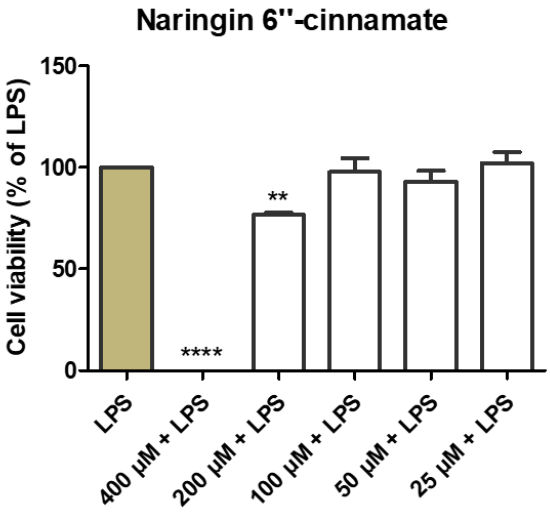

***Figure A-13**- Effect of naringin 6''-cinnamate plus LPS on cell viability.* Raw 264.7 cells were either maintained in control medium or pre-incubated with different concentrations of the naringin 6''-cinnamate and naringin for 1h, and cell viability was measured 24 h after cells treatment using resazurin assay. Data are expressed as percentage of viable cells with respect to LPS and represent the mean ± SEM from 3 independent experiments. **p < 0.01; ****p < 0.0001, significantly different from LPS, as determined by one-way ANOVA followed by *Dunnet's* post-hoc test.