Patrícia da Silva Ferreira

Preparation of new bioactive flavonoid derivatives

Dissertação de Mestrado em Química Farmacêutica Industrial, orientada pelo Professor Doutor Jorge António Ribeiro Salvador e pela Professora Doutora Maria Manuel Cruz Silva e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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"Start by doing what's necessary; then do what's possible;
and suddenly you are doing the impossible"

Francis of Assisi

À minha mãe e irmã

Acknowledgments

Ao longo deste projecto surgiram muitos obstáculos que foram superados com a ajuda de algumas pessoas que não posso deixar de agradecer. Esta dissertação não é apenas minha mas também de todos aqueles que contribuíram de alguma forma para este trabalho.

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Por último, mas não menos importante, a toda a minha família um grande obrigado pelo apoio incondicional, motivação e paciência em todas as fases deste trabalho.

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Abbreviations

AA	arachidonic acid
$ABTS^{\bullet+}$	2,2-azinobis-3-ethylbenzthiazoline-6-sulphonic acid radical scavenging
AUC	area under the curve
Ca^{2+}	calcium
CALB	lipase B from *Candida antarctica*
CAT	catalase
CH_2Cl_2	dichloromethane
^{13}C NMR	carbon-13 nuclear magnetic resonance
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
d	doublet
dd	doublet of doublets
DEPT	distortionless enhancement by polarization transfer
4-DMAP	4-dimethylaminopyridine
$DMPD^{\bullet+}$	N,N-dimethyl-p-phenylenediamine radical scavenging
DNA	deoxyribonucleic acid
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical scavenging
EC	enzyme code
FRAP	ferric ion reducing antioxidant power
GPx	glutathione peroxidase
GSH	glutathione
HCl	hydrochloric acid
5-HETE	5-hydroxyeicosatetraenoic acid
12-HETE	12-hydroxyeicosatetraenoic acid
HIV	human immunodeficiency virus
^{1}H NMR	proton nuclear magnetic resonance
$HO^{\bullet}$	radical hydroxyl
H_2O_2	hydrogen peroxide
5-HETE	5-hydroxyeicosatetraenoic acid

12-HETE	12-hydroxyeicosatetraenoic acid
HX/XO	hypoxanthine-xanthine oxidase
Hz	Hertz
IL-I	interleukin-1
IL-Iβ	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
J	coupling constant
Kcat	turnover number
Km	Michaelis-Menten constant
lipase Ps	lipase from *Pseudomonas cepacia*
LOX	lipoxygenases
5- LOX	5- lipoxygenases
12-LOX	12- lipoxygenases
LT	leukotrienes
LTB_4	leukotrienes B_4
m	multiplet
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NF-κB	nuclear factor kappa B
NMR	nuclear magnetic resonance
$NO^{\bullet}$	nitric oxide radical
NO	nitric oxide
NOS	nitric oxide synthases
NSAIDs	non-steroidal antiinflammatory drugs
$O_2^{\bullet-}$	superoxide ion
O_2	singlet oxigen
PG	prostaglandins
PGE2	prostaglandins E2
PKC	protein kinase C
PLA2	phospholipases A2
ppm	parts per million
ROS	reactive oxygen species
s	singlet

SAR	structure-activity relationship
SOD	superoxide dismutase
t	triplet
t1/2	half-life
TLC	thin-layer chromatography
TNF	tumor necrosis factor
TX	thromboxanes
UV	ultraviolet radiation
XO	xanthine oxidase
δ	chemical shift

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Resumo

Os polifénois são compostos naturais de grande interesse, presentes em diversos produtos do nosso dia-a-dia, devido às diversas atividades biológicas comprovadas, nomeadamente antioxidante, anti-inflamatória e anticancerígena. No entanto a sua aplicabilidade na indústria farmacêutica está condicionada pela baixa estabilidade e solubilidade em meio lipofílico.

Uma das estratégias eficazes utilizadas para melhorar as propriedades destes compostos é através da modificação da sua estrutura, nomeadamente reações de acilação.

Neste trabalho de investigação realizou-se a semi-síntese de novos derivados bioativos através de reações regiosseletivas de acilação pela combinação de métodos enzimáticos e químicos. Assim o objetivo deste trabalho foi o de sintetizar, isolar e caracterizar derivados acilados do resveratrol e da naringenina, com atividade biológica reconhecida.

Desta forma foi possível observar a regiosseletividade das reações enzimáticas e das reações químicas perante substratos com diferentes grupos hidroxilo, levando à obtenção de ésteres com elevada regiosselectividade e bons rendimentos.

Palavras-chave: Compostos polifenólicos, flavonóides, estilbenos, atividade antioxidante, atividade anti-inflamatória, atividade anticancerígena, acilação, naringenina, resveratrol, enzimas, regiosseletividade.

Abstract

Polyphenols are natural compounds of great interest, present in several products of our daily lives, due to the diverse biological activities proven, namely antioxidant, antiinflammatory, and anticarcinogenic. However its applicability in the pharmaceutical industry is conditioned by the low stability and solubility in lipophilic medium.

One of the effective strategies used to improve the properties of these compounds is by modifying their structure through acylation reactions.

In this research, the semi-synthesis of new bioactive derivatives was performed through regiosselective acylation reactions by combination of enzymatic and chemical methods.

Thus the objective of this work was to synthesize, isolate and characterize acylated derivatives of resveratrol and naringenin, with recognizable biological activity.

It was possible to observe the regioselectivity of the enzymatic reactions and the chemical reactions towards to substrates with different hydroxyl groups, leading to the production of esters with high regioselectivity and good yields.

Key words: Polyphenolic compounds, flavonoids, stilbenes, antioxidant activity, antiinflammatory activity, anticarcinogenic activity, acylation, naringenin, resveratrol, enzymes, regioselectivity.

Chapter 1

Introduction

1. Introduction

1.1. Natural polyphenolic compounds

Natural products, from plants and animals, are subject of great interest in drug discovery. They are the source of medicinal preparations which some of them entered on clinical trials and others provide *leads* for compounds that have entered in clinical trials (Harvey *et al.,* 2015).

Polyphenols are plant-derived natural compounds and therefore they are in several products such as fruits, seeds, vegetables, derived foodstuffs and beverages. These compounds have been increasing interest to medicinal chemistry and food industry because of their beneficial effects. In plants, polyphenols contribute to the protection against UV light, defense against herbivory and also attract pollinating insects. *In vitro* and *in vivo* studies show antioxidant, antiinflammatory, antibacterial, bone stimulating and anticancer properties. Furthermore, polyphenols are extremely reactive and good substrates for numerous enzymes including oxidases, peroxidases, glycosidases and esterases. They undergo several enzymatic and chemical reactions during postharvest food, storage and processing (Ambriz-Pérez *et al.,* 2016; Cazzola *et al.,* 2016; Lakey-Beitia *et al.,* 2014; Quideau *et al.,* 2011; Cheynier, 2005).

Despite of all these qualities their application in medicine is limited by their low stability (mainly in neutral and basic media) and poor bioavailability when administered by conventional systemic routes (Cazzola *et al.,* 2016).

Taking into account all of their beneficial effects, studies are being directed to the discovery of new *lead* compounds in the research and development process of new drugs. Structure modification can allow us to explore novel structure-activity relationships in order to obtain new bioactive derivatives.

1.1.1. Classification of polyphenols

These secondary metabolites are known to structurally have, at least, one aromatic ring to which one or more hydroxyl groups are bound to aromatic or aliphatic structures. Polyphenols are divided in two main categories, flavonoids and non-flavonoids.

Among the flavonoids there are the anthoxanthins (flavonol, flavanol, isoflavonoid, flavone, and flavanone) and the anthocyanins while the non-flavonoid compounds include the phenolic acids, stilbenes, curcuminoids, lignans and tannins.

This division is made according to the number and arrangement of their carbon atoms and are commonly found conjugated to sugars and organic acids. Studies show that different polyphenol subgroups differ significantly in stability, bioavailability and physiological functions related to human health (Ambriz-Pérez *et al.,* 2016; Lakey-Beitia *et al.,* 2014; Tsao, 2010; Crozier *et al.,* 2009).

In this work, special attention is given to the flavonoids and stilbenes since they are interesting bioactive molecules.

1.1.2. Flavonoids

Flavonoids are the largest group of polyphenolic compounds which are available in almost all plants and are an integral part of human and animal diets. Being phytochemicals, flavonoids cannot be synthesized by humans and animals. They contribute to the staining of flowering plants and are present in vegetables, fruits, seeds, nuts, grains, spices, different medicinal plants as well as in beverages such as wine (particularly red wine), tea and at, lower levels, in beer. They occur as aglycones, methylated and glycosides derivatives being the last ones the most predominant form in plants. Compared to the glycosylated forms, the aglycones show a higher biological activity *in vitro* (Kumar *et al.,* 2013; Chebil *et al*., 2007; Pietta, 2000).

Flavonoids are frequently hydroxylated in the positions 3, 5, 7, 2, 3′, 4′ and 5′ and the glycosylation sites are at C-3 and C-7, being the carbohydrate L-rhamnose, D-glucose, glucorhamnose, galactose or arabinose (Kumar *et al.,* 2013).

There are several thousands of flavonoids identified which all of them have a benzo-γ-pyrone structure. This diversity is due to the occurrence of several substitution in which primary substituents (hydroxyl, methoxyl, or glycosyl groups) can themselves be substituted (glycosylated or acylated) (Kumar *et al.,* 2013; Cheynier, 2005).

1.1.2.1. Structure of flavonoids

The basic flavonoid are structurally derived from benzo-*γ*-pyrone and consists on 15 carbon atoms arranged in three rings (C6-C3-C6), two of them aromatic which are labeled A, B and C. The two aromatic rings (A and B) are linked by a pyran heterocyclic ring (C ring) (**F**igure 1) (Ribeiro *et al.,* 2015; Crozier *et al.,* 2009).

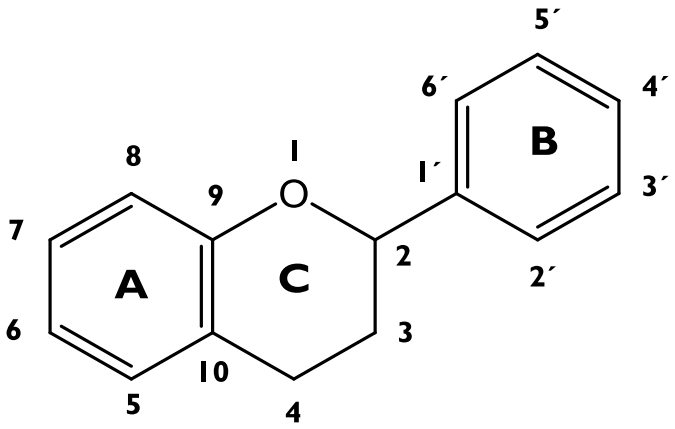


Figure 1. *Basic flavonoid structure.*

1.1.2.2. Classification of flavonoids

The several classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring while individual compounds within a class differ in the pattern of substitution of the A and B rings. So flavonoids can be classified as flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (**F**igure 2) (Lakey-Beitia *et al.,* 2014; Kumar *et al.,* 2013).

I. Flavonols contain a benzo-γ-pyrone ring with phenyl substitution at C-2, a double bond between C-2 and C-3, a hydroxyl group at C-3 and a carbonyl group at C-4, on the C ring.

II. Flavones contain a benzo-γ-pyrone ring with phenyl substitution at C-2, a double bond between C-2 and C-3 and a carbonyl group at C-4, on the C ring.

III. Isoflavones contain a benzo-γ-pyrone ring with phenyl substitution at C-3, a double bond between C-2 and C-3 and a carbonyl group at C-4, on the C ring.

IV. Flavanones contain a benzo-γ-pyrone ring with phenyl substitution at C-2 and a carbonyl group at C-4, on the C ring.

V. Anthocyanidins contain a benzo-γ-pyrone ring with phenyl substitution at C-2, on the C ring but possess flavylium salt structure. They are the free form, anthocyanins are the glycosides of anthocyanidins.

VI. Flavanols contain a benzo-γ-pyrone ring with phenyl substitution at C-2 and a hydroxyl group at C-3, on the C ring.

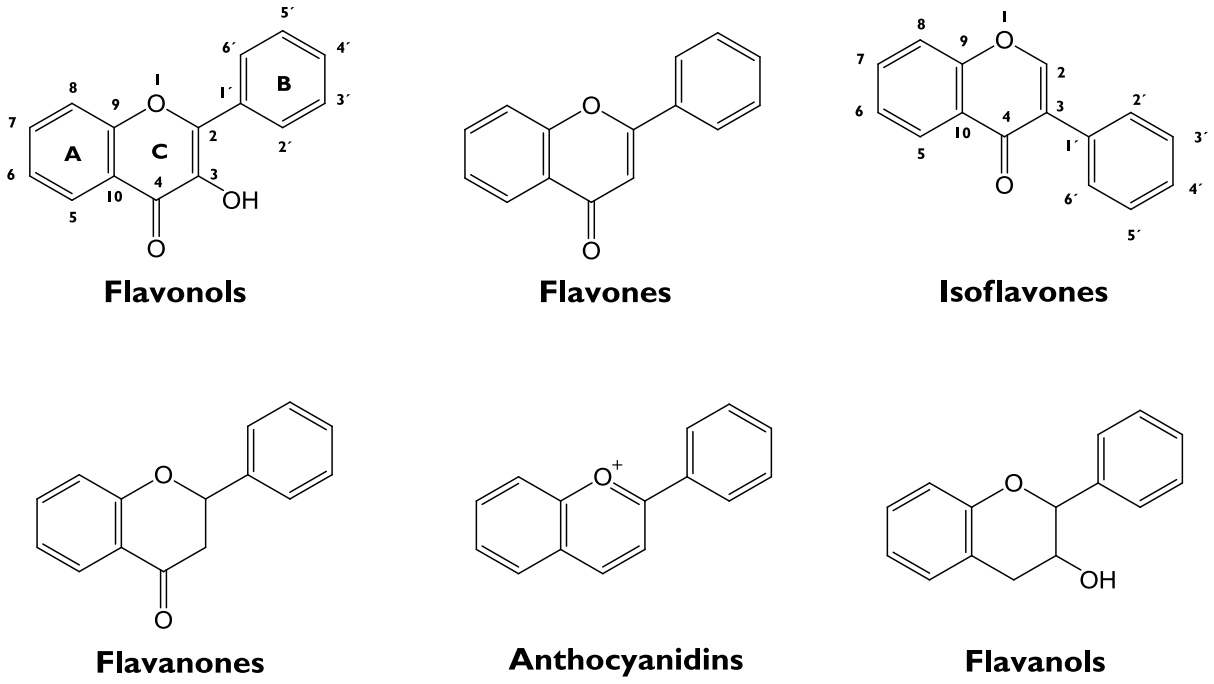


Figure 2. *Basic structure of the major subclasses of flavonoids.*

1.1.3. Stilbenes

Stilbenes are a class of polyphenolic compounds accessible in some plants. As the enzyme responsible for the biosynthesis of stilbenes, stilbene synthase, is not expressed ubiquitously, these compounds are found in a limited but heterogeneous group of plant families. They are present in higher quantity in grapes (such as in grape products including grape juices and wine) and in peanuts. Alike in flavonoids, the hydroxyl groups of stilbenes can also be subject

of substitutions with sugars, methyl, methoxy and other residues (Reinisalo *et al.,* 2015; Rivière *et al*., 2012; Xiao *et al*., 2008).

1.1.3.1. Structure of stilbenes

The basic stilbene structure is the 1,2-diphenylethylene nucleus which consists in two aromatic rings (A and B) linked by an ethylene bridge (C6-C2-C6). Since the double bond turns the structure more rigid, there are only two possible configurations: the stereoisomers (*E*)-stilbene (*trans*-stilbene) which is not sterically hindered and the (*Z*)-stilbene (*cis*-stilbene) which is sterically hindered and therefore less stable, being the first ones the most common form (**F**igure 3) (Rivière *et al*., 2012; Likhtenshtein; 2010; Crozier *et al.,* 2009; Xiao *et al*., 2008).

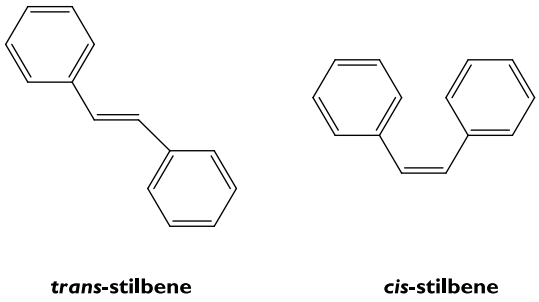


Figure 3. *Basic structure of trans-stilbene and cis-stilbene (Adapted from LIKHTENSHTEIN, G. -* ***Stilbenes: Applications in Chemistry, Life Sciences and Materials Science****. Weinheim: Wiley-VCH Verlag GmbH & Co, 2010. ISBN 978-3-527-32388-3).*

In plants, after being synthesized stilbenes can undergo several modifications like glycosylation and oligomerization to form complex polyphenolic compounds. Stilbenes can be classified as the monomeric and the oligomeric stilbenes. The last ones are formed by the linkage between homogeneous or heterogeneous monomeric stilbenes by C-C or C-O-C bonds with two, four, six or eight linkage points.

The majority of stilbenes are derivatives of *trans*-resveratrol which is considered the basic unit monomer. Resveratrol is also the most studied because it exhibits more potential as an antioxidant and an anticancer agent than the *cis* form. Due to their biological activities, stilbenes are considered good *lead* compounds for the development of new drugs (Keylor *et al.,* 2015; Pangeni *et al.,* 2014; Rivière *et al*., 2012; Chong *et al.,* 2009; Shen *et al.,* 2009).

1.1.4. Antioxidant activity of polyphenols

Oxidation refers to the transference of electrons from one atom to another. However it can occur the transference of unpaired single electrons generating free radicals. So the definition of free radical refers to an atom or molecule containing one or more unpaired electrons in valence shell or outer orbit, being capable of independent existence. The odd number of electron(s) makes it unstable, short lived and highly reactive. The mechanism of action of these radicals is to withdraw electrons from other compounds to obtain stability because of their high reactivity. On the other hand, these products themselves become radicals beginning a chain reaction cascade which finally leads to damages in the cells (Phaniendra *et al.,* 2015; Gülçin, 2012).

The most important free radicals are the oxygen derivatives, called reactive oxygen species (ROS) which includes superoxide ion ($O_2^{\bullet-}$), radical hydroxyl ($HO^{\bullet}$) peroxyl ($ROO^{\bullet}$); alkoxyl ($RO^{\bullet}$); nitric oxide ($NO^{\bullet}$) and also including the non-radicals singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Gülçin, 2012).

The formation of free radicals in the body occurs by endogenous mechanisms as a normal part of metabolism within the mitochondria and in endoplasmic reticulum (where the oxygen consume is higher) and induced by environmental factors like smoking, certain pollutants, organic solvents and pesticides. The beneficial effects of free radicals are involved in several physiological functions when at low or moderate levels. However at higher concentration free radicals generates oxidative stress, leading to cell damage in several disease such as diabetes mellitus, neurodegenerative diseases, cancer, cardiovascular diseases, cataracts, rheumatoid arthritis, asthma, among others. The overproduction of ROS is caused by the imbalance between the oxidants and antioxidants (enzymatic and non enzymatic), leading to damages on lipids, proteins, DNA, polyunsaturated fatty acids and carbohydrates (Phaniendra *et al.,* 2015; Gülçin, 2012).

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Antioxidants include enzymatic proteins such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione transferase (GSTs) and peroxiredoxin (Prdx) present in human plasma and erythrocytes and nonenzymatic molecules like α-tocopherol, ascorbic acid, ubiquinol, β-carotene, among others (Gülçin, 2012; Procházková *et al*., 2011).

Polyphenols are strong antioxidants. The intake of products enriched on these compounds has been linked to a lower risk of some diseases associated to oxidative stress. The

antioxidant activity of polyphenols and their analogues depends on the number and position of the hydroxyl groups bound to the aromatic ring, the binding site and the type of substituents. Their mechanisms of action are the scavenging of ROS by donating a hydrogen atom or electron,

H-atom transfer $ArOH + R^{\bullet} \rightarrow ArO^{\bullet} + RH$

Single-electron tranfer $ArOH + R^{\bullet} \rightarrow ArOH^{\bullet+} + R^{-}$

the chelation of metal ions such as iron(II)/copper(I) and iron(III)/copper(II) ions that are involved in the conversion of $O_2^{\bullet-}$ and H_2O_2 into highly aggressive $OH^{\bullet}$ through Haber-Weiss/Fenton-type reactions,

Haber-Weiss reaction

$$Fe^{3+} + O_2^{-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

result

$$O_2^{\bullet-} + H_2O_2 \rightarrow OH^{-} + OH^{\bullet} + O_2$$

Fenton reaction $Fe^{2+}(Cu^{+}) + H_2O_2 \rightarrow OH^{-} + OH^{\bullet} + Fe^{3+}(Cu^{2+})$

the inhibition of enzymes responsible for the formation of radicals such as xanthine oxidase (XO) and protein kinase C (PKC), the upregulation of endogenous antioxidants and exerting a synergistic antioxidant action through the regeneration of essencial vitamins like *α*-tocopherol *(*Gülçin, 2012; Quideau *et al.,* 2011; Tsao *et al.,* 2010 Young *et al.,* 2001; Pietta, 2000).

$$\alpha\text{-}TO^{\bullet} + ArOH \rightarrow \alpha\text{-}TOH + ArO^{\bullet}$$

Polyphenols can also act as prooxidants since the free radical formed in response to the donation of an electron or hydrogen atom, when in enough concentration, can itself exert potential prooxidants activities.

The mechanism involved is by reducing iron(III) or copper(II) ions that they chelate The cathecol moiety suffers desprotonation and oxidation by the transference of one electron to metals such as copper or iron, but mostly copper(II) because of their lower standard reduction potential (Cu^{2+}/Cu^{+} → 0.15 V versus Fe^{3+}/Fe^{2+} → 0.77 V). This leads to the formation of the semiquinone radical anions that reacts with other free-radical species, including O_2 to afford oxidizing ortho-quinones and $O_2^{\bullet-}$. Then the copper(I) ions participate in the reduction of $O_2^{\bullet-}$ in H_2O_2 and in the convertion of hydrogen peroxide into DNA-damaging $OH^{\bullet}$, through the Fenton-type reaction as shown in figure 4. The electrophilic quinone can also induce covalent DNA damage as well as protein and peptide covalent modifications (Quideau *et al.,* 2011; Tsao *et al.,* 2010).

