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