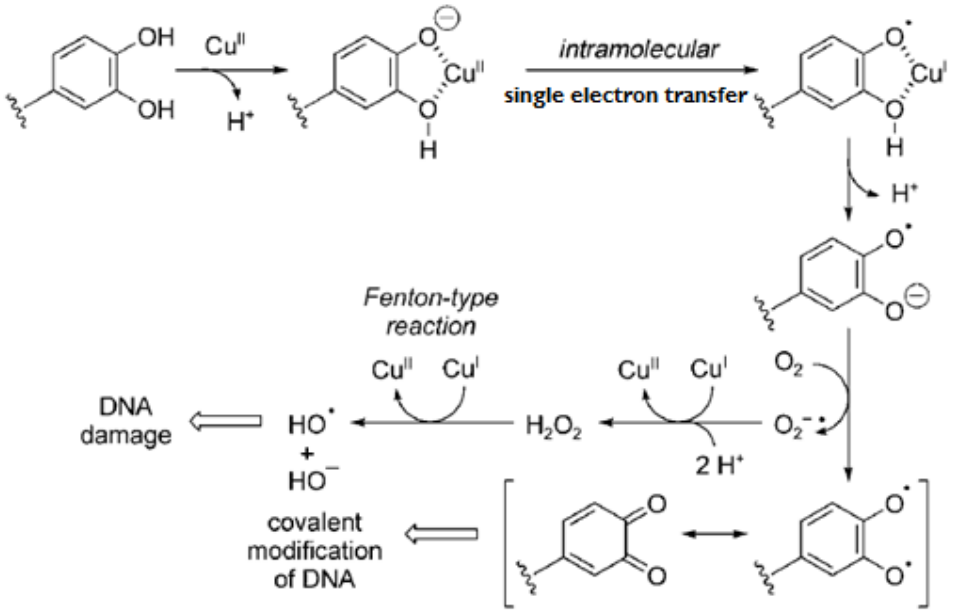


Figure 4. *Proposed prooxidation mechanism for copper(II)-mediated DNA damage by polyphenols (Adapted from QUIDEAU, S. [et al.] (2011) - Plant Polyphenols : Chemical Properties, Biological Activities, and Synthesis. "Angewandte Chemie International Edition" 50 (2011) 586–621).*

1.1.4.1. Antioxidant activity of flavonoids

The antioxidant activity depends on the arrangement of functional groups in the flavonoid structure, the configuration and the total number of hydroxyl groups (Kumar *et al.,* 2013; Procházková *et al*., 2011).

The mechanisms of flavonoids included in the antioxidant activity are: the scavenging of ROS (**F**igure 5); the suppression of ROS formation either by inhibition of enzymes or by chelating

trace elements involved in free radical generation (**F**igure 9) and upregulation or protection of antioxidant defenses. Often the effects caused by flavonoids are a result of the combination of two or more mechanisms (Kumar *et al.,* 2013).

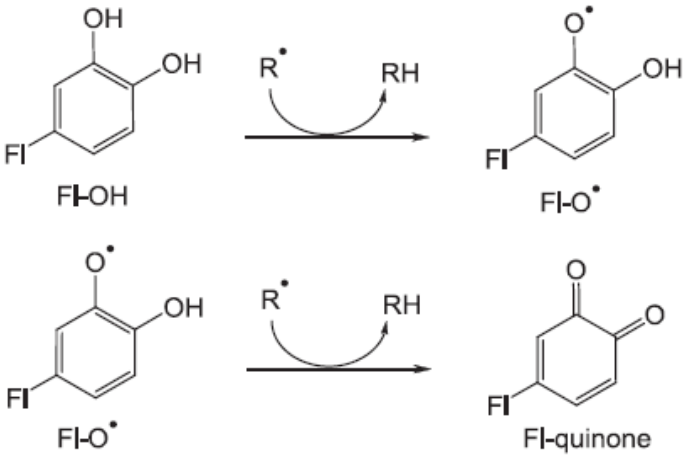


Figure 5. *Scavenging of* ROS by flavonoids. *Where R• represents superoxide anion and radical peroxyl, alkoxyl and hydroxyl and Fl-O• represents the aroxyl radical (In PROCHÁZKOVÁ D. [et al.] (2011) - Antioxidant and prooxidant properties of flavonoids. "Fitoterapia" 82:4 (2011) 513–523).*

The presence of the hydroxyl groups on the B ring is the major determinant of ROS scavenging, whereas substitution of the rings A and C has little impact on $O_2^{\bullet-}$ scavenging (Procházková *et al*., 2011).

1.1.4.1.1. SAR of flavonoids as antioxidants

Several studies have allowed to establish SAR between the flavonoids and their antioxidant activity. The main structural feature that flavonoids require for efficient radical scavenging are: an ortho-dihydroxy (catechol) structure in the B ring, which confers higher stability to the radical form and participates in electron delocalization (**F**igure 6); the 2,3 double bond in conjugation with a 4-oxo function in the C ring, which is responsible for electron delocalization from the B ring (**F**igure 7) and the 3- and 5-OH groups with a 4-oxo function in A and C rings (**F**igure 8) (Croft, 1998; Rice-evans *et al.,* 1996).

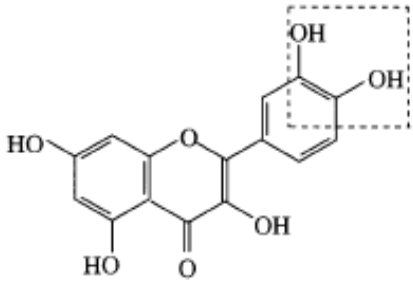


Figure 6. *An ortho-dihydroxy (catechol) moiety in the B ring (In CROFT, K. D. (1998) - The Chemistry and Biological Effects of Flavonoids and Phenolic Acids. "Annals of the New York Academy of Sciences" 854 (1998) 435–442).*

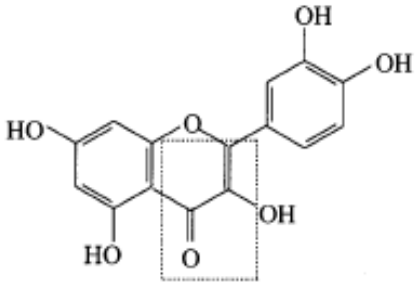


Figure 7. *2,3 Double bond in conjugation with a 4-oxo function in the C ring (In CROFT, K. D. (1998) - The Chemistry and Biological Effects of Flavonoids and Phenolic Acids. "Annals of the New York Academy of Sciences" 854 (1998) 435–442).*

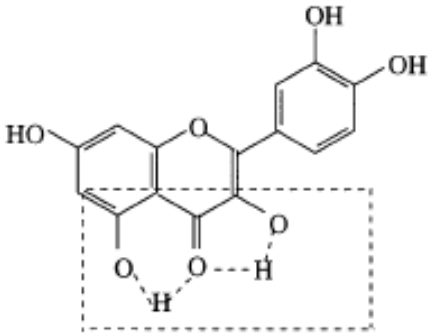


Figure 8. *Hydroxyl groups at positions 3 and 5 (In CROFT, K. D. (1998) - The Chemistry and Biological Effects of Flavonoids and Phenolic Acids. "Annals of the New York Academy of Sciences" 854 (1998) 435–442).*

For instance, according to what was mentioned above, quercetin, a flavonol, satisfies all the parameters and has therefore a strong antioxidant activity. Glycosylation reduces their antioxidant activity when compared to the corresponding aglycons (Rice-Evans *et al.,* 1996).

Another pathway of the flavonoids as antioxidant is the chelation of metal ions. The proposed binding sites, to capture these metal ions, in the flavonoids are the catechol moiety in B ring, the 3-OH with 4-oxo groups in the heterocyclic ring (C ring) and the 4-oxo with 5-OH groups between the C and the A rings (**F**igure 9) (Pietta, 2000).

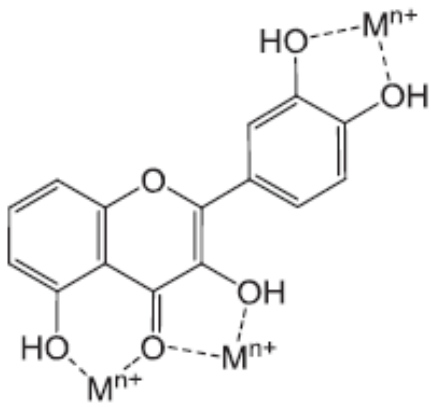


Figure 9. *Binding sites for trace metals (In PROCHÁZKOVÁ D. [et al.] (2011) - Antioxidant and prooxidant properties of flavonoids. "Fitoterapia" 82:4 (2011) 513–523).*

However it was demonstrated that the main contribution to metal chelation is the catechol moiety, due to the more pronounced bathochromic shift produced by comparing the chelation of copper between quercetin and kaempferol (similar structure to quercetin except the lack of the catechol group in the B ring) (Pietta, 2000).

In certain situations, flavonoids can also act as prooxidants. This activity is proportional to the total number of hydroxyl groups in their structure. Hanasakl *et al.* (1994) demonstrated that series of mono- and dihydroxyflavonoids have no prooxidant activity while several hydroxyl groups, principally in the B ring, increased significantly the production of hydroxyl radicals in Fenton reaction. (Procházková *et al*., 2011).
Studies showed that the same flavonoid compound could behave both as an antioxidant and a prooxidant. Some characteristics, on flavonoids, that are essential to increase the antioxidant activity may also enhance oxidative stress. Van Acker *et al.* (1996) pointed out

that the most active antioxidants are likely to be prooxidants. This should be taken into account when designing therapeutically flavonoids of interest (Amic *et al*., 2007; Pietta, 2000).

1.1.4.2. Antioxidant activity of stilbenes

Stilbenes are characterized to have strong antioxidant activity. On the other hand they also have, just like flavonoids, prooxidant activity. The antioxidant activity depends on the redox properties of their phenolic hydroxyl groups and the potential for electron delocalization across the chemical structure (Gambini *et al.,* 2015; Gülçin, 2012).

1.1.4.2.1. SAR of stilbenes as antioxidants

Studies about resveratrol and its derivatives showed the SAR in order to provide a better antioxidant activity. In these studies, the authors established that the number and the position of hydroxyl groups, the double bond and the stereoisomery are essential for the powerful antioxidant activity of resveratrol against different ROS (**F**igure 10). The scavenging of ROS by resveratrol and its derivatives is mostly by the transference of an H-atom. As the *para*-hydroxyl group is more acidic than the other two *meta*-hydroxyl groups, it increases the antioxidant activity by stabilizing the semiquinone radical-anion intermediate via resonance through the *trans* double bond.

The authors also revealed that the complete methylation reduced significantly the activity, indicating that the presence of phenolic hydroxyl group is required. The double bond in the stilbenic skeleton and its stereoisomery are also important being the *cis*-form significantly less effective than *trans*-resveratrol (He *et al.,* 2013).

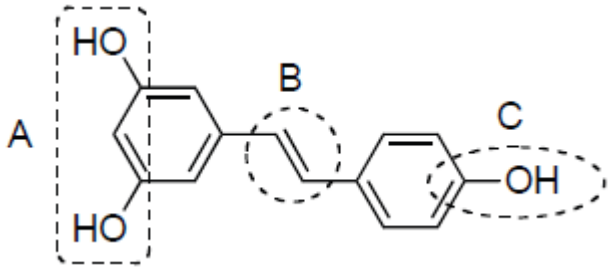


Figure 10. *The meta-hydroxyl groups (A), the para-hydroxyl group (C) and tranisomery of the double bond (B) are essential for a strong antioxidant activity (In HE, S. (2013) - From Resveratrol to Its Derivatives : New Sources of Natural Antioxidant. "Current Medicinal Chemistry" 20 (2013) 1005-1017).*

1.1.5. Antiinflammatory activity of polyphenols

Inflammation is a biological defense mechanism caused by disruption of homeostatic balance in response to the presence of biological, chemical or physical agents in the body. Some of these agents may be pathogens (bacteria, fungi, and viruses), trauma (shock or burns), toxic compounds (pollutants) as well as reactions of the immune system (hypersensitivity) (Ambriz-Pérez *et al*., 2016).

The inflammatory response depends on the balance between the pro- and antiinflammatory signals. When the equilibrium is broken a more widespread inflammatory response takes place. This reaction includes endothelial and epithelial cells and the recruitment of inflammatory cells (neutrophils, monocytes, macrophages and, in some cases, lymphocytes). On the other hand, these cells release mediators that amplify the inflammatory response and recruit additional cells. So it is important to find molecules that act effectively in the several steps of inflammation (Ribeiro *et al.,* 2015).

The conventional treatment of inflammation is through the use of non-steroidal antiinflammatory drugs (NSAIDs), however they can act as inhibitors of ciclooxigenase-1 (COX-1) and ciclooxigenase-2 (COX-2). Therefore the search for natural products, such as polyphenols, with more selectivity and less side effects are growing in the pharmaceutical industries as an alternative to the drugs used nowadays (Ambriz-Pérez *et al.,* 2016).

In order to have the best SAR several studies, using different targets of inflammation, were made and concluded that certain requirements are necessary on polyphenolic compounds: a planar ring system; an unsaturation in the C ring as ketonic carbonyl at C-4 and/or a double bond between the C-2 and C-3; a hydroxyl group in the B ring and in C-5 and C-7 of the A ring and the non-glycosylated molecules. The mechanisms involved in their antiinflammatory activity are not clear but there is a correlation between the high intake of food rich in polyphenols and a downregulation of the inflammatory response. It is believed that polyphenols exert antiinflammatory activities by inhibiting the synthesis of proinflammatory mediators, modification of eicosanoid synthesis, inhibition of the activity of the immune cells or inhibition of nitric oxide synthases (NOS) and COX-2 via its inhibitory effects on nuclear factor kappa B (NF-κB) (Ambriz-Pérez *et al.,* 2016).

1.1.5.1. Mediators of the inflammatory process

1.1.5.1.1. Metabolites of arachidonic acid

Arachidonic acid (AA) is a polyunsaturated fatty acid, a common constituent of cell membranes and the main precursor of eicosanoids. When cells are stimulated by activation of their receptors (by mechanical trauma, cytokines and growth factors) AA is released from the phospholipid membrane by phospholipases A2 (PLA2) and is metabolized into a huge range of compounds, called eicosanoids, by series of enzymes including cyclooxygenases (COX) and lipoxygenases (LOX) (**F**igure 11) (Ribeiro *et al.,* 2015).

COX catalyzes the synthesis of prostaglandins (PG) and thromboxanes (TX) from AA. This enzyme exists in two different isoforms, COX-1 and COX-2. Specifically, COX-1 is a constitutive enzyme expressed in a variety of tissues, while COX-2 is an inducible enzyme expressed primarily in cells involved in inflammation, such as macrophages, fibroblasts and endothelial cells. COX expression is induced by numerous stimuli such as growth factors (Platelet-derived growth factor and epidermal growth factor) and proinflammatory cytokines (Interleukin-1 Beta (IL-1β) and tumor necrosis factor alpha (TNF-α)) (Ribeiro *et al.,* 2015).

LOX are responsible for the production of hydroxy acids and leukotrienes (LT) from AA. Among the different isoforms the 5-LOX and 12-LOX are involved in allergic and inflammatory disorders. 5-LOX produces 5-hydroxyeicosatetraenoic acid (5-HETE) and LTs and 12-LOX synthesizes 12-hydroxyeicosatetraenoic acid (12-HETE) (García-Lafuente *et al.,* 2009).

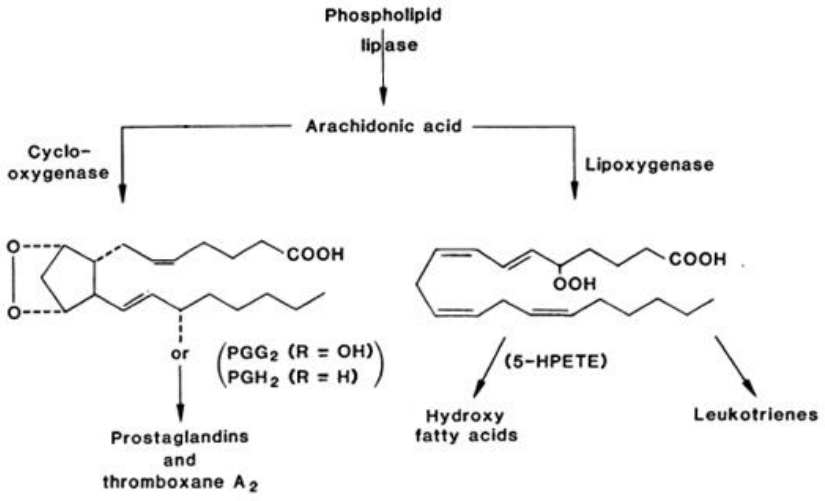


Figure 11. *Arachidonic acid cascade (Adapted from KUEHL, F. A. [et al.] (1980) - Prostaglandins, Arachidonic Acid, and Inflammation. "Science" 210 (1980) 978–984).*

The eicosanoids have an important role in the inflammatory process. There are several types of PG and depending on the class, they can cause vasodilation, vascular hyperpermeability, inhibition of T-lymphocyte activation and proliferation, reduction of the production of several inflammatory cytokines, chemoattraction for neutrophils, reduction of macrophage activation, reduction of $NO^{\bullet}$ production, inhibition of platelet aggregation, among others (Ribeiro *et al.,* 2015).

TXA_2, one of the most important TX, in inflammation acts as vasoconstrictor, mediates the activation and aggregation of platelets and promotes the contraction of smooth muscle (Ribeiro *et al.,* 2015).

LTB_4, one of the most important LT, promote chemotaxis, neutrophil and macrophages chemoattraction, stimulate the leukocyte adhesion to endothelial cells by upregulating leukocyte integrin expression and generate ROS (Ribeiro *et al.,* 2015).

5-HETE promote chemotaxis, leukocyte adhesion and increase the levels of Ca^{2+} intracellular (Ribeiro *et al.,* 2015).

1.1.5.1.2. Nitric oxide

Louis Ignarro, Ferid Murad and Robert Furchgott, in 1998, won the Nobel Prize for Physiology or Medicine for their contribution to the discovery of NO as a endothelium-derived relaxing factor (EDRF), responsible for maintaining vasomotor tone and systemic blood pressure (Rawlingson, 2003).

NO is a pleiotropic, short-lived free radical, which is widely utilized as a signaling molecule in cells throughout the body, carrying out numerous roles including the regulation of local vascular tone and blood flow. Thus, in general, NO will cause local vasodilation and consequently increases the oxygen delivery. NO is the metabolic by-product of the conversion of L-arginine to L-citrulline by a family of enzymes called NOS (Lundberg *et al.,* 2015; Nagy *et al.,* 2007).

There are three different NOS isoforms which are classified according to whether they are constitutively expressed like endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) or induced in response to inflammatory processes like inducible nitric oxide synthase (iNOS). Both eNOS and nNOS synthesize NO in moderate amounts that has a tonic physiological function, while activation of iNOS produces much greater amounts of NO, being part of the innate immune system by killing bacteria, viruses and fungi (Lundberg *et al.,* 2015).

1.1.5.1.3. Cytokines

Cytokines are small and soluble proteins produced and secreated by a wide variety of cells (leukocyte, endothelial and epithelial cells and fibroblasts) triggered by different factors like trauma, stress, radiation, UV light, cell–cell contact, among others. They regulate the intensity and duration of the inflammatory response (Ribeiro *et al.,* 2015).

Several cytokines can target the same receptor and a single cytokine can have multiple, even contradictory, effects and so they are characterized by their redundancy and pleiotropy (Ribeiro *et al.,* 2015).

Two cytokines play a crucial role during the inflammatory process, TNF and IL-1, which have strong inflammatory properties. TNF cause the activation of endothelial cells which will increase the expression of adhesion molecules (important for recruiting inflammatory cells to the sites of inflammation), increase the procoagulant activity of endothelial cells, the mobilization and effector function of neutrophils and their adhesiveness for endothelial cells and increase fever and the production of IL-1, IL-6 and TNF via stimulation of macrophages. IL-1 share many activities with TNF including the activation of the endothelial cells, induction of coagulation and fever and also participate in 17 T-helper cells differenciation (McManus *et al.,* 2014; Khan, 2008).

1.1.5.2. Antiinflammatory activity of flavonoids

Many mechanisms have been described to explain the antiinflammatory activity of flavonoids: the antioxidant and radical scavenging activities; the regulation of cellular activities of inflammation-related cells (including T and B cells, macrophages, neutrophils, mast cells or basophils); the modulation of proinflammatory enzyme activities (like PLA2, COX, LOX and NOS); the modulation of the production of other proinflammatory molecules (such as TNF-α and IL-1β) and the modulation of the expression of proinflammatory genes (García-Lafuente *et al.,* 2009; Kim *et al*., 2004).

1.1.5.2.1. SAR of flavonoids as antiinflammatory

Kim *et al*. (2004) compared several flavonoids on their effects on the enzymes involved in the production of eicosanoids and in the expression of proinflammatory molecules. It was

observe that the important moieties are the 2,3-double bound on the C ring, the 5,7-hydroxyl groups on the A ring and the 4'- or 3',4'-hydroxyl groups on the B ring.
Loke *et al.* (2008) compared quercetin with is structural analogues luteolin, kaempferol and taxifolin which have specific OH functional group substitutions in order to understand their SAR (**F**igure 12), concerning their inhibitory actions on LTB_4.

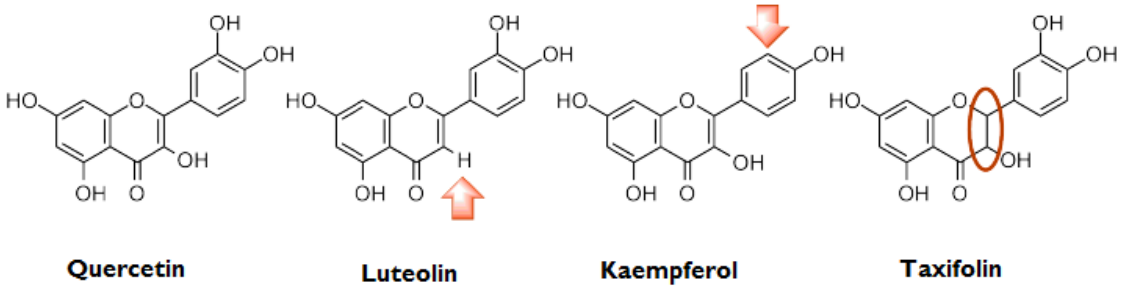


Figure 12. *Structural differences between quercetin and the various analogues (Adapted from COUTINHO, M. A. S. [et al.] (2009) - Flavonoides: Potenciais Agentes Terapêuticos para o Processo Inflamatório.* "Revista Virtual de Química" *1:3 (2009) 241-256).*

In this study they showed that the hydroxyl group in C-3´ in the B ring, is a requisite for the inibitory activity on LTB_4 and that the absence of this group reduced its effects over 60 %, kaempferol. The absence of the OH in the position 3 on the C ring as in luteolin had minimal effect on this action. Finally, the nonexistence of the 2,3-double bound on the C ring, taxifolin, leads to a decrease in the inhibitory activity of LTB_4 (Loke *et al.*, 2008).
Comparative studies between aglycon and glycoside pairs also showed that the addition of sugar residues clearly reduced the antiinflammatory activity (Loke *et al.,* 2008).

Briefly, among the structural factors that influence antiinflammatory activity of flavonoids are the 2,3-double bound on the C-ring, the number and position of hydroxyl groups and the absence of glycosilation of the molecule.

1.1.6. Anticarcinogenic activity of polyphenols

Cancer is not a single disease, it manifests in several subtypes each with its own distinct histopathological and biological features. A common aspect in all cancers is the uncontrolled cell proliferation which offers the possibility of therapeutic approaches to the disease. There

are several mechanisms involved in the development of cancer including hereditary genetic mutations, extrinsic factors, also called environmental factors (UV light, ionizing radiation, carcinogens, smoking, among others) and intrinsic processes, including those that result in random mutations due to errors in DNA replication, being the last one the major cause of variations in cancer incidence between tissues. Tomasetti *et al.,* (2015) showed that 65% of variation in cancer risk among tissues is explanined by the total number of stem-cells division and so just only a third of the variation is attributable to the other factors (Tomasetti *et al*., 2015; Wodarz *et al.,* 2015; Wu *et al.,* 2015; Marjanovic *et al.,* 2013).

In order to overcome the toxicity associated with the current radio-/chemotherapies, new compounds are continuously being studied as anticancer agents. Some epidemiological studies suggest that diet particularly rich in fruits and vegetables have cancer preventive properties. These effects are related to the polyphenols which demonstrate to have antitumor activities both in animal models such as in humans, both alone or in combination with current chemotherapy/radiotherapy (Mileo *et al.,* 2016).

The mechanisms of polyphenols involved in the anticarcinogenic activity involve by modulation of oxidative stress (several studies have established that the levels of copper are elevated in cancer patients), induction of apoptotic death cells and inhibition of cell cycle, cell proliferation and angiogenesis (Mileo *et al.,* 2016; Spatafora *et al.,* 2012).

1.1.6.1. Anticarcinogenic activity of flavonoids

In recent years, much attention has been given to flavonoids to their abilities to inhibit oxidative stress, cell proliferation, metastasis and angiogenesis, to promote the cell cycle arrest and to increase apoptosis (Benacente-García *et al.,* 2008; Nijveldt *et al.,* 2001).

It has been stated that flavonoids, as antioxidants, can inhibit carcinogenesis. The damage caused by ROS could be one of the reasons for the development of cancer. ROS can interact with DNA and causes changes that if occurs in critical genes, such as oncogenes or tumor suppressor genes, may result in cancer (Nijveldt *et al.,* 2001).

Although most flavonoids appear to be nontoxic to humans and animals, they have been shown to inhibit proliferation in many kinds of cancerous cell lines. Benacente-García *et al.,* (2008) compared the flavanone, flavone, and flavonol and the number of substituents on the A and B rings in diferent cell lines. They demonstrated that the presence of a 2,3-double bond, on the C ring and the number and position of hydroxyl groups (at least three adjacent hydroxyl groups) increased the antiproliferative activity.

Flavonoids also arrest cell-cycle progression at either G1/S or G2/M boundaries, have an effect on apoptosis, and exert anti-invasive and anti-angiogenic properties being therefore considered good anticarcinogenic agents (Benavente-García *et al.,* 2008; Kanadaswami *et al*., 2005).

1.1.7. Relevant biological activities of selected polyphenols

The biological activities of polyphenols are due to its chemical structure. It was observe that the number, positions and types of substitutions are very important to safeguard their beneficial effects (Chebil *et al.,* 2007).

In the glycosylated form, the sugar moiety attached influences the pharmacokinetic behavior, in particular, the absorption, distribution and metabolism. The glycosides are commonly hydrolyzed to their aglycones to produce effects *in vivo* to a certain way they are like prodrugs. The linkage of sugar moieties allow to dramatically improve the solubility of aglycones.

In general O-glycosylation reduces the bioactivity in certain activities, including antioxidant, antidiabetes, anti-inflammatory, antibacterial, antifungal, antitumor, anticoagulante and others. Nevertheless, O-glycosylation can also improve certain types of biological benefits such as anti-HIV, tyrosinase inhibition, antirotavirus, antistress, antiobesity, anticholinesterase potential, antiadipogenic and antiallergic (Xiao *et al.,* 2014).

1.1.7.1. Naringenin and naringin

Naringenin (4´,5,7-trihydroxyflavanone) is found especially in many *Citrus* fruits and has been shown to possess antioxidant, anti-inflammatory, antiproliferative and antimutagenic properties. Naringin (4´,5,7-trihydroxyflavanone-7-rhamnoglucoside), a glycoside of naringenin, is responsible for the bitterness of grapefruit juices (**F**igure 13). In the human body, naringin, when administered, loses the sugar once it is cleaved off in the gastrointestinal tract and liver to yield the aglycone naringenin (Davies *et al.,* 2013; Cavia-Saiz *et al*., 2010).

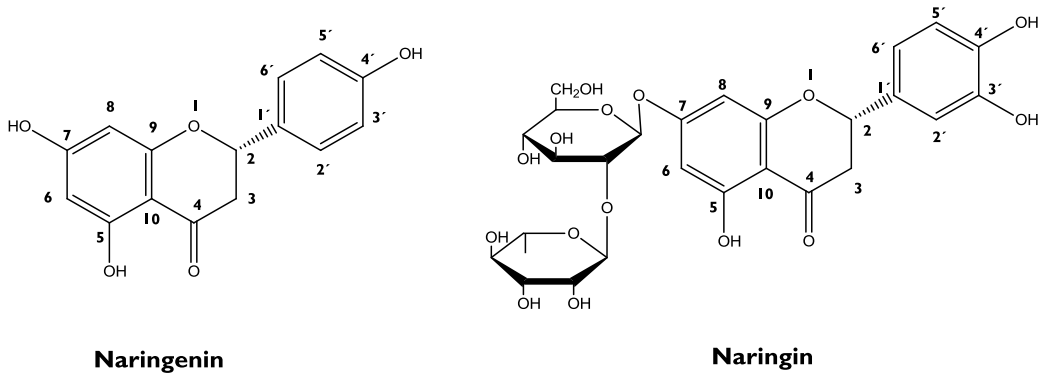


***Figure 13**. Chemical structures of naringenin and naringin.*

As previously described glycosylation decreases the antioxidant activity of flavonoids. Cavia-Saiz *et al.* (2010) compared the antioxidante activity of both flavanones, naringenin and naringin *in vitro*. They showed that naringenin exhibited the higher antioxidant capacity (by $ABTS^{\bullet+}$ and FRAP methods) and $HO^{\bullet}$ and $O_2^{\bullet-}$ scavenger efficiency than naringin. Naringenin also have the ability to inhibit oxidases, such as XO. Russo *et al.* (2000) suggested that because of the 4-oxo function, some flavonoids can compete with the xanthine for the active site of the enzyme.

On the other hand, in the same study their prooxidant effect was evaluated, *in vitro*. The Glutathione (GSH), an antioxidant, plays an important role in the defense of the cellular system so depletion of GSH increases vulnerability to the damage caused by free radicals. Pre-treatment with naringin or naringenin, in high concentrations, did not prevent the oxidation of GSH (Cavia-Saiz *et al*., 2010).

Ye *et al.* (2009) studied the effect of hydroxylation in antioxidant activity using naringin as the initial substrate. Naringin was hydroxylated in the B-ring to 3'-hydroxyl naringin (3'-OHN) and 3',5'-dihydroxyl naringin (3',5'-DOHN). With the same concentration, naringin did not show radical scavenging when a hydroxyl group was introduced and the antioxidative capacity of 3'-OHN was dramatically increased, 68.6 times higher than naringin. The second hydroxyl group was introduced to form 3',5'-DOHN and the antioxidative activity increased comparative to 3'-OHN but not too much. So this confirmed that the differences in radical scavenging activity between flavonoids were attributed to their structural differences in hydroxylation, glycosylation and methoxylation.

Naringin and naringenin also exert antiinflammatory effects. Shiratori *et al.* (2005) showed that naringin and naringenin, when injected intravenously, suppressed NO concentration and reduced PGE_2 levels in aqueous humor on endotoxin-induced uveitis in rats, being naringenin

more effective than naringin. Hamalainen *et al.* (2007) also reported that naringenin inhibited NF-*κ*B activation and iNOS expression in activated macrophages. Once again, the inhibition of NO production by naringenin was higher than naringin which had no effect.

Naringenin and naringin have also anticarcinogenic activities. Naringenin has been reported to induce cytotoxicity in several cell lines such as in breast cancer (MCF-4, MDA-MB-231), stomach (KATOIII, MKN-7), liver (HepG2, Hep3B, and Huh7) cervix (Hela, Hela-TG), pancreas (PK-1), colon (Caco-2) and in leukemia (HL60, NALM-6, Jurkat, and U937). Naringenin showed higher citotoxicity in leukemia cells and lower in Caco-2, compared with the other cell lines. Naringenin also induced apoptosis, in a dose-dependent manner, in both Caco-2 and HL60 cell lines and inhibit cell proliferation at a concentration higher than 0.71 mM, on colon cells HT29 (Davies *et al.,* 2013).
Treatment with naringin showed a decrease in the tumor weight, more than 60%, in rats with Walker 256 Carcinosarcoma (Camargo *et al.,* 2012), induction of cell apoptosis and G1 cycle arrest in triple-negative breast cancer (TNBC) (Li *et al*., 2013) and inhibiton of proliferation and growth of human breast cancer cells, *in vitro,* in MDA-MB-435 cell line (So *et al*., 1996).

1.1.7.2. Resveratrol

Resveratrol (3, 4´, 5-*trans*-trihydroxy-stilbene) is predominant in grapes and in peanuts and it is also an active principle found in Japanese and Chinese folk medicines used to treat ailments related to the liver, skin, heart, and lipid metabolism (**F**igure 14). Resveratrol contributes to the so called French Paradox. In an epidemiological study by the World Health Organization (WHO) showed that the french population consumes a rich diet in saturated fat and yet the number of people with cardiovascular disease is significantly lower than in other countries probably due to the regular intake of red wine. Further investigations revealed resveratrol as the main component of red wine exerting a protective effect against cardiovascular diseases (Wang *et al.,* 2016; Keylor *et al.,* 2015; Pangeni *et al.,* 2014).

Resveratrol is known to have antioxidant, antiinflammatory, anticarcinogenic activities, among others (Wang *et al.,* 2016).

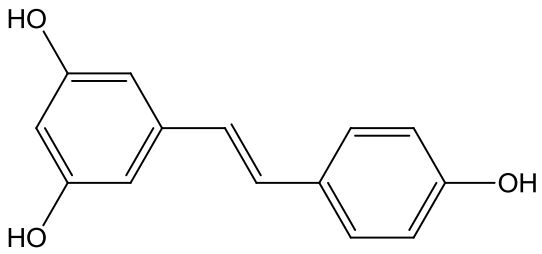


Figure 14. *Chemical structure of resveratrol.*

Resveratrol is characterized to have both free radical scavenger and powerful antioxidant activity. The antioxidant activity depends on the redox properties of their phenolic hydroxyl groups and the potential for electron delocalization across the chemical structure (Gülçin, 2012).

Gülçin (2010) studied the effect *in vitro* of resveratrol as antioxidant and radical scavenging by employing several antioxidant assays. He showed that resveratrol inhibited more (89.1%) of the lipid peroxidation of linoleic acid emulsion at 30 μg/mL concentration compared to the others antioxidants BHA (83.3%), BHT (82.1 %), α-tocopherol (68.1%) and trolox (81.3%) with the same concentration. In addition, resveratrol had effective $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$, $O_2^{\bullet-}$ and H_2O_2 scavenging activities.

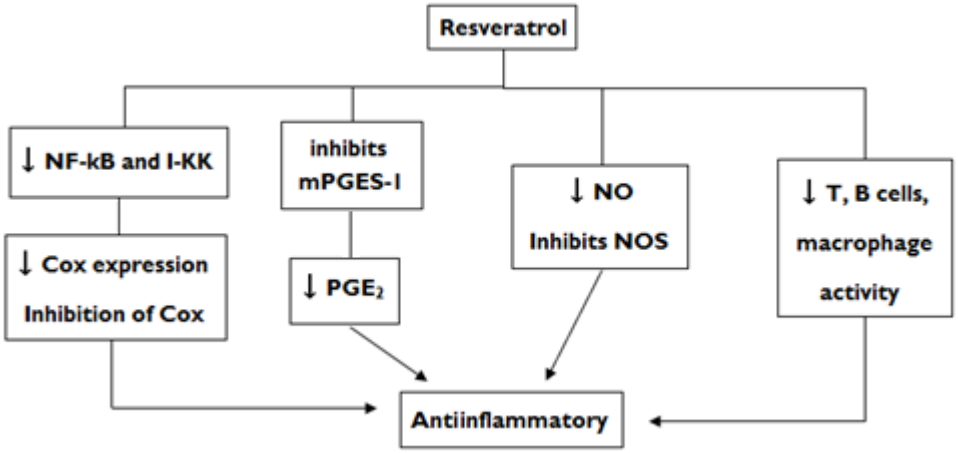
Wang *et al.,* (2016) showed the effects of resveratrol on human erythrocytes using an *in vitro* model of erythrocyte oxidative damage induced by hypoxanthine-xanthine oxidase (HX-XO). It decreases the formation of ROS induced by HX-XO, preserved erythrocyte antioxidant enzymes (SOD, CAT and GPx) activities and increased GSH level.

On the other hand, resveratrol can act as prooxidant, it can lead to the production of $O_2^{\bullet-}$, H_2O_2, and a complex mixture of semiquinones and quinones which can become cytotoxic. Also the oxidized resveratrol can generate complexes with copper that can fragment DNA (Gambini *et al.,* 2015).

Resveratrol showed an antiinflammatory activity both *in vivo* and *in vitro*. The mechanisms involved in this activity are centered mostly on COX-1 and COX-2. Resveratrol is known to inhibit the expression of those two enzymes, to reduce the production of PGE_2, to inhibit the expression of microsomal PGE_2 synthase-1 (mPGES-1), to reduced the formation of ROS and suppress the activity of T- and B-cells and macrophages (**S**cheme 1) (Udenigwe *et al.,* 2008).

An *in vivo* studies comproved the inhibition of COX-2 in rats treated with resveratrol in male albino Wistar rats injected with 1,2-dimethylhydrazine, a colon carcinogen, and in

female Sprague Dawley rats injected with 7,12-dimethylbenz(a)anthracene, a mammary carcinogenesis (Aluyen *et al.,* 2012).

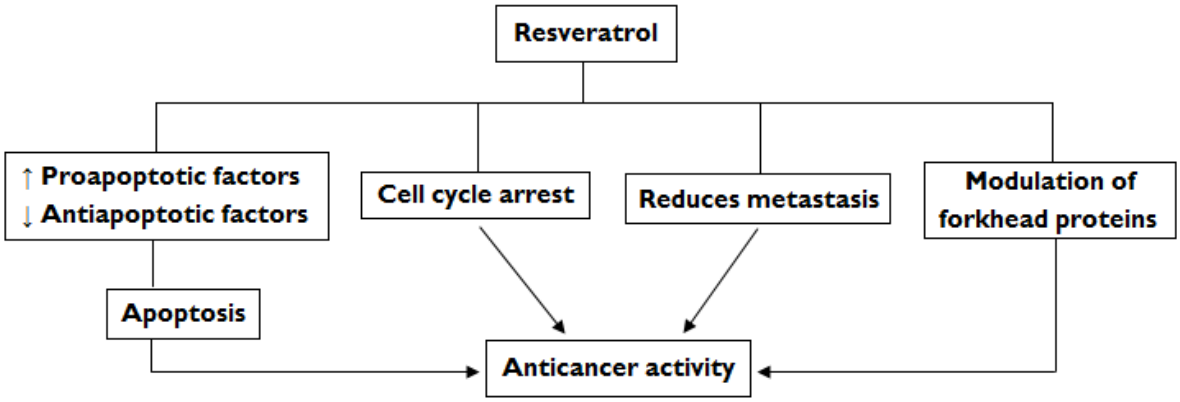


Scheme 1. *Schematic representation of mechanisms of antiinflammatory properties of resveratrol (Adapted from UDENIGWE, C. C. [et al.] (2008). - Potential of resveratrol in anticancer and anti-inflammatory therapy. "Nutrition Reviews" 66:8 (2008) 445–454).*

Resveratrol has also chemopreventive and chemotherapeutic properties. This is of a great interest because it affects several cancers at the initiation, promotion and progression. Usually the chemopreventive properties of resveratrol has been associated with its antioxidant activity (Sirerol *et al.,* 2016).

Resveratrol has been the subject of several studies, in fact in many *in vitro* and *in vivo* carcinogenesis assays with several types of cancers like breast, lung, colon, skin (nonmelanoma skin cancer and melanoma), prostate, ovarian, liver, oral cavities, thyroid, and leukemia. Jang in 1997 showed that topical application of resveratrol reduced the number of skin tumours per mouse by up to 98%. This fact opened doors to new studies all over the world (Sirerol et al., 2016; Baur *et al.,* 2006).

Resveratrol has been shown to induce death cells in mouse xenograft models of human neuroblastoma, SK-N-AS, NGP and SH-SY5Y (Ginkel *et al.,* 2007) and in human colorectal cancer cells, DLD1 and HT29 (Trincheri *et al.,* 2006). It inhibited the cancer progression, *in vitro,* in estrogen-positive (MCF-7) and estrogen-negative (MDA-MB-231) breast cancer cells (Su *et al.,* 2007) and inhibited the progression and growth on prostate cancer in transgenic adenocarcinoma mouse prostate (Harper *et al.,* 2007). The mechanisms involved in this activity are represented in **s**cheme 2.



***Scheme* 2.** *Schematic representation of mechanisms of anticancer properties of resveratrol (Adapted from UDENIGWE, C. C. [et al.] (2008). - Potential of resveratrol in anticancer and anti-inflammatory therapy. "Nutrition Reviews" 66:8 (2008) 445–454).*

Despite all of these references, the anticancer activity of *trans*-resveratrol is compromised due to weak and nonspecific effects on many biological targets. So it is important to develop derivatives with enhanced properties (Ogas, 2013).

1.2. Biocatalysis

1.2.1. Biocatalysis: principles and applications

In order to obtain good yields, some reactions require the use of catalysts (molecules that take part in the reaction making it faster by reducing the magnitude of the energy barrier) which are not consumed or altered throughout the reaction. One of the most proficient catalysts are enzymes (proteins that are mainly used in pharmaceutical products and fine chemicals) when specificity of the reaction is one of the major problems (Choi *et al*., 2015; Illanes, 2008).

Enzymes are needed in all living systems to catalyze the chemical reactions necessary to preserve the functionality and integrity of the biological systems, so they are able to act in a wide range of reactions, many of these, difficult to reproduce by chemical synthesis. In recent years the use of enzymes in reactions and consequently their applications in different areas (pharmaceutical, food, cosmetic, textile industries) has been increased exponentially, due to its distinct properties and to advances in protein engineering technology (Choi *et al*., 2015; Illanes, 2008).

Thus biocatalysis is defined as the use of isolated enzymes or whole-cell biotransformation, under artificial conditions, to promote reactions (Patel, 2016; Illanes, 2008).

Nowadays there is great concern for the environment, its protection has to be ensured. Pharmaceutical industry is one of the areas that causes more pollution so, safer and greener alternatives have to be used in order to preserve the environment such as biocatalytic processes instead of conventional chemical synthesis (Choi *et al*., 2015).

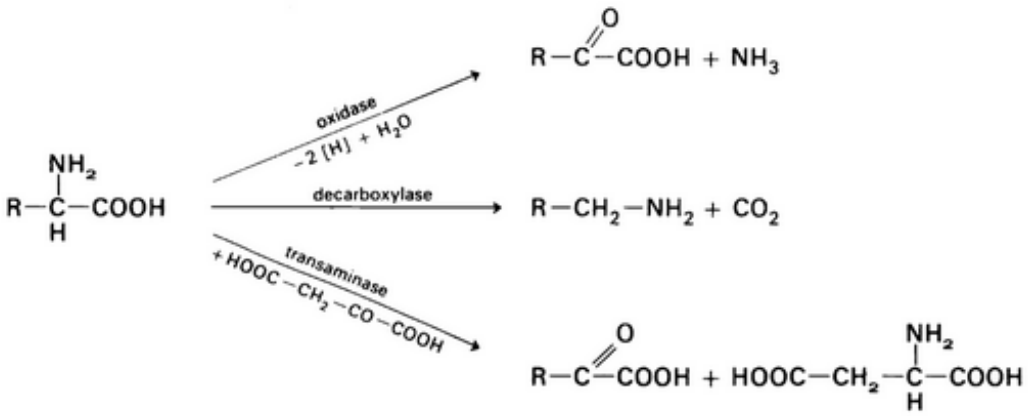
Biocatalysis has many characteristics that suit the requirements of green chemistry: the use of catalysts from natural sources that are biodegradable and non-harmful; the moderate conditions and the reduce waste of the reactions and the high selectivity (chemoselectivity, regioselectivity, and streoselectivity) and consequently the formation of products with higher purity compared to conventional chemistry. However, the enzymes are complex molecules that are intrinsically fragile and costly to produce (Patel, 2016; Choi *et al*., 2015; Illanes, 2008).

1.2.2. Enzyme specificity

Enzymes are catalysts that have specificity in relation to the substrate they use and the reaction they catalyze. Specificity is expressed as the rate at which a substrate is transformed to product rather than the affinity of substrate binding (Hedstrom, 2010).

1.2.2.1. Reaction Specificity

The enzymes have the ability to catalyze one reaction in the middle of so many others thermodynamically possible, being this particularity designated reaction specificity. In this case the enzyme decreases the activation energy of the reaction that will be catalyzed, restricting itself to that. The type of reaction is controlled by the position of acidic and basic residues, hydrophobic moieties, proton donors, proton acceptors, among other factors in the enzyme active site and by the orientation of the substrate on the enzyme.
For example, from the same substrate we can have different products depending on the enzymes used. Each of the enzymes catalyzes different reactions giving origin to the respective products (Hedstrom, 2010; Karlson, 1975).



There are exceptions, this specificity is not absolute. Enzymes have the ability to catalyze several reactions in the same catalytic site, some of them not biologically important, called promiscuous reactions. The development of the area of biocatalysis can be achieved by optimization of the promiscuous activities (Hedstrom, 2010).

1.2.2.2. Substrate Specificity

The substrate specificity describes the high ability of the enzymes to catalyze the conversion of the substrate into the respective product. This is determined by the specificity constant *Kcat*/*Km*, where *Kcat* value is the turnover number (that measures the amount of product formed per enzyme molecule when all of the enzyme is bound to the substrate) and *Km* is a complex kinetic constant and an apparent dissociation constant of all enzyme-bound substrate complexes. So different substrates compete for the same enzyme presenting different values of *Kcat*/*Km* and therefore this measure describes the catalytic efficiency of the enzyme towards a given substrate (Hedstrom, 2010).

What determines the specificity of an enzyme to a certain substrate is the structure of its active site. To occur an enzymatic reaction the active site should not be only complementary with the substrate. Structural complementary between the active site and the transition state of the reaction and the accumulation of noncovalent forces such as hydrogen bonding, steric, electrostatic, van der Waals and hydrophobic between the active site and the substrate are two properties that determine the specificity (Hedstrom, 2010).

1.2.3. Enzyme selectivity

One of the advantages of biocatalysis over chemical synthesis is that it catalyzes reactions with higher chemo-, regio- and stereoselectivity.

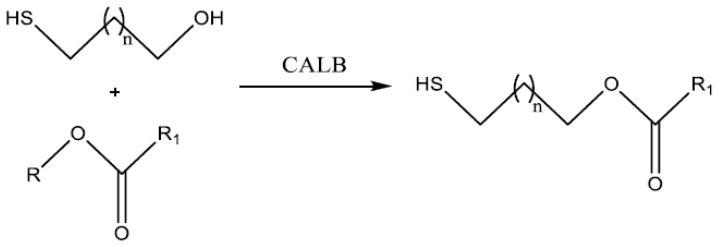
In pharmaceutical industry chirality is one of the important factors for the efficiency and safety of many drug products thus justifying the development of conditions to increase the activity and selectivity of biocatalysts (Patel, 2011).

1.2.3.1. Substrate selectivity

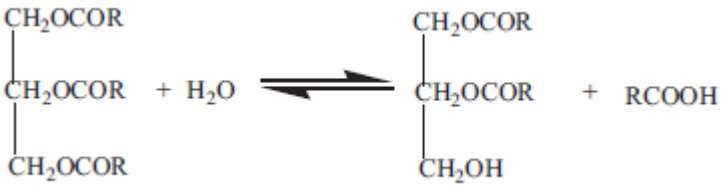
The selectivity of an enzyme is the ability to distinguish two competing substrates with similar structures. Thus the selectivity of an enzyme for two substrates A and B is expressed by the ratio of the specificity constants of each of the substrates $(Kcat/Km)_A/(Kcat/Km)_B$ (Reymond, 2006).

The selectivity for the substrate can be of three types:

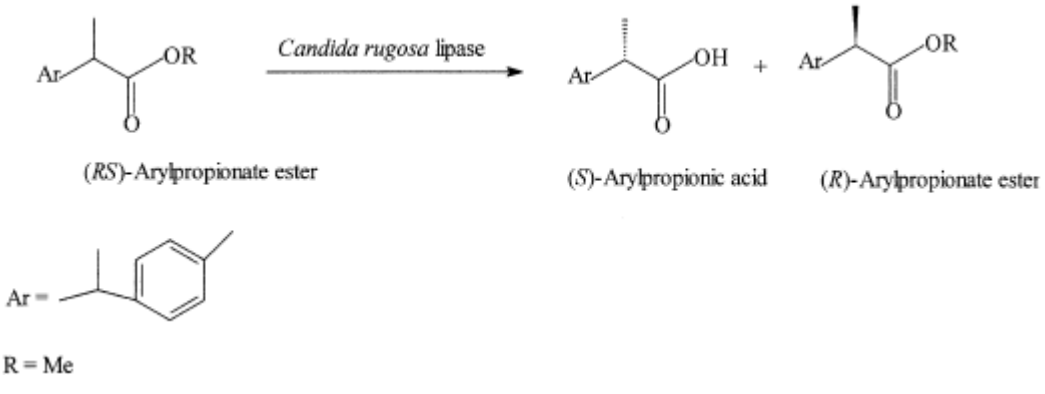
Chemoselectivity – Preferential reaction of an enzyme to one of two or more different functional groups. An example of this property is the study by Hedfords *et al.* (2010) which showed a selectivity for the enzyme lipase B from *Candida antarctica* (CALB) 10^5 times higher for alcohols rather than for thiols in transacylation reactions.



Regioselectivity – Preferential reaction of an enzyme to one of two or more similar functional groups on the same substrate. For example, the preferencial formation of 2,3-diglyceride from triglyceriden by a 1,3-specific lipases (Kapoor *et al*., 2012).



Stereoselectivity – Preferencial reaction of an enzyme to one of the stereoisomers. For example, the production of S-ibuprofen from a racemic mixture of α-methyl-4-[isobutyl]phenylacetic acid (ibuprofen) using lipase from *Candida cylindracea* (Muralidhar *et al.,* 2002).



1.2.4. Enzyme classification and nomenclature

Although researches assign enzymes with names according to their own preference there was a need to develop a system of classification and nomenclature for them. So the International Union of Biochemistry and Molecular Biology (IUBMB) developed a system of classification in which enzymes were divided into six major classes (**T**able 1), wich of them have subclasses and sub-subclasses according with the reactions they catalyze (McDonald *et al*., 2015; Simpson, 2012).

Each enzyme in the Enzymes List must have two names and an Enzyme Commission or Enzyme Code (EC) number. The first of the two names is the recommended name (for everyday use), the second is the systematic name (more precise chemically) which is used when the enzyme must be identified without ambiguity. The EC number characterizes the reaction type as to class (first digit), subclass (second digit) which describes the nature of the group involved in the reaction, and sub-subclass (third digit) that gives information about the type of reaction being catalyzed. The fourth digit is the serial number of the enzyme in its sub-subclasse. For example, the EC number for protease tripeptide aminopeptidase is 3.4.11.4 where EC3 indicates a hydrolase, EC3.4 indicates that it acts on peptide bonds and cleaves off the amino acids from the amino end EC3.4.11, while acting on tripeptides as substract EC3.4.11.4 (Khan *et al*, 2015; Drauz, 2012; Simpson, 2012).

Table 1. *Enzyme classes and the types of reaction they catalyze.*

Class number	Class name	Reaction scheme
1	Oxidoreductases	$AH_2 + B^+ \longrightarrow A + BH + H^+$ or $AH_2 + B \longrightarrow A + BH_2$
2	Transferases	$AX + B \longrightarrow A + BX$
3	Hydrolases	$A—B + H_2O \longrightarrow AH + BOH$
4	Lyases	$A{=}B + X—Y \longrightarrow \begin{matrix} A—B \\ \vert \quad \vert \\ X \quad Y \end{matrix}$
5	Isomerases	$A \longrightarrow B$
6	Ligases	$A + B + NTP \longrightarrow A—B + NDP + P$ or $A + B + NTP \longrightarrow A—B + NMP + PP$

Class 1. Oxidoreductase

These enzymes catalyze oxidation-reduction reactions resulting in the transfer of hydrogen, oxygen and/or electrons between a donor and an acceptor. This class includes dehydrogenases (enzymes that catalyze the transfer of hydrogen), oxidases (enzymes that catalyze the transfer of two electrons from a donor to oxygen (acceptor)), oxygenases (enzymes that catalyze the incorporation of oxygen into a substrate, in this case the incorporation of one oxygen is called monooxygenase and the incorportation of both oxygen atom are called dioxygenases) and peroxidases (enzymes that catalyzed the transfer of eletrons into H_2O_2 (acceptor)).

Class 2. Transferases

These enzymes transfer functional groups (methyl, glycosyl, sulphate, phosphate groups) from one substrate (donor) to another (acceptor). This class has been dividided into 10 subclasses based on the type of group involved.

Class 3. Hydrolases

These enzymes catalyze the hydrolytic cleavage of bonds such as C–O, C–N, C–C, C-S and some other bonds, including phosphoric anhydride bonds. In theory all hydrolases are esterases since that is due to the transfer of a specific group to water acting as an acceptor.
This class has been dividided into 13 subclasses based on the nature of the bond hydrolysed.

Class 4. Lyases

These enzymes cleave the bond C–C, C–O, C–N and other bonds by elimination reactions resulting in the formation of double bonds or rings or conversely added groups to double bonds.
This class has been dividided into 8 subclasses based on the type of bond cleaved.

Class 5. Isomerases

These enzymes catalyze geometric or structural changes within one molecule. According to the type of isomerism involved, they may be called *racemases*, *epimerases*, cis–trans-*isomerases*, *isomerases*, *tautomerases*, *mutases* or *cycloisomerases*.
This class has been dividided into 6 subclasses based on the type of isomerization reactions.

Class 6. Ligases

These enzymes catalyze the combination of two molecules coupled with hydrolysis of a diphosphate bond in ATP or a similar triphosphate.
This class has been dividided into 6 subclasses based on the type of bonds they catalyze (McDonald *et al*., 2015; Simpson, 2012; Shanmugam, 2009).

Hydrolases are the enzymes used in this work more specifically lipases. Thus we will clarify the catalytic mechanism, properties and applications of this enzyme.

1.2.5. Hydrolases: lipases sources and applications

As previously said, the enzymes are classified according to the reactions that catalyze. Hydrolases catalyze the hydrolytic cleavage of C–O, C–N, C–C, C-S and other bonds, so in this context, lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are a kind of enzyme that belongs to this class.
Under normal conditions, lipases catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil–water interface. In addition to these reactions in certain cases the lipases act in the synthesis like in water-restricted environments. In this case the thermodynamic equilibrium moves in the direction of the synthesis and so the reverse reaction, esterification, or even several transesterification reactions can occur. Lipases catalyze a wide range of reactions including hydrolysis, esterification, acidolysis, alcoholysis, interesterification and aminolysis (Anobom *et al.,* 2014; Thakur 2012).
Lipases are produced, with different catalytic properties, by animals (pancreatic, hepatic and gastric), plants and microorganisms (bacterial, fungal and yeast). Being the lipases from

microorganisms the most used class of enzymes in biotechnological applications and organic chemistry due to their stability, selectivity and broad substrate specificity.
As these enzymes have great effectiveness, stability and versatility they are used in several commercial areas, namely in food, cosmetic, detergent, pharmaceutical, textile, paper, fine chemical, oil chemical and in biodiesel industries (Anobom *et al.,* 2014; Thakur 2012).

1.2.5.1. Structure of lipases

Although each lipase has its own identity these have some similar structural elements, namely the tertiary folding α/β-hydrolase, containing α-helices and β-sheets and an active site with the catalytic triad, consisting of serine-aspartate/glutamate-histidine residues and the oxyanion hole (Rauwerdink *et al.,* 2015).

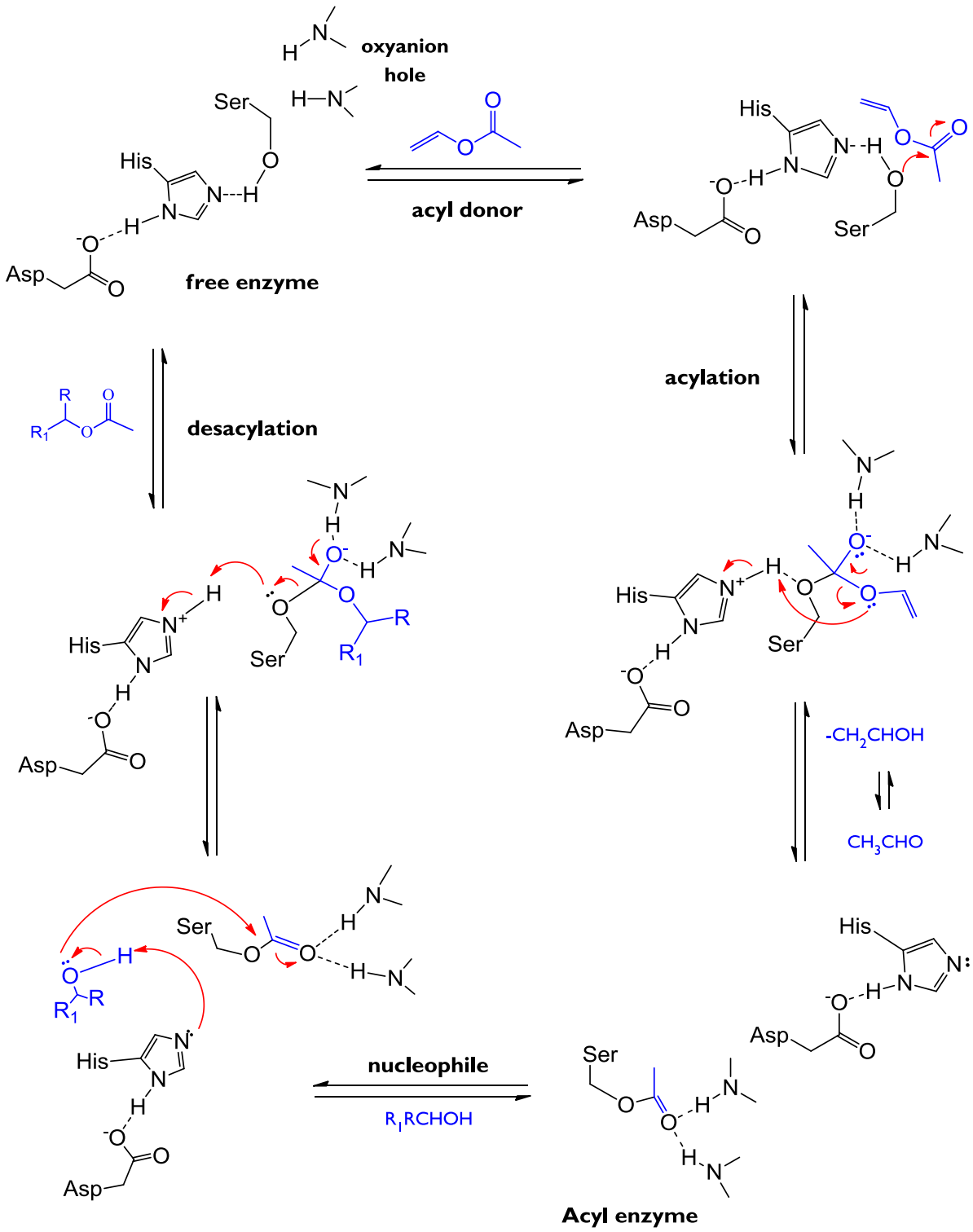
Many lipases are activated after binding to a hydrophobic substrate interface. These enzymes contain a mobile peptide chain called lid that covers the access of the substrate to the catalytic center, creating a hydrophobic environment inside the lid in contact with the active site whereas the outside is hydrophilic, in contact with solvent. Studies using the crystallography of structures showed that the lid can adopt two conformations: the closed and the opened states. In aqueous environment, the active site is covered by the lid and therefore the enzyme surface is mainly hydrophilic leading to the inactivation of the enzyme. In the presence of a hydrophobic solvent or at hydrophobic interface, the conformation of the enzyme changes and the lid opens, exposing the catalytic site to the substrate leading to the activation of the enzyme. The configurational rearrangement of the enzyme is known as interfacial activation.
The mechanisms of activation by which the lid moves are still poorly understood owing to the structural differences in lid from lipase to lipase (Rauwerdink *et al.,* 2015; Anobom *et al.,* 2014; Mukherjee, 2014; Rehm *et al.,* 2010).

1.2.5.2. Enzymatic mechanism of lipases

The catalytic mechanism of lipases in their catalytic action is the ping-pong bi-bi mechanism, involving an acyl-serine enzyme intermediate. In this case either the formation or the release of the intermediate may be rate limiting (Rauwerdink *et al.,* 2015).
In the acylation step, the oxygen atom of the serine attacks the carbonyl group of the ester linkage forming the first tetrahedral intermediate which establishes hydrogend bonds with

backbone nitrogen atoms in the oxyanion hole. In Nature, the ester are tri, di or mono acyl glicerols. At the same time the catalytic histidine, acts as a base, and deprotonates serine, stabilizing the negative charge formed by the attack. The reformation of the tetrahedral intermediate leads to the formation of the carbon-oxygen double bond and consequently to the release of the alcohol (first product) and the acyl enzime intermediate which contains the alkyl chain of the first substrate covalently bonded. Histidine acts as an acid and the ethanol is released (**S**cheme 3).

The second step is the deacylation step that is basically the inverse of the acylation. In this case the nucleophilic attack is made by the alcohol (in Nature the nucleophile is water) to the intermediate acyl enzyme, once again, forming the second tetrahedral intermediate with the aid of the histidine, acting as a base. The rearrangement leads to the release of the second product, an ester, and the enzyme in its intact form (**S**cheme 3) (Rauwerdink *et al.,* 2015; Anobom *et al.,* 2014; Mukherjee, 2014).



Scheme 3. *Enzymatic mechanism of lipases in the presence of an irreversible acyl donor (vinyl acetate), which isomerizes to the carbonyl product, shifting the reaction equilibrium.*

1.2.6. Biocatalysis in organic solvents

Despite the importance of water in many biotransformation reactions, enzymes are not restricted to catalyze reactions in aqueous media. On the contrary, the many advantages observed in the use of organic solvents allowed this area to be investigated in the production of several industrial products (Kumar *et al.,* 2016).

In aqueous media the removal of water is an expensive process due to the high boiling point and low vapor pressure. On the other hand, undesired reaction as hydrolysis, racemization, polymerization and decomposition often occurs in water thus limiting many of the reaction of interest. Therefore the use of organic solvents will overcome certain disadvantages observed in the use of the conventional solvent, such as: the increased solubilility of non-polar substrates and products; the reversal of the thermodynamic equilibrium in favor of synthesis instead of hydrolysis; in some cases can affect the regiosseletivity and the enantioselectivity of the reaction and suppresses unwanted water-dependent side reactions.

Despite of these advantages, it is never possible to predict if the organic solvent is ideal for that particular biotransformation, making the reactions unpredictable (Kumar *et al.,* 2016; Grunwald, 2014; Kapoor *et al*., 2012; Torres *et al.,* 2004).

1.2.6.1. Lipase behavior in organic solvents

The availability and stability of the lipases in both organic and aqueous media gives them great applicability in several areas. As stated above, under natural conditions, they are able to catalyze hydrolysis reactions of the ester bond and in non-aqueous conditions catalyze the reverse reaction of ester synthesis (Sharma *et al.,* 2014).

The majority of lipases are active and stable in anhydrous organic solvents. As lipases undergoe an interfacial activation, they have a lower catalytic activity in aqueous media than in biphasic systems like aqueous-organic solvent (Kumar *et al.,* 2016).

Lipases behave differently in different organic solvents and/or organic media in different reaction systems. The activity and catalytic potential of the enzymes in organic media does not depend only on the octanol-water partition coefficients (log P) but also the cumulative effect of the dielectric constant, dipole moment, hydrogen binding, polarizability and the denaturing capacity of the enzyme (Kumar *et al.,* 2016; Grunwald, 2014).

As previously stated the use of organic solvents alters the selectivity of the enzyme, in particular regioselectivity and enantioselectivity. The conformation of the enzyme in these solvents appears to be more rigid, allowing to control some of the catalytic properties of the

enzyme, such as substrate seletivity. The exchange of one solvent for another can affect the regioselectivity of the lipases in a given reaction for example, in a study by Rubio et al. (1991) showed that the transesterification of 1,4-dibutyryloxy-2-octylbenzene into butanol catalyzed by lipase from *Pseudomonas cepacia* resulted in the formation of 4-butyryloxy-2-octyl phenol in the case of the use of hydrophobic solvents while in hydrophilic solvents favored the formation of 4-butyryloxy-3-octyl phenol (Sharma *et al.,* 2014; Kapoor *et al*., 2012).

The inversion of enantioselectivity was demonstrated by Hirose et al., (1992) that showed the hydrolysis of several prochiral 1,4-dihydropyridine derivatives catalyzed by lipase from *Pseudomonas sp.* in different organic solvents. They showed that using the ciclohexane solvent it gave the (*R*)-enantiomer (ee~90%) and using the iso-propyl ether gave the (*S*)-enantiomers (ee~90%) (Kapoor *et al*., 2012).

The applicability of the use of organic solvents in biotransformations is increasing, however, many of these solvents do not apply to the principles of green chemistry since they compromise the health and safety of humans and the environment. Thus the substitution of these harmful solvents by other more friendly solvents such as ionic liquids and supercritical fluids has helped in solving these problems (Patel, 2016; Grunwald, 2014).

Another issue associated with the use of organic solvents is due to the fact that many enzymes are easily inactive and denatured in these media. So the isolation of enzymes that have been secreted by microorganisms tolerant to organic solvents is one of the methods developed in order to stabilize the enzymes in these solvents (Kumar *et al.,* 2016).

1.2.7. Combination of enzymatic and non-enzymatic reactions

Enzymatic reactions have several advantages over chemical reactions and vice versa (**F**igure 15). Although these two disciplines are independent of each other they both have the same goal of obtaining small molecule products that are essential components of our everyday lives. Sometimes the question is what will be the best approach to get a particular product. In this case the choice of the best method has to take into account the overall yield of the product, the cost of its production, the environmental impact of the process and its operational simplicity. The high selectivity, catalytic efficacy, compliance with the principles of green chemistry and acceptance of unnatural substrates makes the enzymatic reactions the most favorable. However, this does not happen since even, with these advantages, the

biological catalysts still offer a limited number of reactions compared to synthetic organic chemistry (Kumar *et al.,* 2016; Wallace *et al.,* 2014).

Since the two approaches are complementary in terms of their positive and negative aspects, instead of choosing just one we try to combine them in their strengths (Wallace *et al.,* 2014).

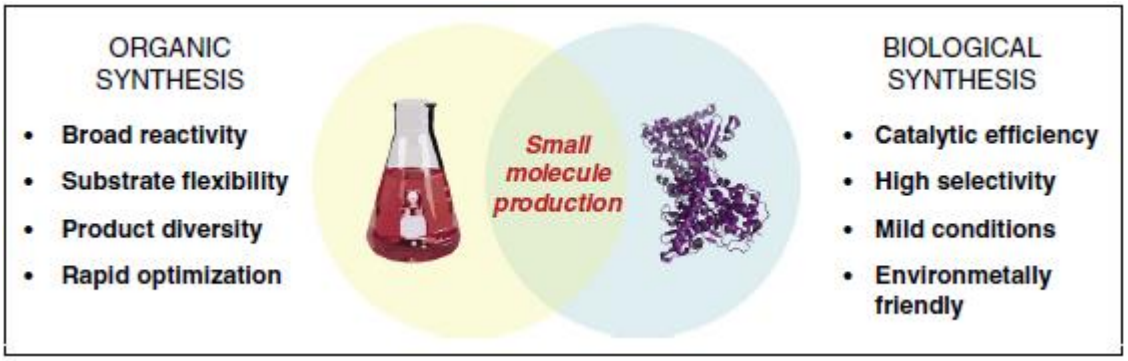


Figure 15. *Advantages related to organic and biological synthesis (Adapted from WALLACE, S. [et al.] (2014) - Opportunities for merging chemical and biological synthesis. "Current Opinion in Biotechnology" 30 (2014) 1–8).*

Thus in this work the combination of enzymatic reactions with non-enzymatic reactions is incorporated into the final product.

1.3. Structural modification of polyphenols

1.3.1. Structural modification of flavonoids and stilbenes

The majority of the studies describe the biological activities of polyphenols *in vitro* not taking into account the bioavailability and metabolism factors that occur *in vivo*. In the human body, these factors may partially account for the lack of biological activity (Manach *et al.,* 2004).

On the other hand, the use of polyphenols in several domains is limited by their weak stability and solubility in organic or aqueous solventes and so to enhance their properties several studies were made in order to modify its structure by chemical, enzymatic or chemo-enzymatic reactions (Amri, 2012; Chebyl *et al.,* 2007).

The clarification of SAR of these compounds is an asset to the development of new derivatives. The simple structure and the several manipulation sites turns these molecules (naringenin and resveratrol) the most interesting to chemical modification (Pezzuto *et al.,* 2013).

Over the years, several analogs have been synthesized and tested. Many of them showed much greater activity than the parent compound in terms of potency and specificity (Pezzuto *et al.,* 2013).

1.3.1.1. Acylation of flavonoids and stilbenes

Acylation upgrades lipophilicity of the molecule and improves absorption and cell permeability, leading to an improvement of their bioavailability. These modification became a solution to the low water solubility and bioavalability and so the interest on acylated derivatives has increased (Vlachogianni *et al.,* 2014).

Some characteristics are needed to keep in the structure of polyphenols in order to preserve the biological effects observed so great attention must be given to the number, positions and types of substitutions occurring during the acylation reaction. This is more critical for aglycone because the substitution takes place on the hydroxy groups of A, B and C rings while for glycosides, the acylation occurs on the glycosylated moiety (Chebil *et al.,* 2007).

A study compared the pharmacokinetics of resveratrol and its prodrug 3,5,4´-Tri-O-acetylresveratrol, in rats, and showed that after resveratrol was transformed into its prodrug, its pharmacokinetic properties were improved such as the $t_{1/2}$ has been prolonged

and the AUC has increased. This can be explained by the fact that resveratrol is metabolized into glucuronic acid and sulfate conjugations being the reason of its low bioavailability. The hydroxyl groups are the main points where glucuronidation and sulfation take place, so acetylation of these groups protects them of these modifications during the metabolism (Liang *et al.,* 2013).

This study concluded that acetylated derivatives can be obtained from natural sources, chemically using acetic anhydride or enzymatically using lipases or transferases (Vlachogianni *et al.,* 2014). These derivatives have the same or stronger activity than the parent compound, for example, the 3,5-Diacetyl-resveratrol showed a stronger effect as antioxidant than resveratrol, it exhibited a powerfull effect in non-enzymatic linoleic acid peroxidation (Vlachogianni *et al.,* 2014) and exerts a more powerfull inhibitory effect on platelet activating factor (which is a strong phospholipid mediator that is implicated in the initiation of the inflammatory process) (Fragopoulou *et al.,* 2007). 3,5,4´-Tri-O-acetylresveratrol showed that was able to protect live cells after gamma irradiation on mices (Koide *et al.,* 2011); exhibited the antiproliferative effect on HepG2, human hepatoma cell line, (inhibited 47% of cells proliferation after 48h), at the same concentration of resveratrol (inhibited 26%) (Colin *et al.,* 2008); induced the accumulation of human colon cancer cells in early S phase of the cell cycle; enhance 5-fluorouracil-mediated inhibition of colon cancer cell proliferation (Colin *et al.,* 2009) and also exhibited the same effect antimelanogenic of resveratrol (Park *et al.,* 2014).

Chapter 2

Thesis objectives

2. Thesis objectives

The applicability of the polyphenols in the pharmaceutical industry is conditioned by the low stability and low solubility in lipophilic medium. One solution to this problem is the acylation of the hydroxyl groups, in which in many cases may even increase their biological properties.

The objectives of this work were to obtain acylated derivatives of polyhydroxylated substrates, resveratrol and naringenin, through regiosselective acylation reactions by combination of enzymatic and chemical methods.

Therefore, the goals of this work are to obtain acylated compounds through selective biocatalytic transformations and to further acylate them by a chemical reaction.

After the synthesis of these new derivatives, isolation by chromatographic column and characterization by NMR, ^{1}H NMR, ^{13}C NMR and DEPT, will be performed.

In summary, in this work we will obtain a set of novel compounds with relevant biological activity.

Chapter 3

Results and discussion

3. Results and discussion

As previously stated polyphenols have relevant biological activity, however their applicability is limited due to the low solubility in lipophilic media and the low bioavailability in the organism. The modification of these compounds, in particular through acylation reactions, allows the improvement of these properties.

The acylation of the hydroxyl groups of the substrates used, naringenin and resveratrol, was obtained by enzymatic and chemical methods, using lipases as biocatalysts, or only by chemical means.

3.1. Regiosselective enzymatic acylations

For each substrate, the best enzymatic reaction conditions were selected, according to the yields obtained. Identification of the products was made by ^{1}H NMR analysis, ^{13}C NMR analysis and DEPT.

The signal in the region of 7.260 ppm in the ^{1}H NMR spectrum and the triplet in the 77 ppm region in the ^{13}C NMR spectrum are relative to the deuterated chloroform.

3.1.1. Enzimatic acylation of naringenin

Naringenin is found especially in many *Citrus* fruits and has been shown to possess antioxidant, antiinflammatory, antiproliferative and antimutagenic properties.

This substrate has 3 phenolic hydroxyl groups that can undergo acylation (**F**igure 13). The most accessible hydroxyl group is that at C-4´ and therefore is more susceptible to acylation. On the other hand, the hydroxyl group at C-5 is the least likely to undergo acylation because of the greater steric hindrance.

In the enzyme catalyzed reactions, acetone was used as solvent, vinyl acetate as acylating agent, and CALB as biocatalyst, at 50°C and 200 rpm. The mono-acylated derivative was obtained as main product, easily isolated by chromatographic column and characterized by NMR along with the starting substrate, naringenin, for comparison.

^{1}H NMR and ^{13}C NMR and DEPT spectra of naringenin

^{1}H NMR (δ ppm): The spectrum of naringenin provides signals characteristic of its structure and therefore will allow us to observe the slight differences of these peaks in the acylated products. The signal δ=11.94 is attributed to the hydrogen of the hydroxyl group in the carbon C-5, involved in an intramolecular hydrogen bond with the carbonyl of carbon C-4, the singlet at δ=10.45 refers to the hydrogen of the hydroxyl group at C-7 and the signal δ=9.30 is the hydrogen of the hydroxyl group at C-4´. The hydrogens belonging to the aromatic group of the B ring are at δ=7.11 (2H, d, J=8.63 Hz, H-2´ and H-6´) and δ=6.62 (2H, d, J=8.63 Hz, H-2´ and H-6´). The singlet at δ=5.71 is attributed to the hydrogens at C-6 and C-8 of the A ring. In relation to C ring we observed a double doublet at δ=5.23 (1H, dd, H-2), δ=3.05 (1H, dd, H-3_{trans}), and δ=2.54 (1H, dd, H-3_{cis}) (**F**igure 16).

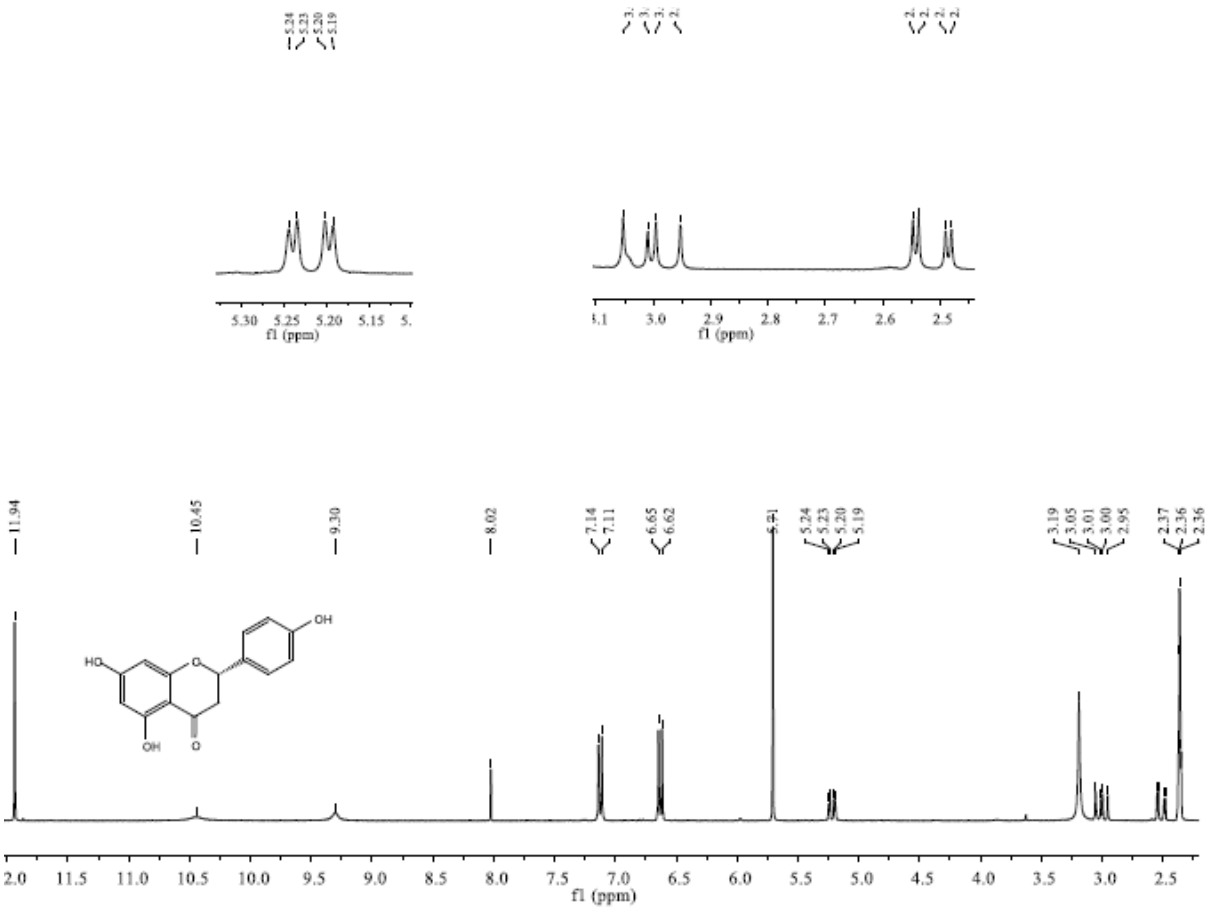


Figure 16. *^{1}H NMR spectrum of naringenin.*

^{13}C NMR (δ ppm): 40.67 (C-3); 77.10 (C-2); 93.71 (C-8); 94.62 (C-6); 100.56 (C-10); 114.04 (C-3´ and C-5´); 126.96 (C-2´and C-6´); 127.69 (C-1´); 156.89 (C-4´); 162.02 (C-9); 162.67 (C-5); 165.96 (C-7); 195.27 (C-4) (**F**igure 17).

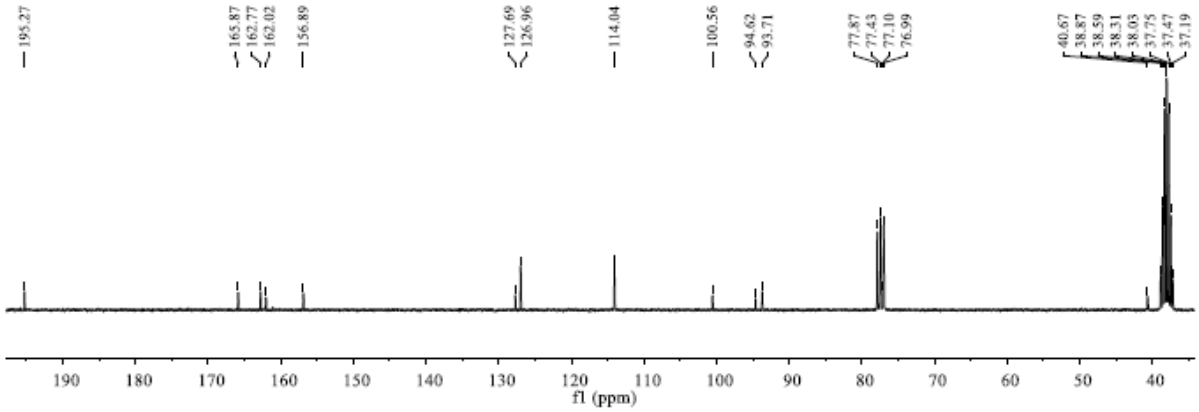


Figure 17. *^{13}C NMR spectrum of naringenin.*

The DEPT 135 spectrum gives information about the type of carbons present in the structure. Thus of 15 carbons present in naringenin, 7 are quaternary, 1 is secondary and 7 are tertiary (**F**igure 18).

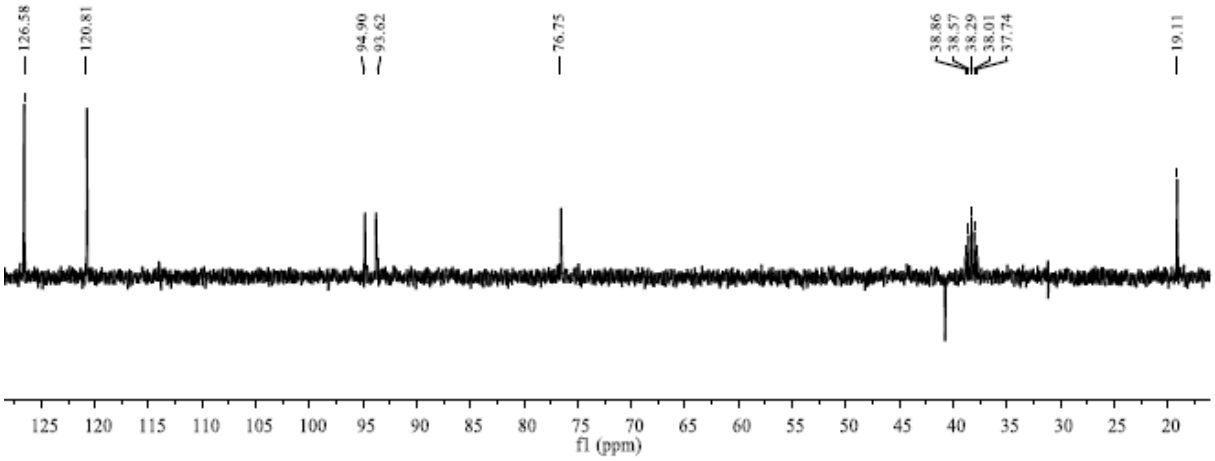


Figure 18. *DEPT 135 spectrum of naringenin.*

^{1}H NMR spectrum of product obtained

By analyzing the two spectra ^{1}H NMR from the initial substrate and the acylated derivative, we found signal loss at δ=9.30 ppm relative to the hydroxyl group at C-4´and the appearance of a singlet at δ=2.33 ppm relative to the CH_3 of the acetyl group in C-4´ (**F**igure 19).

As this reaction has already been described by Kyriakou and their co-workers (2012), we herein analyzed the ^{1}H NMR spectrum for confirmation of the structure of the acylated derivative obtained.

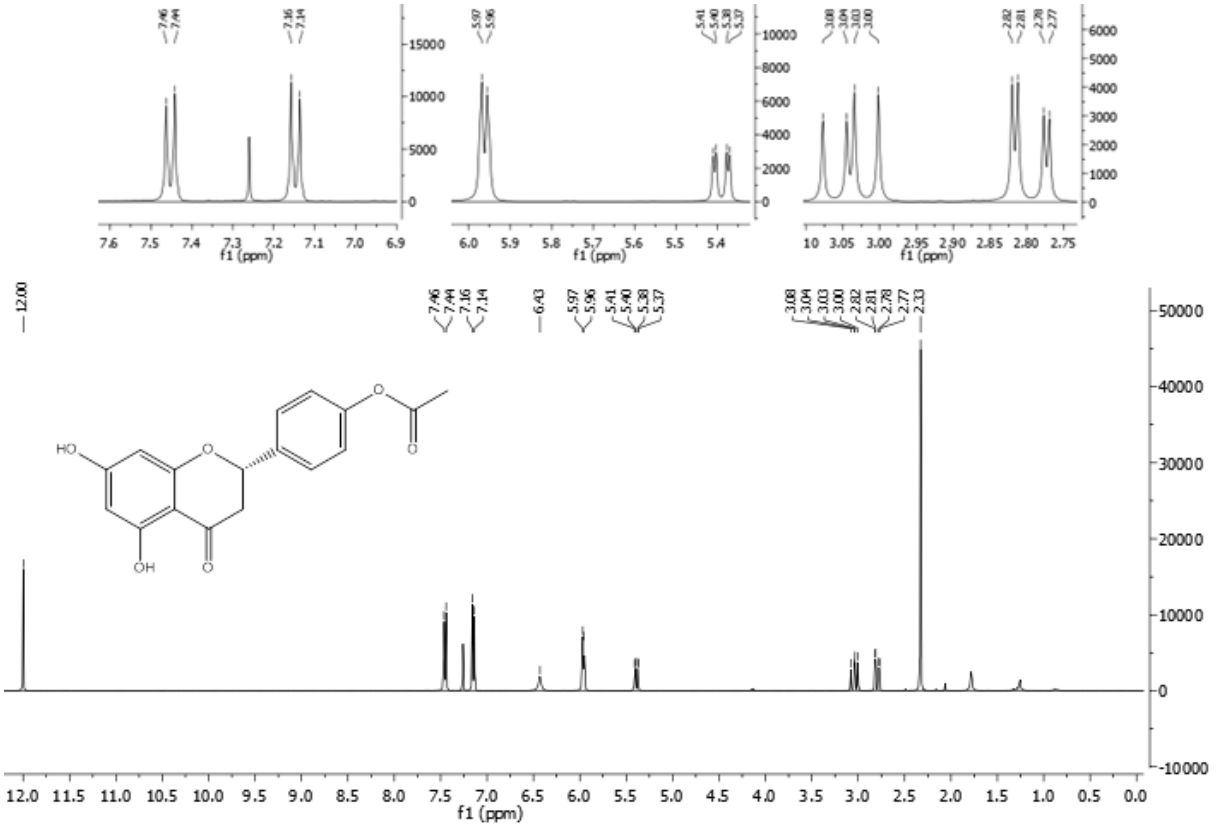


Figure 19. *^{1}H NMR spectrum of 4´-acetyl naringenin.*

Given the information of the structural analysis, the reaction product was caracterized as 4´-acetyl naringenin (**F**igure 20).

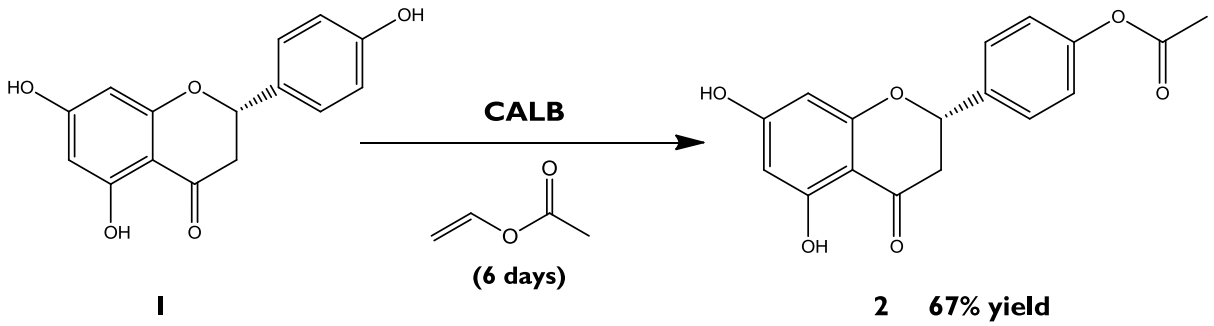


Figure 20. *Enzymatic acylation reaction of naringenin with vinyl acetate.*

3.1.2. Enzimatic acylation of resveratrol

Resveratrol has 3 phenolic hydroxyl groups that can undergo acylation (**F**igure 14). The most accessible hydroxyl group, just like in naringenin, is that at C-4´ and therefore is more susceptible to enzymatic acylation.

In the enzyme catalyzed reactions, toluene was used as solvent, vinyl acetate as acylating agent, and lipase PS as biocatalyst, at 50°C and 200 rpm. The diacylated derivative was obtained as main product, easily isolated by chromatographic column and characterized by NMR along with the starting substrate, resveratrol, for comparison.

^{1}H NMR and ^{13}C NMR and DEPT 135 spectrum of resveratrol

^{1}H NMR (δ ppm): The spectrum of resveratrol provides signals characteristic of its structure and therefore will allow us to observe the slight differences of these peaks in the acylated products. The resveratrol spectrum shows signals relating to 7 aromatic hydrogens, 3 doublets at δ=6.20, 6.73 and 7.18 ppm and 1 triplet at δ=5.96 ppm. The 2 doublets at δ=6.56 and 6.60 ppm correspond to the α and β hydrogens with coupling values of J^{3}=16.5 Hz and J^{3}=16.3 Hz, characteristic of the *trans* stereoisomer.
The hydrogens of the hydroxyl groups are at δ=8.90 ppm, integrating 1 hydrogen, and δ=9.24 ppm, integrating 2 hydrogens (**F**igure 21).

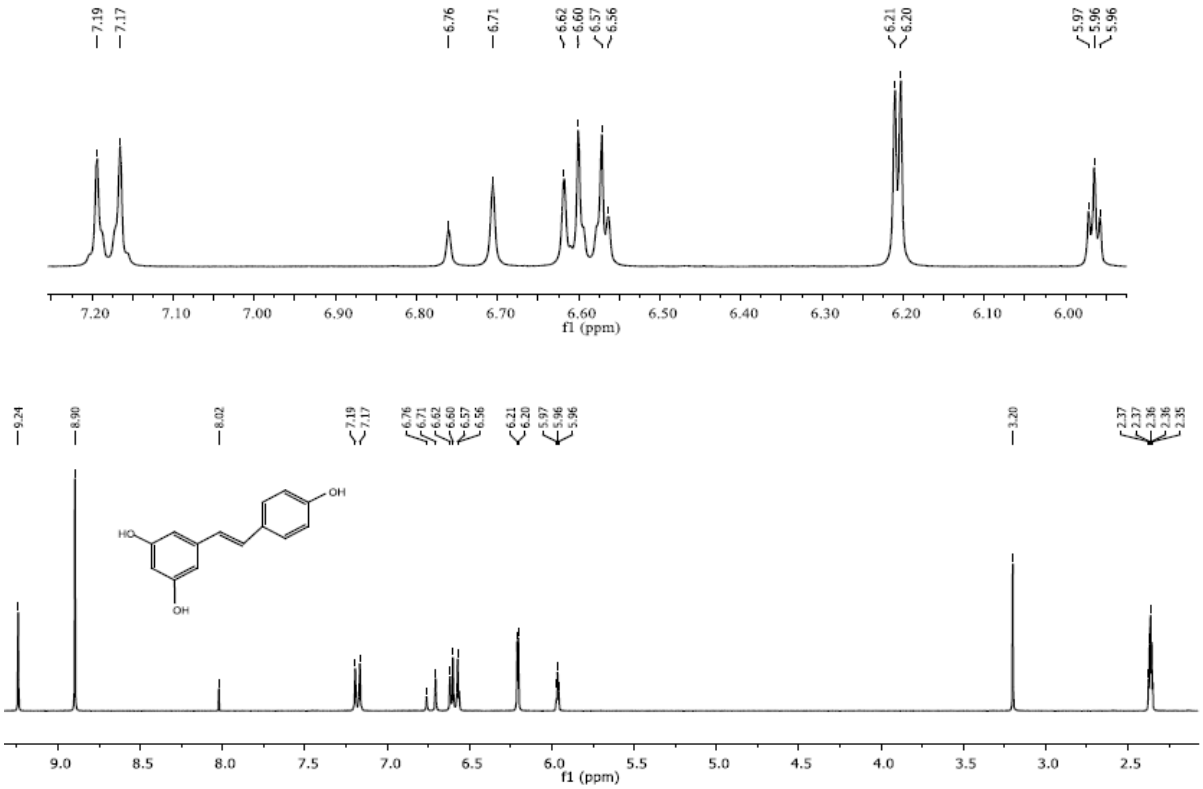


Figure 21. *^{1}H NMR spectrum of resveratrol.*

^{13}C NMR (δ ppm): 100.57 (C-4); 103.05 (C-2 and C-6); 114.33 (C-3′ and C-5′); 124.51 (C-7); 126.56 (C-2′ and C-6′); 126.69 (C-8); 138.13 (C-1); 156.33 (C-4′); 157.60 (C-3, C-5) (**F**igure 22).

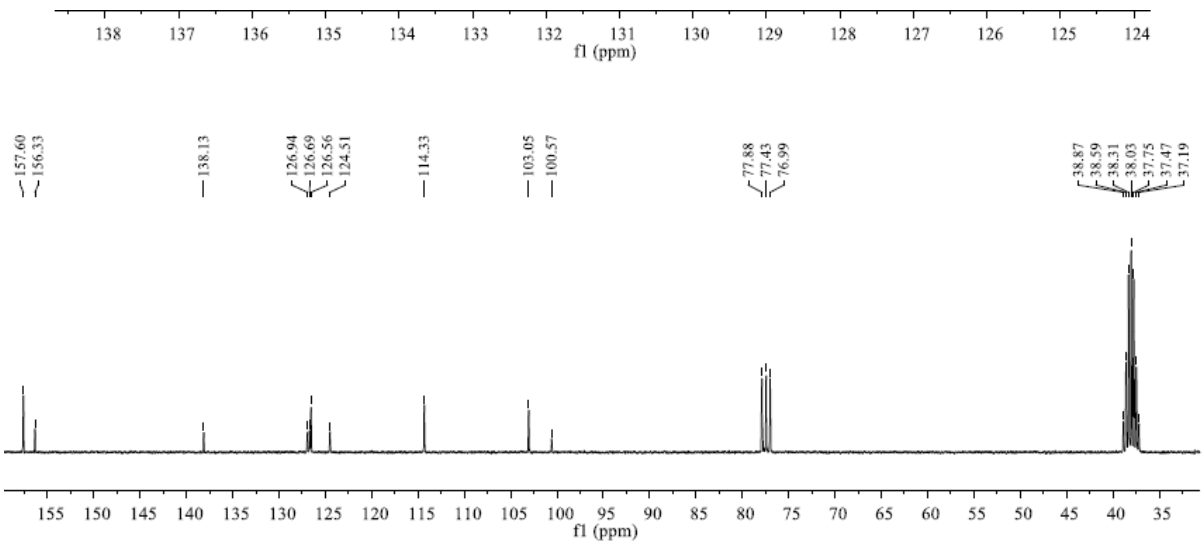


Figure 22. *^{13}C NMR spectrum of resveratrol.*

Based on the DEPT 135 spectrum of 14 carbons present in resveratrol, 5 are quaternary and 9 are tertiary (**F**igure 23).

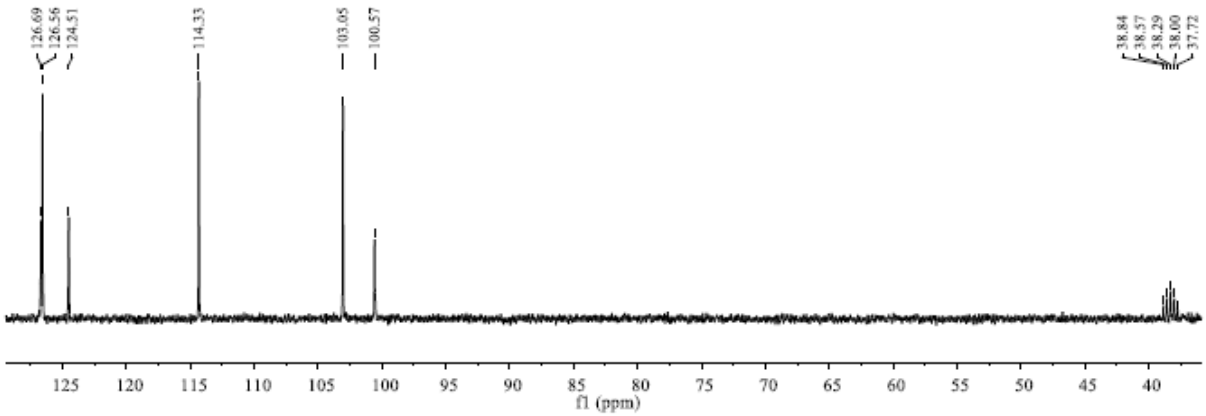


Figure 23. *DEPT 135 spectrum of resveratrol*

^{1}H NMR spectrum of product obtained

By analyzing the two spectra ^{1}H NMR from the initial substrate and the acylated derivative we found signal loss corresponding to two hydrogens of the hydroxyl groups at C-4´ and C-7 and the appearance of two singlets at δ=2.31 and 2.32 ppm relative to the CH_3 of the acetyl groups in C-7 and C-4´ (**F**igure 24).

As this procedure has already been described by Barradas (2013), we herein analyzed the ^{1}H NMR spectrum for confirmation of structure of the acylated derivative obtained.

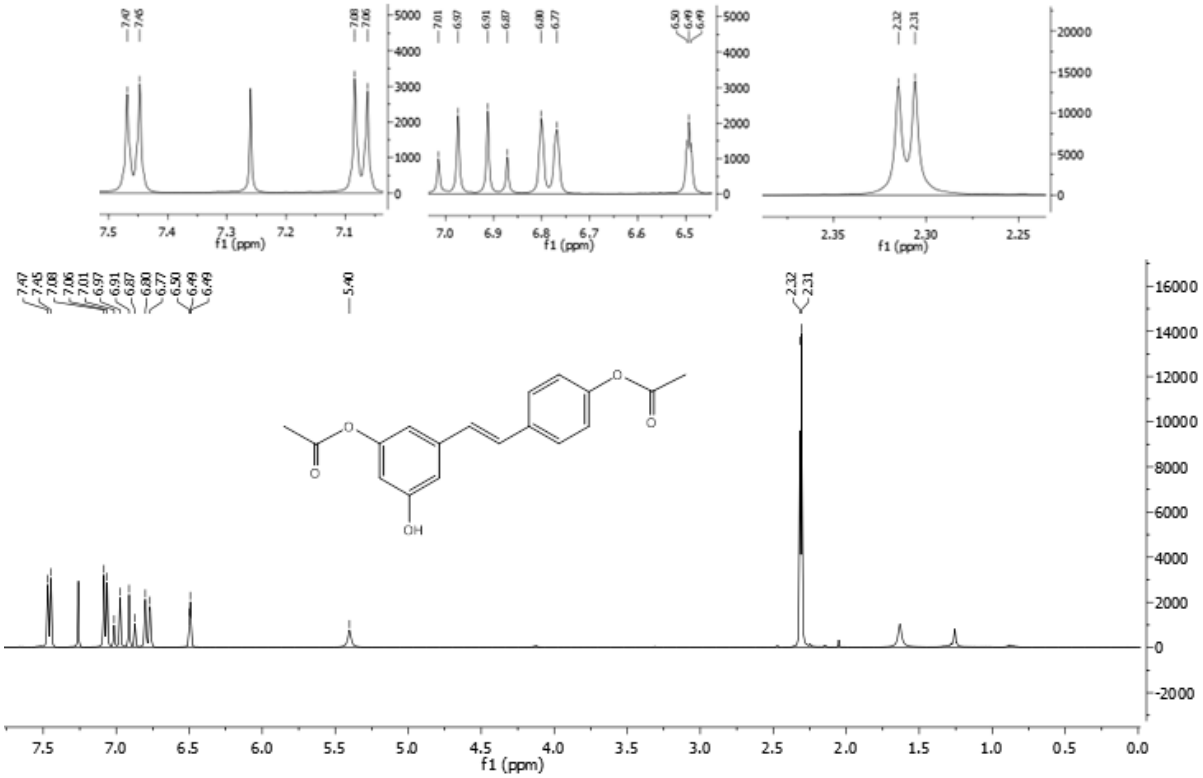


Figure 24. *^{1}H NMR spectrum of 3,4´-diacetyl resveratrol.*

Given the information of the structural analysis, the reaction product was caracterized as 3,4´-diacetyl resveratrol (**F**igure 25).

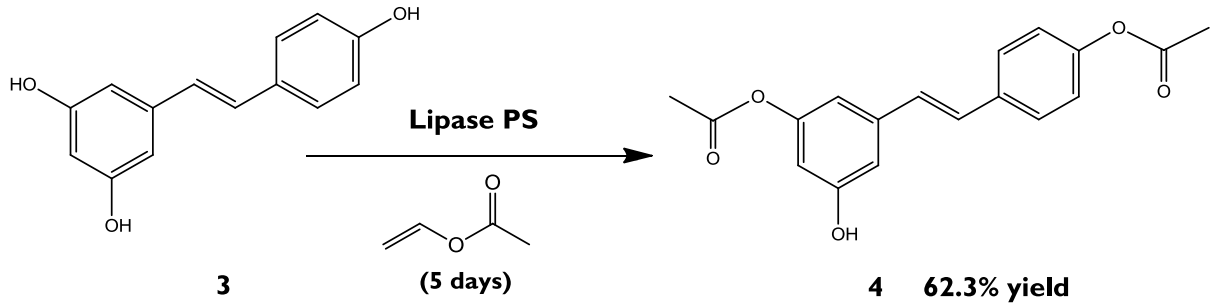


Figure 25. *Enzymatic acylation reaction of resveratrol with vinyl acetate.*

3.2. Chemical acylations

3.2.1. Chemical acylation of naringenin

In the chemical acylation reactions, dichloromethane was used as solvent, acetic anhydride as acylating agent and 4-DMAP as catalyst, with stirring at room temperature. The diacylated derivative was obtained as main product, easily isolated by chromatographic column and characterized by NMR.

^{1}H NMR and ^{13}C NMR and DEPT 135 spectra of product obtained

By analyzing the two ^{1}H NMR spectra from the initial substrate and the acylated derivative we found signal loss at δ=10.47 and 9.30 ppm corresponding of two hydrogens of hydroxyl groups in C-4´ and C-7 and the appearance of two singlets at δ=2.32 and 2.38 ppm relative to CH_3 of the acetyl groups in C-7 and C-4´ (**F**igure 26).

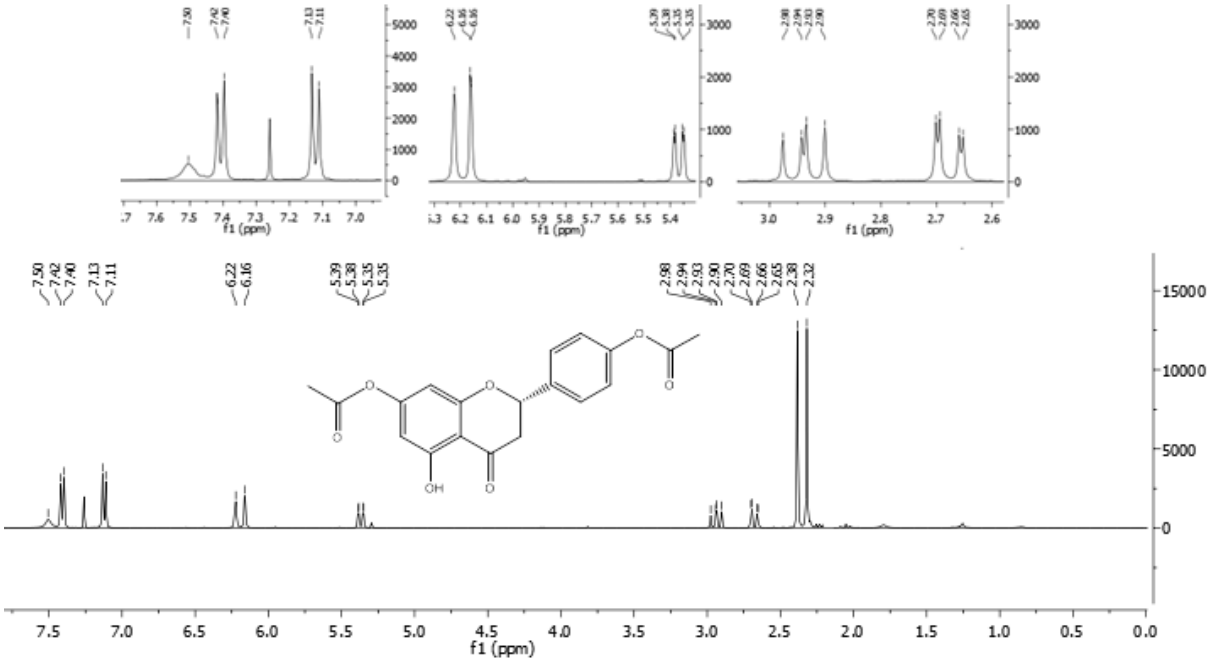


Figure 26. *^{1}H NMR spectrum of 7,4´-diacetyl naringenin.*

By analyzing the two spectra ^{13}C NMR from the initial substrate and the acylated derivative we found the appearance of extra signals relative to the CH_3 at δ=21.14 and 21.24 ppm and

to the carbonyl of the acetyl groups at δ=169.73 and 170.40 ppm. Slight changes occur in other carbons, but it is not significant (**F**igure 27).

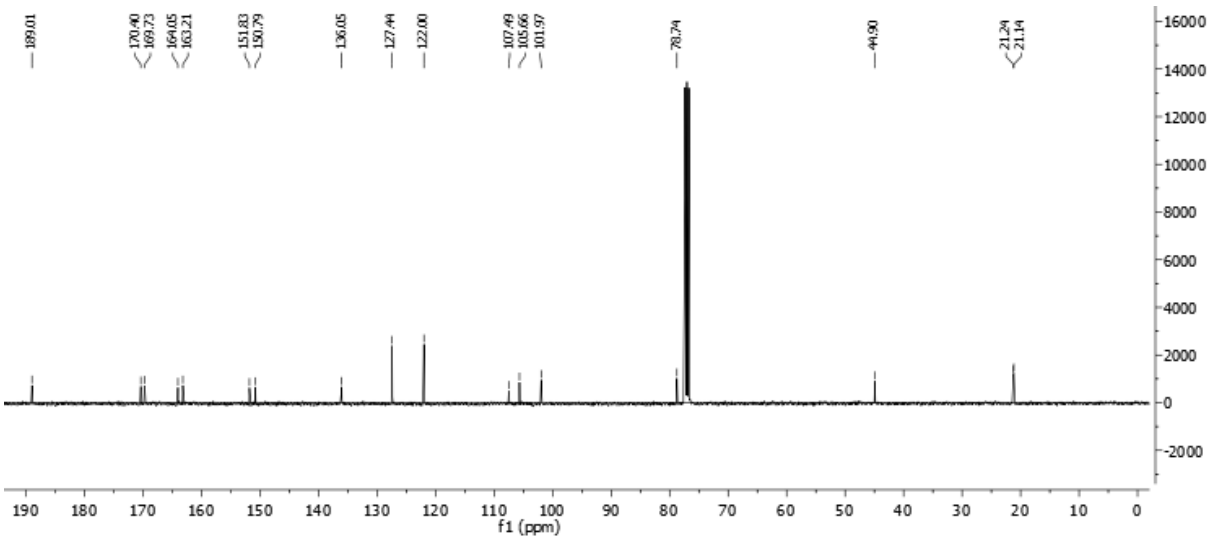


Figure 27. *^{13}C NMR spectrum of 7,4´-diacetyl naringenin.*

By analyzing the two spectra DEPT 135 form the initial substrate and the acylated derivative we found the appearance of two extra signals relative to the CH_3 of the acetyl groups at 21.14 and 30.97 ppm (**F**igure 28).

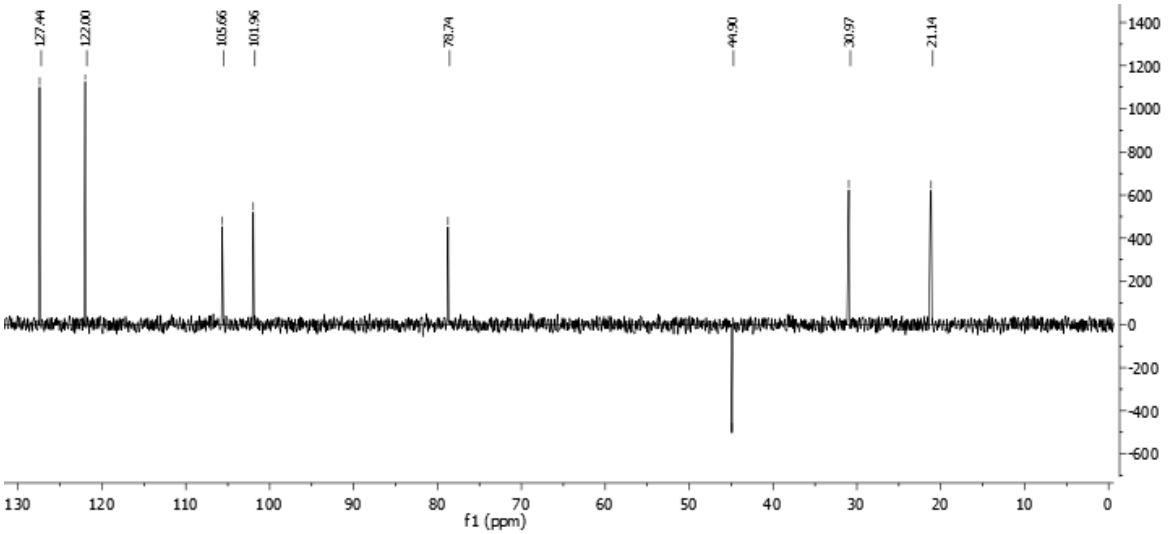


Figure 28. *DEPT 135 spectrum of 7,4´-diacetyl naringenin.*

Given the information of the structural analysis, the reaction product was caracterized as 7,4´-diacetyl naringenin (**F**igure 29).

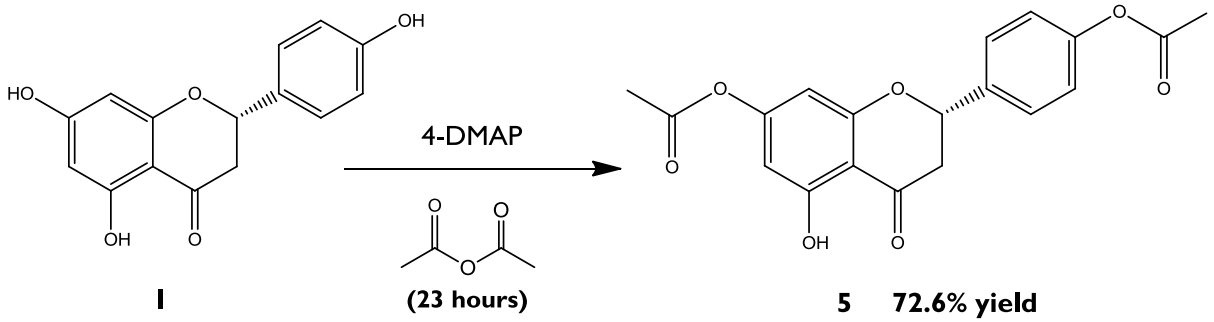


Figure 29. *Chemical acylation reaction of resveratrol with acetic anhydride.*

3.2.2. Chemical acylation of 4´-acetyl naringenin

In the chemical acylation reactions, dichloromethane was used as solvent, benzoyl chloride as acylating agent and bismuth (III) trifluoromethanesulfonate as catalyst, with stirring at room temperature. The diacylated derivative was obtained as main product isolated by chromatographic column and characterized by NMR.

^{1}H NMR and ^{13}C NMR and DEPT 135 spectra of product obtained

By analyzing the three ^{1}H NMR spectra from the initial substrate, the first acylated derivative **(2)** and the diacylated product, we found signal loss at δ=6.43 ppm corresponding to one hydrogen at C-5 and the appearance of 3 signals at δ=7.54, 7.65 and 8.21 ppm corresponding to the benzoyl group at C-5 (**F**igure 30).

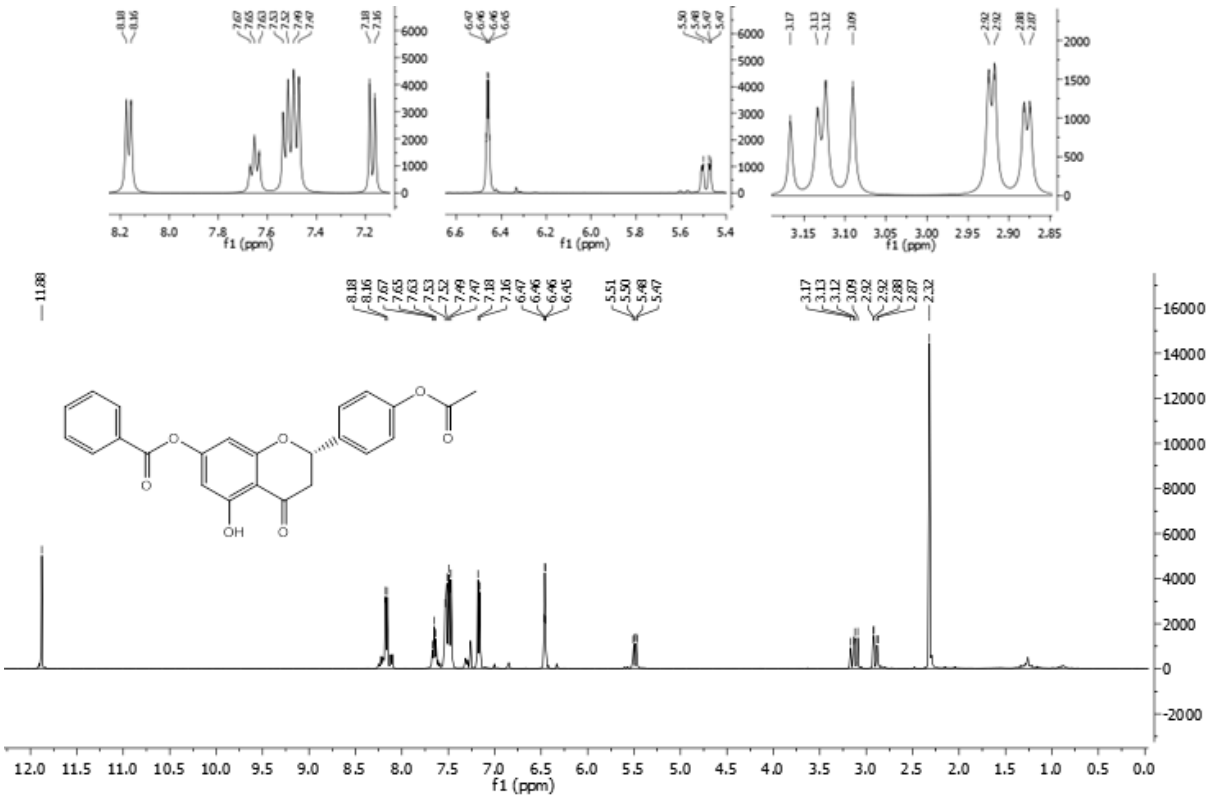


Figure 30. *[1]H NMR spectrum of 4´- acetyl-7-benzoyl naringenin.*

By analyzing the ^{13}C NMR spectrum from the diacylated product we found the appearance of extra signals, 1 relative to the carbonyl group at 164.03 ppm and 3 relative to the aromatics on the benzoyl group at 128.69, 130.3 and 134.01 ppm. Slight changes occur in other carbons but it is not significant (**F**igure 31).

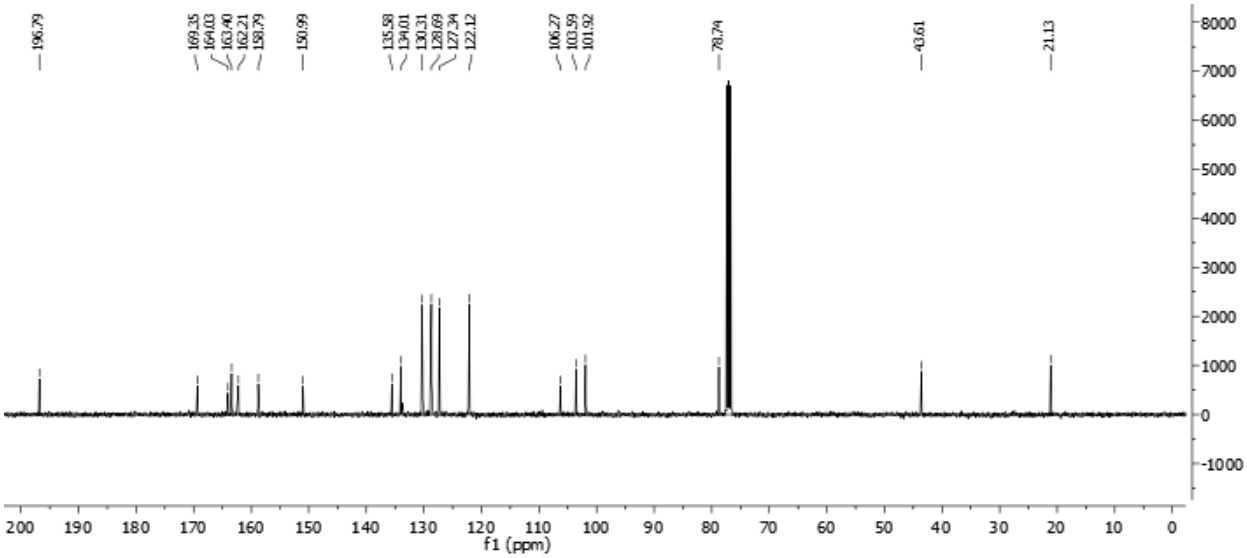


Figure 31. *[13]C NMR spectrum of 4´- acetyl-7-benzoyl naringenin.*

By analyzing the DEPT 135 spectrum from the diacylated product we found the appearance of three extra signals relative to 5 CH from the benzoyl group (**F**igure 32).

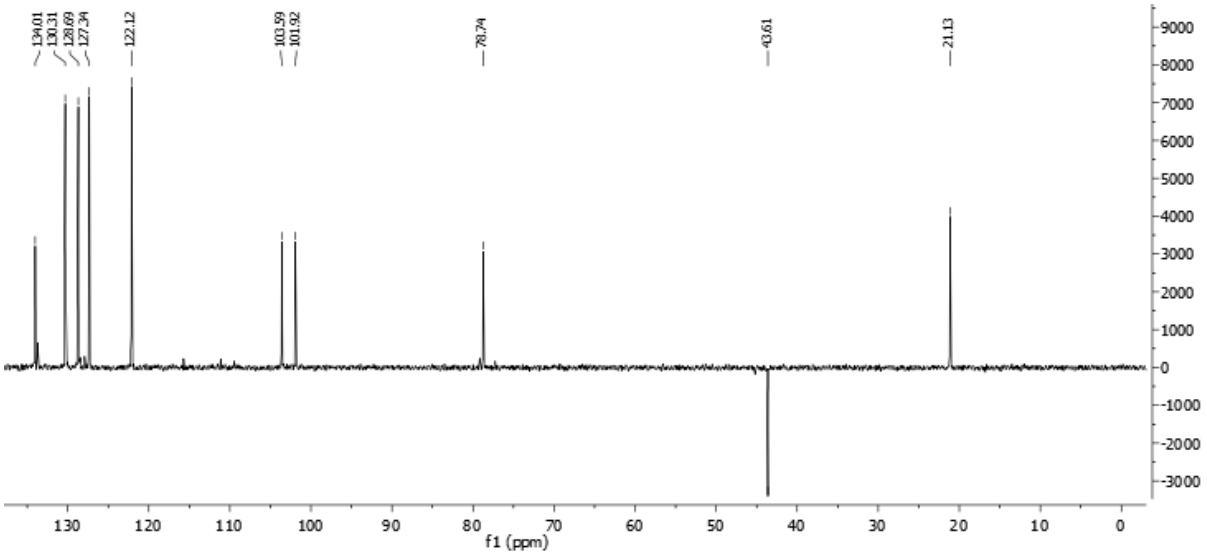


Figure 32. *DEPT 135 spectrum of 4´- acetyl-7-benzoyl naringenin.*

Given the information of the structural analysis, the reaction product was caracterized as 4´-acetyl-7-benzoyl naringenin (**F**igure 33).

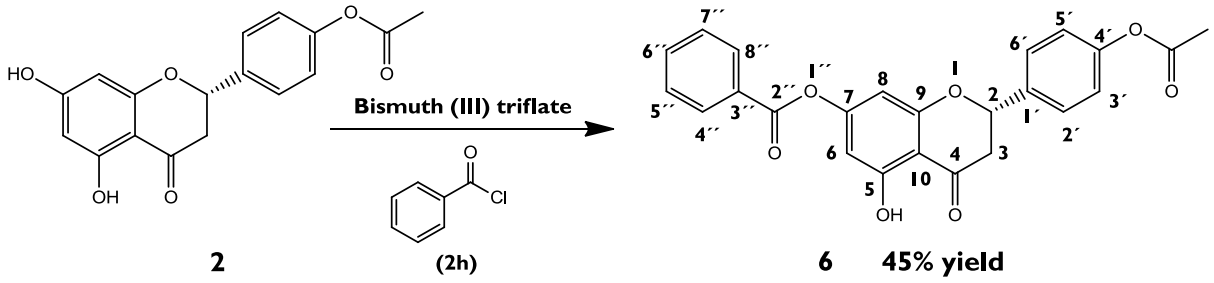


Figure 33. *Chemical acylation reaction of 4´-acetyl naringenin with benzoyl chloride.*

3.2.3. Chemical acylation of 7,4´-diacetyl naringenin

In the chemical acylation reactions, dichloromethane was used as solvent, benzoyl chloride as acylating agent and bismuth (III) trifluoromethanesulfonate as catalyst, with stirring at

room temperature. The triacylated derivative was obtained as main product, isolated by chromatographic column, and characterized by NMR.

^{1}H NMR and ^{13}C NMR and DEPT 135 spectra of product obtained

By analyzing the three ^{1}H NMR spectra from the initial substrate, the first acylated derivative **(5)** and the triacylated product we found signal loss at δ=7.50 ppm corresponding to one hydrogen at C-5 and the appearance of 3 signals at δ=7.53, 7.66 and 8.17 ppm corresponding to the benzoyl group at C-5 (**F**igure 34)

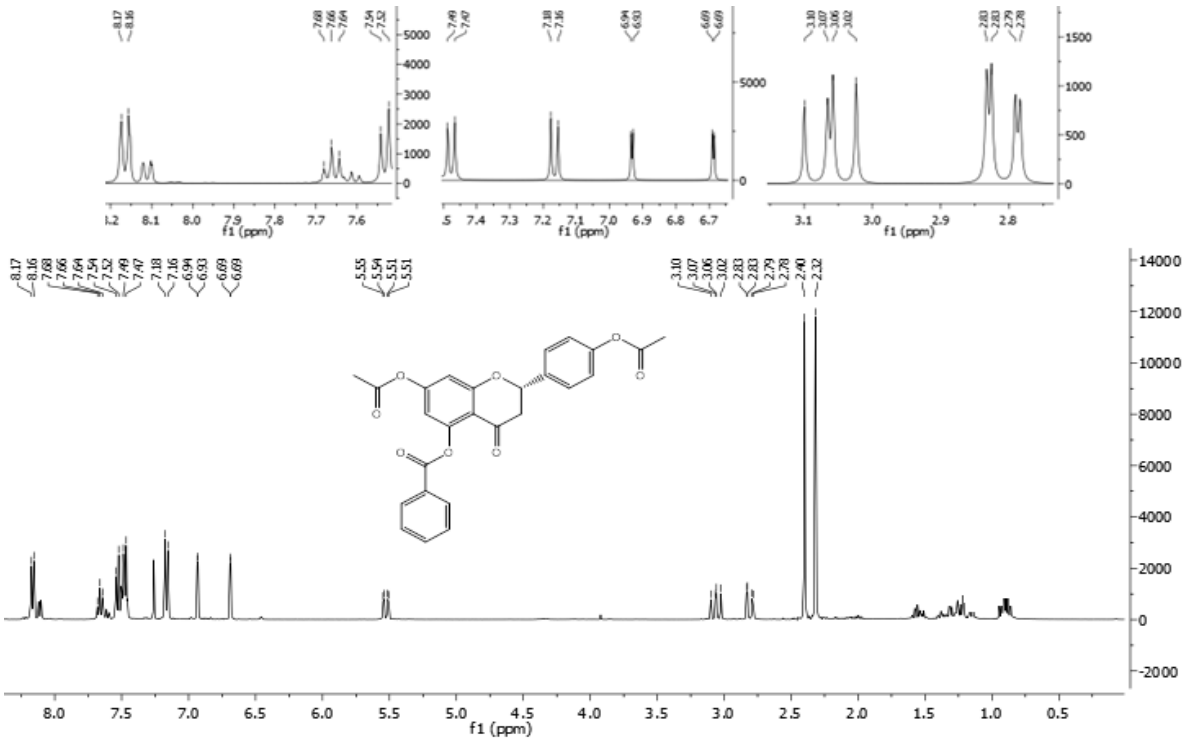


Figure 34. *^{1}H NMR spectrum of 5-benzoyl-7,4´-diacetyl naringenin.*

By analyzing the ^{13}C NMR spectra from the initial substrate, the first acylated derivative **(5)** and the triacylated product we found the appearance of extra signals, 1 relative to the carbonyl group at 163.82 ppm and 3 relative to the aromatics from the benzoyl group at 128.74, 130.32 and 134.13 ppm. Slight changes occur in other carbons, but it´s not significant (**F**igure 35).

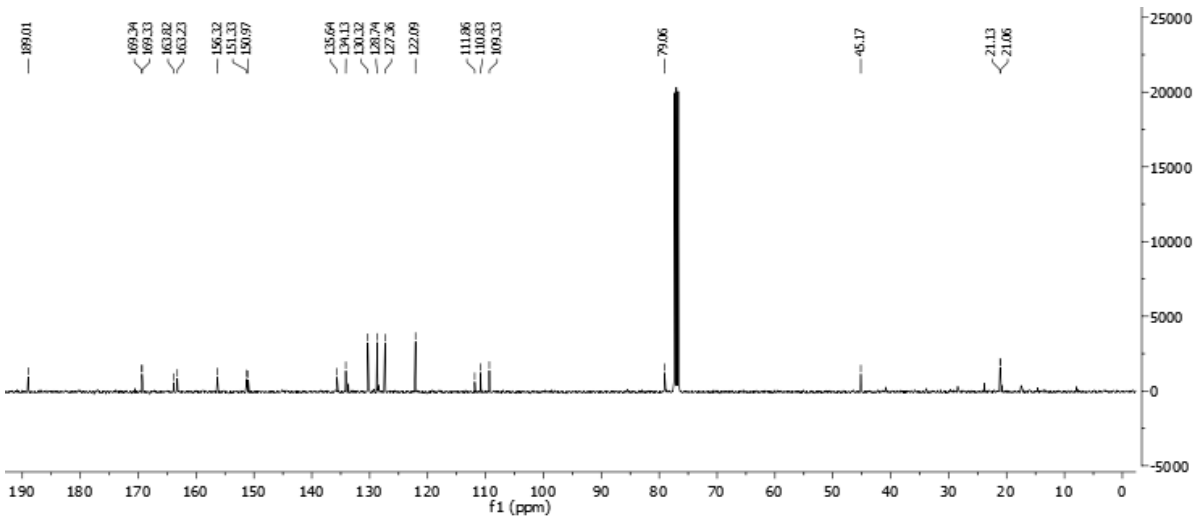


Figure 35. *^{13}C NMR spectrum of 5-benzoyl-7,4´-diacetyl naringenin.*

By analyzing the DEPT 135 spectra from the initial substrate, the first acylated derivative **(5)** and the triacylated product we found the appearance of three extra signals relative to 5 CH from the benzoyl group (**F**igure 36).

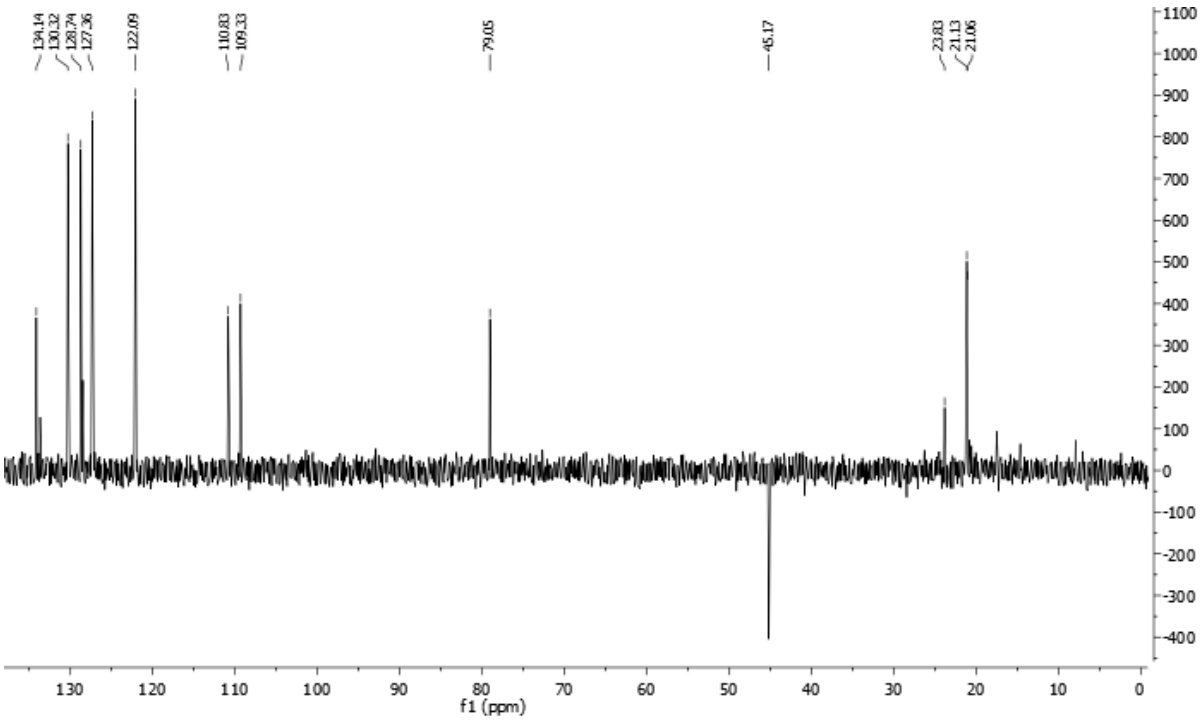


Figure 36*. DEPT 135 spectrum of 5-benzoyl-7,4´-diacetyl naringenin.*

Given the information of the structural analysis, the reaction product was caracterized as 5-benzoyl-7,4´-diacetyl naringenin (**F**igure 37).

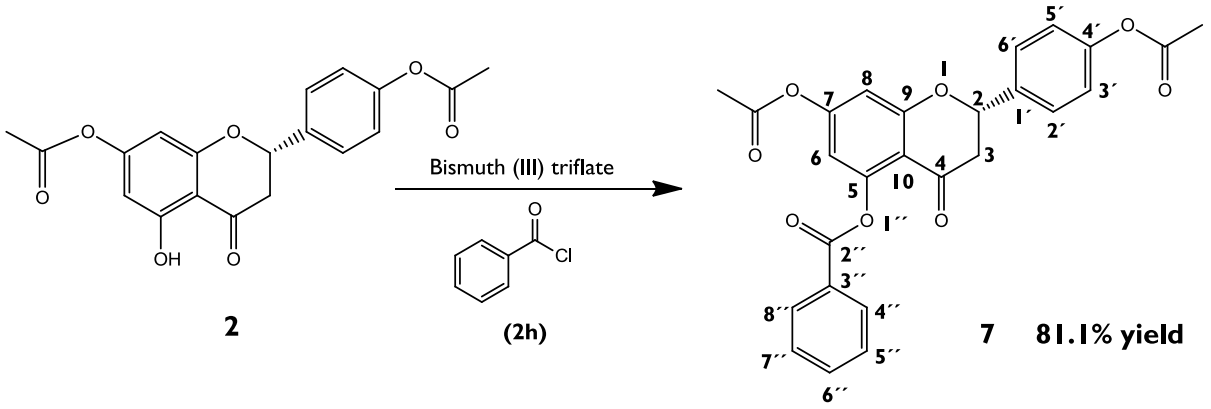


Figure 37. *Chemical acylation reaction of 7, 4´- diacetyl naringenin with benzoyl chloride.*

3.2.4. Chemical acylation of 3,4´-diacetyl resveratrol

In the chemical acylation reactions, dichloromethane was used as solvent, benzoyl chloride as acylating agent and bismuth (III) trifluoromethanesulfonate as catalyst, with stirring at room temperature. The triacylated derivative was obtained as main product, isolated by chromatographic column and characterized by NMR.

^{1}H NMR and ^{13}C NMR and DEPT 135 spectra of product obtained

By analyzing the ^{1}H NMR spectra from the initial substrate, the first acylated derivative **(4)** and the triacylated product we found signal loss at δ=5.40 ppm corresponding to one hydrogen at C-5 and the appearance of 3 signals at δ=7.48, 7.59 and 8.15 corresponding to the benzoyl at C-5 (**F**igure 38).

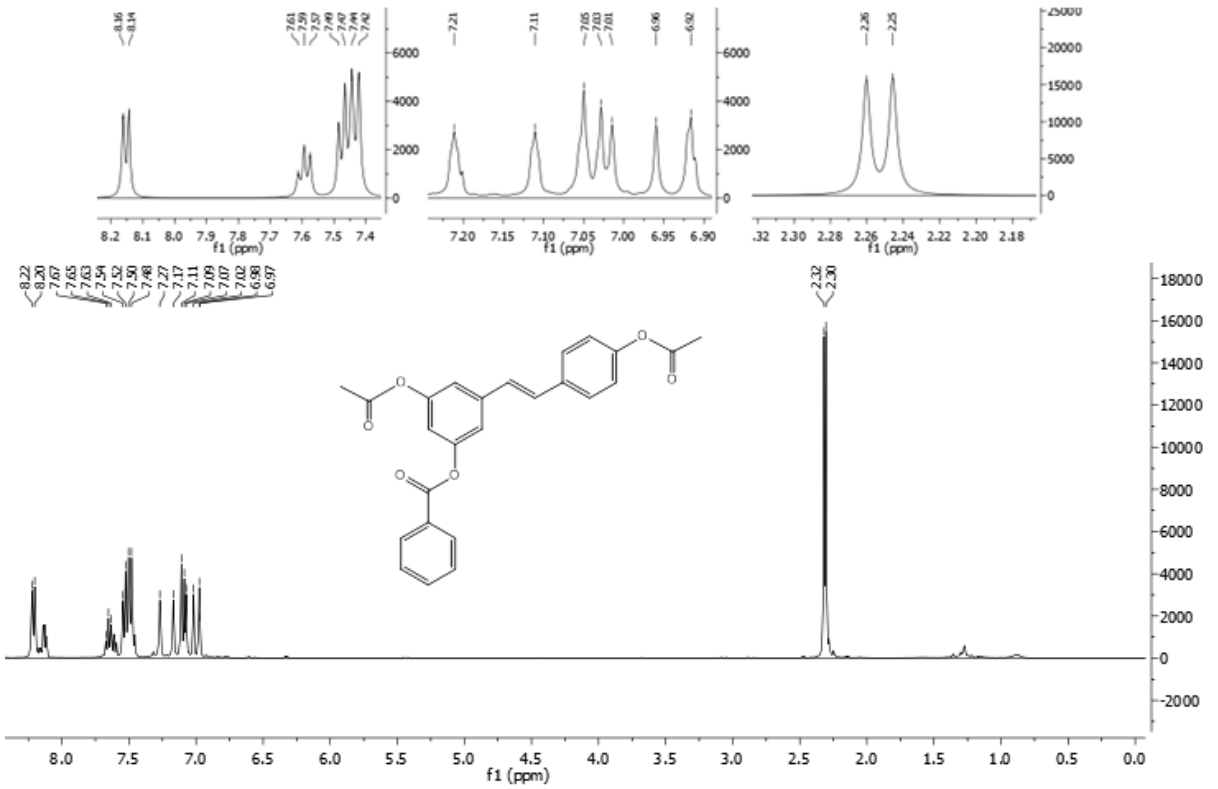


Figure 38. *^{1}H NMR spectrum of 5-benzoyl-3,4´-diacetyl resveratrol.*

By analyzing the ^{13}C NMR spectrum of the triacylated product we found the appearance of extra signals, 1 relative to the carbonyl group at 164.79 ppm and 6 relatives to the aromatics from the benzoyl group at 128.49, 128.66, 129.70, 130.20, 130.23 and 133.78 ppm. Slight changes occur in other carbons, but it is not significant (**F**igure 39).

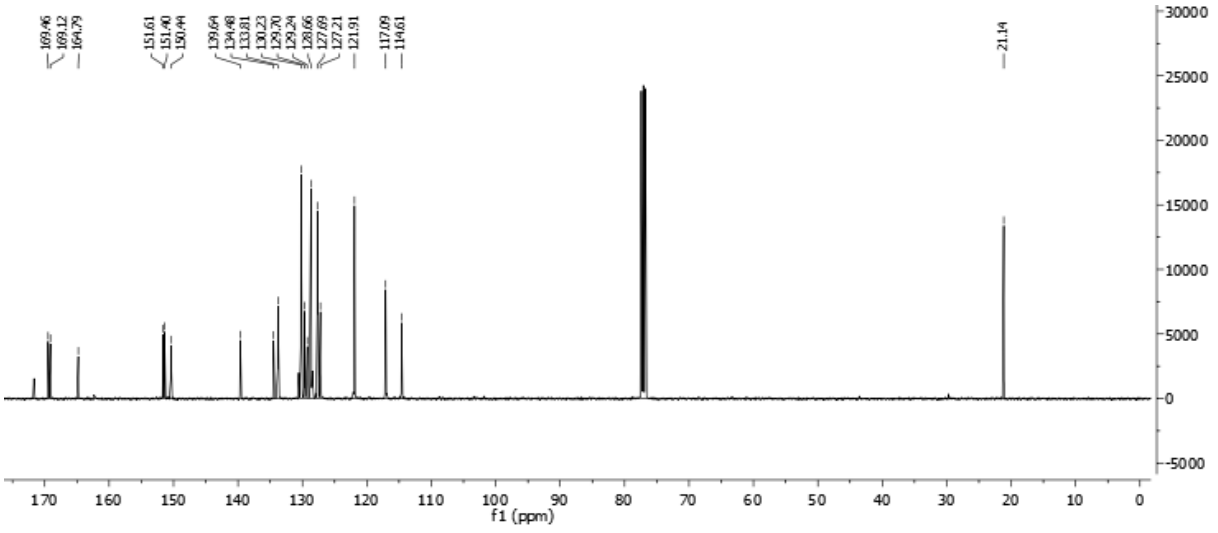


Figure 39. *^{13}C NMR spectrum of 5-benzoyl-3,4´-diacetyl resveratrol.*

By analyzing the DEPT 135 spectrum from the triacylated product we found the appearance of three extra signals relative to 5 CH from the benzoyl group (**F**igure 40).

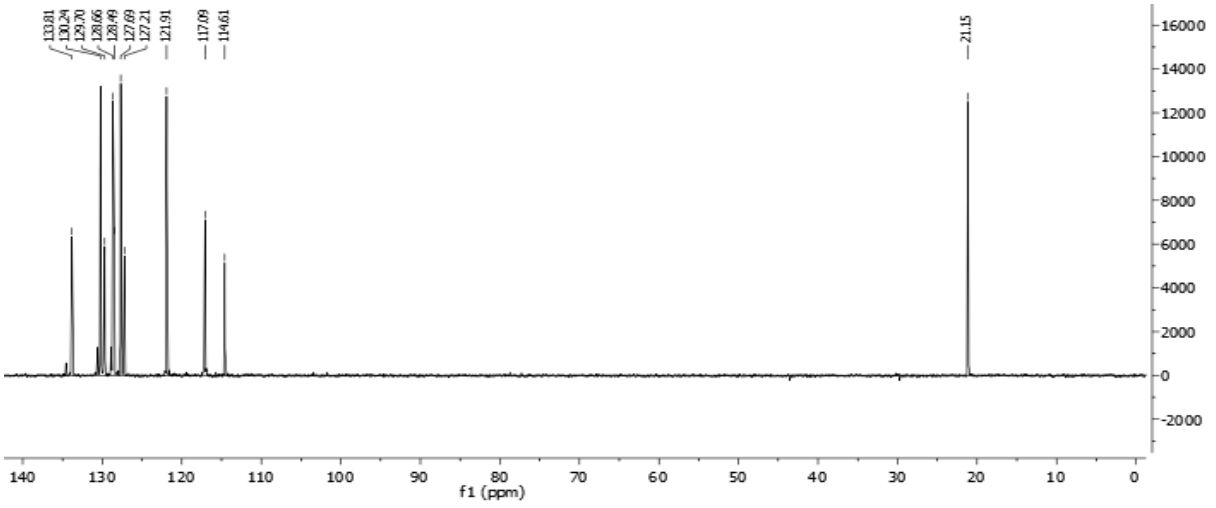


Figure 40. *DEPT 135 spectrum of 5-benzoyl-3,4´-diacetyl resveratrol.*

Given the information of the structural analysis, the reaction product was caracterized as 5-benzoyl-3,4´-diacetyl resveratrol (**F**igure 41).

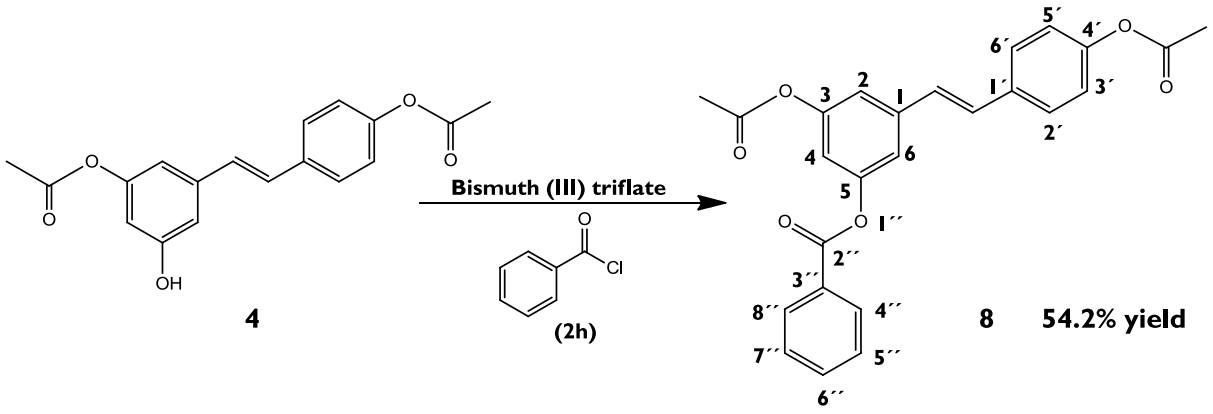


Figure 41. *Chemical acylation reaction of 3,4´-diacetyl resveratrol with benzoyl chloride.*

Of the 6 synthesized compounds 4 are new: 7, 4´-diacetyl naringenin; 4´-acetyl-7-benzoyl naringenin; 5-benzoyl-7,4´-diacetyl naringenin and 5-benzoyl-3,4´-diacetyl resveratrol.

For the first time, acetylated compounds were obtained by regioselective chemical acylation using benzoyl chloride and bismuth (III) triflate as the acylating agent and catalyst, respectively.

Chapter 4

Conclusion

4. Conclusion

Polyphenolic compounds are widely distributed in Nature and due to the diverse proven biological activities, namely antioxidant, anti-inflammatory and anticarcinogenic, have deserved special attention and continue to be studied nowadays. One of the strategies to combat the limitation of these compounds is through structural modifications, namely acylation reactions that allow them to become more hydrophobic by fatty acid linkage. These reactions can be obtained by chemical, enzymatic or chemo-enzymatic means.

In this research we used acylation by combining both methods. In the enzymatic reactions, lipases have been used to allow regioselective acylation reactions with gentle reaction conditions appling to the principles of Green Chemistry. In chemical reactions, regioselectivity was also succeed without using the protection and deprotection reactions, thus reducing costs and reaction time.

In this work we aimed to combine the advantages of both techniques to obtain bioactive compounds.

Thus, enzymatic acylation reactions of substrates such as naringenin and resveratrol under the action of lipases in organic medium were studied, using vinyl acetate as the irreversible acyl donor. Having obtained a diacylated derivative of resveratrol using lipase PS and a monoacylated derivative of naringenin with CALB. These results have once again proven the great ability of lipases to recognize and differentiate hydroxyl groups on the same substrate, leading to the esters with high regioselectivity and yields.

On the other hand, we proceeded to the study of chemical acylation reactions of naringenin, acyl derivative from naringenin, and acyl derivative from resveratrol. The regioselectivity was also obtained with the chemical reactions involving acetic anhydride and benzoyl chloride as donors of the acyl group, thus forming a diacylated and monoacylated derivative also with high regioselectivity and good yields.

This can be explained by the stereochemical hindrance at C-5 carbon, by the presence of a carbonyl group at the C-4 neighbor carbon.

In summary, 6 compounds were synthetized, from which 4 are new compounds, to the best of our knowledge.

Thus, this work allowed to obtain new acylated derivatives with the aim of improving not only the solubility but also the stability of these compounds and to some extent explore the structure-activity for the identification of structural characteristics necessary for a biological activity, obtaining thus novel compounds biologically active

Chapter 5

Experimental procedure

5. Experimental procedure

5.1. Instrumentation

The spectrum of proton nuclear magnetic resonance, ^{1}H-NMR, were obtained on a Varian Unity 400 spectrometer at 400 MHz. The spectrum of Carbon-13 nuclear magnetic resonance, ^{13}C-NMR, were obtained on a Bruker Avance III 100 MHz. The characterization of the methyl, methylene and methine was performed by DEPT. The chemical shifts are given in δ scale (ppm) and are relative to deuterated chloroform ($CDCl_3$) as solvent.
The enzymatic reactions were performed in a New Brunswick Scientific Incubator Shaker Classic Series C24 at 50°C and 200 rpm.

5.2. Chromatography

The thin layer chromatography was performed on silica gel 60 F_{254} Merck using mixtures of petroleum ether / ethyl acetate (2:1) as eluent.

5.3. Reagents and solvents

All commercially available compounds were used as supplied by the manufacturer.
The naringenin and resveratrol substrates were obtained from TCI.
The lipases used, bismuth III trifluoromethanesulfonate, 4-dimethylaminopyridine, vinyl acetate, dichloromethane, ethanol, sodium hydroxide and acetic anhydride were obtained from Sigma-Aldrich Co.
Toluene, acetone, anhydrous sodium sulfate, silica gel 60 (column chromatography) and TLC silica gel 60 F254 were obtained from MERK Co.
The benzoyl chloride was obtained from B.D.H. Laboratory and the petroleum ether and ethyl acetate, used in column chromatography, were obtained from VWR Chemicals BDH Prolabo.
The reagent used to reveal TLC's was the komarovsky reagent, a mixture of sulphuric acid (50%) and methanolic *p*-benzaldehyde (2%), mixed immediately before use. The sulphuric acid and methanol were obtained from Sigma-Aldrich Co and the aldehyde from TCI.

Enzymatic acylations

In all of these reactions the substrate, the solvent, the acylation agent and the enzyme were added into flasks closed and were shaked in a New Brunswick Scientific Incubator Shaker Classic Series C24 at 50°C and 200 rpm.
All of these reactions were monitored by TLC.

5.3.1. Enzymatic acylation of naringenin

4´-Acetyl naringenin **2**

To a solution of naringenin (**1**, 100 mg; 0.36 mmol) in acetone (10 mL), the vinyl acetate (5 mL) and CALB were added (200 mg). The reactional mixture was shaked at 50°C in 200 rpm. After 2 days more CALB was added (150 mg). After 6 days the enzyme was filtered and the solvent evaporated. The 4´-acetyl naringenin (**2**, 75.3 mg, 67 %; white solid) was isolated by column chromatography, petroleum ether/ethyl acetate with polarity gradient of 4:1 to 3:1.

RMN ^{1}H (400 MHz, CDCl3) δ ppm: 2.33 (3H, s, CH_3 (OAc-4´)); 2.80 (1H, dd, J= 17.13 Hz and 2.95 Hz, H-3_{cis}); 3.05 (1H, dd, J=17.41 and 13.12 Hz, H-3_{trans}); 5.39 (1H, dd, J=12.83 and 2.92 Hz, H-2); 5.96 (2H, dd, J=7.59 Hz, 1.93 H-6, H-8); 6.43 (1H, s, OH); 7.15 (2H, d, J=8.56 Hz, H-3´, H-5´); 7.45 (2H, d, J=8.56 Hz, H-2´, H-6´); 12.0 (1H, s, OH).

5.3.2. Enzymatic acylation of resveratrol

3,4´-Diacetyl resveratrol **4**

To a solution of resveratrol (**3**, 100 mg; 0.44 mmol) in toluene (5 mL), the vinyl acetate (1.5 mL) and lipase PS (120 mg) were added. The reactional mixture was shaked at 50°C in 200 rpm. After 2 days more lipase PS was added (70 mg). After 5 days the enzyme was filtered and the solvent evaporated. The 3,4´-diacetyl resveratrol (**4**, 85.6 mg, 62.3%; white solid) was isolated by column chromatography, petroleum ether/ethyl acetate with polarity gradient of 4:1 to 2:1.

RMN ^{1}H (400 MHz, CDCl3) δ ppm: 2.31, 2.32 (each 3H, 2s, CH_3 (3-OAc), CH_3 (4´-OAc)); 5.40 (1H, s, H-5); 6.49 (1H, t, J=1.76 Hz, H-4); 6.77, 6.80 (2H, s, H-2, H-6); 6.89 (1H, d, J=16.30 Hz, Hα); 6.99 (1H, d, J=16.30 Hz, Hβ); 7.08 (2H, d, J=8.56 Hz, H-3´, H-5´); 7.45 (2H, d, J=8.56 Hz, H-2´, H-6´).

5.4. Chemical acylation

All these reactions were obtained by stirring at room temperature and were monitored by TLC.

5.4.1. Chemical acylation of naringenin

7, 4´-Diacetyl naringenin **5**

To a solution of naringenin (**1**, 100 mg; 0.36 mmol) in CH_2Cl_2 (4 mL), 4-DMAP and acetic anhydride (0.4 mL) were added. The reaction was allowed to stir at room temperature.
After 23 hours the organic layer was washed with a solution of HCl (5%), $NaHCO_3$ concentrated, watter (3x) and NaCl concentrated. After drying with anhydrous sodium sulfate the organic phase was filtered and evaporated.
The 7,4´-diacetyl naringenin (**5**, 93.1 mg, 72.6%; white solid) was isolated by column chromatography, petroleum ether/ethyl acetate with polarity gradient of 4:1 to 1:1.

RMN ^{1}H (400 MHz, CDCl3) δ ppm: 2.32, 2.38 (each 3H, 2s, CH_3 (7-OAc), CH_3 (4´-OAc)); 2.68 (1H, dd, J=16.74 Hz and 2.67 Hz, H-3_{cis}); 2.94 (1H, dd, J=16.47 and 13.49 Hz, H-3_{trans}); 5.37 (1H, dd, J=13.43 and 2.56 Hz, H-2); 6.21 (2H, dd, J=25.87 Hz, and 1.72 H-6, H-8); 7.12 (2H, d, J=8.53 Hz, H-3´, H-5´); 7.41 (2H, d, J=8.53 Hz, H-2´, H-6´); 7.50 (1H, s, OH).

RMN ^{13}C (100 MHz, CDCl3) δ ppm: 21.14, 21.24 (2C, CH_3 (OAc-4´), CH_3 (OAc-7)); 44.90 (C-3); 78.74 (C-2); 101.97 (C-8); 105.66 (C-6); 107.49 (C-10); 122.00 (C-3´, C-5´); 127.44 (C-2´, C-6´); 136.05 (C-1´); 150.79 (C-4´); 151.83 (C-7); 163.21 (C-5); 164.05 (C-9); 169.73, 170.40 (COMe); 189.01 (C-4).

5.4.2. Chemical acylation of 4´-acetate naringenin

4´-Acetyl-7-benzoyl naringenin **6**

To a solution of 4´-acetyl of naringenin (**2**, 50 mg; 0.16 mmol) in CH_2Cl_2 (3mL), bismuth (III) trifluoromethanesulfonate (12mg) and benzoyl chloride (0.5 mL) were added. The reaction was allowed to stir at room temperature.

After 2 hours the organic layer was washed with a solution of HCl (5%), $NaHCO_3$ concentrated, watter (3x) and NaCl concentrated. After drying with anhydrous sodium sulfate, the organic phase was filtered and evaporated.

The 4´-acetyl-7-benzoyl naringenin (**6**, 30.1 mg, 45%; oil) was isolated by column chromatography, petroleum ether/ethyl acetate with polarity gradient of 8:1 to 2:1.

RMN ^{1}H (400 MHz, CDCl3) δ ppm: 2.32 (3H, s, CH_3 (4´-OAc)); 2.90 (1H, dd, J= 17.24 Hz and 2.96 Hz, H-3_{cis}); 3.13 (1H, dd, J = 17.28 and 13.28 Hz, H-3_{trans}); 5.49 (1H, dd, J=13.28 and 2.72 Hz, H-2); 6.45, 6.46 (2H, d, J=1.91 Hz, H-6, H-8); 7.17 (2H, d, J=8.62 Hz, H-3´, H-5´); 7.48 (2H, d, J=8.62 Hz, H-2´, H-6´); 7.53 (2H, m, C-5´´, C-7´´); 7.65 (1H, t, J= 7.43 Hz, H-6´´); 8.17 (2H, d, J=7.33 Hz, H-4´´, H-8´´); 11.88 (1H, s, OH).

RMN ^{13}C (100 MHz, CDCl3) δ ppm: 21.13 (CH_3 (OAc-4´)); 43.61 (C-3); 78.74 (C-2); 101.91 (C-8); 103.59 (C-6); 106.27 (C-10); 122.12 (C-3´, C-5´); 127.34 (C-2´, C-6´); 128.69 (C-5´´, C-7´´); 130.31 (C-3´´, C-4´´, C-8´´); 134.01 (C-6´´); 135.58 (C-1´); 150.99 (C-4´); 158.79 (C-5); 162.21 (C-7); 163.40 (C-9); 164. 03 (C-OBz); 169.35 (COMe); 196.79 (C-4).

5.4.3. Chemical acylation of 7, 4´- diacetyl naringenin

5-Benzoyl-7,4´-diacetyl naringenin **7**

To a solution of 7,4´-diacetyl naringenin (**5**, 50 mg; 0.14 mmol) in CH_2Cl_2 (3mL), bismuth (III) trifluoromethanesulfonate (12mg) and benzoyl chloride (0.5 mL) were added. The reaction was allowed to stir at room temperature.

After 2 hours the organic layer was washed with a solution of HCl (5%), $NaHCO_3$ concentrated, watter (3x) and NaCl concentrated. After drying with anhydrous sodium sulfate, the organic phase was filtered and evaporated.

The 5-benzoyl-7,4´-diacetyl naringenin (**7**, 52.3 mg, 81.1%; oil) was isolated by column chromatography, petroleum ether/ethyl acetate with polarity gradient of 8:1 to 3:1.

RMN ^{1}H (400 MHz, CDCl3) δ ppm: 2.32, 2.40 (each 3H, s, CH_3 (7-OAc), CH_3 (4´-OAc)); 2.81 (1H, dd, J=16.82 Hz and 2.83 Hz, H-3_{cis}); 3.06 (1H, dd, J=16.92 and 13.55 Hz, H-3_{trans}); 5.53 (1H, dd, J=13.52 and 2.62 Hz, H-2); 6.69, 6.93 (2H, d, J=2.25 Hz, H-6, H-8); 7.17 (2H, d, J=8.63 Hz, H-3´, H-5´); 7.48 (2H, d, J=8.63 Hz, H-2´, H-6´), 7.53 (2H, m, C-5´´, C-7´´); 7.66 (1H, t, J=7.46 Hz, H-6´´); 8.17 (2H, d, J=8.10 Hz, H-4´´, H-8´´).

RMN ^{13}C (100 MHz, CDCl3) δ ppm: 21.06, 21.13 (2C, CH_3 (OAc-4´), CH_3 (OAc-7)); 45.17 (C-3); 79.06 (C-2); 109.33 (C-8); 110.33 (C-6); 111.86 (C-10); 122.09 (C-3´, C-5´); 127.36 (C-2´, C-6´); 128.74 (C-5´´, C-7´´); 130.32 (C-3´´, C-4´´, C-8´´); 134.13 (C-6´´); 135.64 (C-1´); 150.97 (C-4´); 151.33 (C-5); 156.32 (C-7); 163.23 (C-9); 163.82 (COBz); 169.32, 169.34 (COMe); 189.01 (C-4).

5.4.4. Chemical acylation of 3,4´-diacetyl resveratrol

5-Benzoyl-3,4´-diacetyl resveratrol **8**

To a solution of 3,4´-diacetyl resveratrol (**4**, 50 mg; 0.16 mmol) in CH_2Cl_2 (3mL), bismuth (III) trifluoromethanesulfonate (12mg) and benzoyl chloride (0.5 mL) were added. The reaction was allowed to stir at room temperature.

After 2 hours the organic layer was washed with a solution of HCl (5%), $NaHCO_3$ concentrated, watter (3x) and NaCl concentrated. After drying with anhydrous sodium sulfate, the organic phase was filtered and evaporated.

The 5-benzoyl-3,4´-diacetyl resveratrol (**8**, 36.1 mg, 54.2%; oil) was isolated by column chromatography, petroleum ether/ethyl acetate with the polarity gradient of 8:1 to 2:1.

RMN ^{1}H (400 MHz, CDCl3) δ ppm: 2.30, 2.32 (each 3H, s, CH_3 (7-OAc), CH_3 (4´-OAc)), 6.98 (1H, t, J= 2.02 Hz, H-4); 7.02 (1H, d, Hα); 7.07 (1H, d, Hβ); 7.10 (2H, d, J=8.87, H-3´, H-5´); 7.17, 7.27 (2H, s, H-4, H-2); 7.49 (2H, d, J=8.87 Hz, H-2´, H-6´), 7.54 (2H, m, C-5´´, C-7´´); 7.65 (1H, t, J=7.46 Hz, H-6´´); 8.21 (2H, d, J=8.31 Hz, H-4´´, H-8´´).

RMN ^{13}C (100 MHz, CDCl3) δ ppm: 21.14 (2C, CH_3 (OAc-4´), CH_3 (OAc-3)); 114.61 (C-4); 117.07, 117.09 (C-2, C-6); 121.91 (C-3´, C-5´); 127.21, 127.69 (CHα, CHβ); 128.49, 128.66 (C-7´´, C-5´´); 129.24, 129.35 (C-6´, C-2´); 129.70, 130.20, 130.23 (C-3´´, C-4´´, C-8´´); 133.78 (C-6´´); 134.52 (C-1´); 139.64 (C-1); 150.44 (C-4´); 151.40, 151.61 (C-3, C-5); 164.79 (COBz); 169.12, 169.46 (COMe).

Chapter 6

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6. Bibliography

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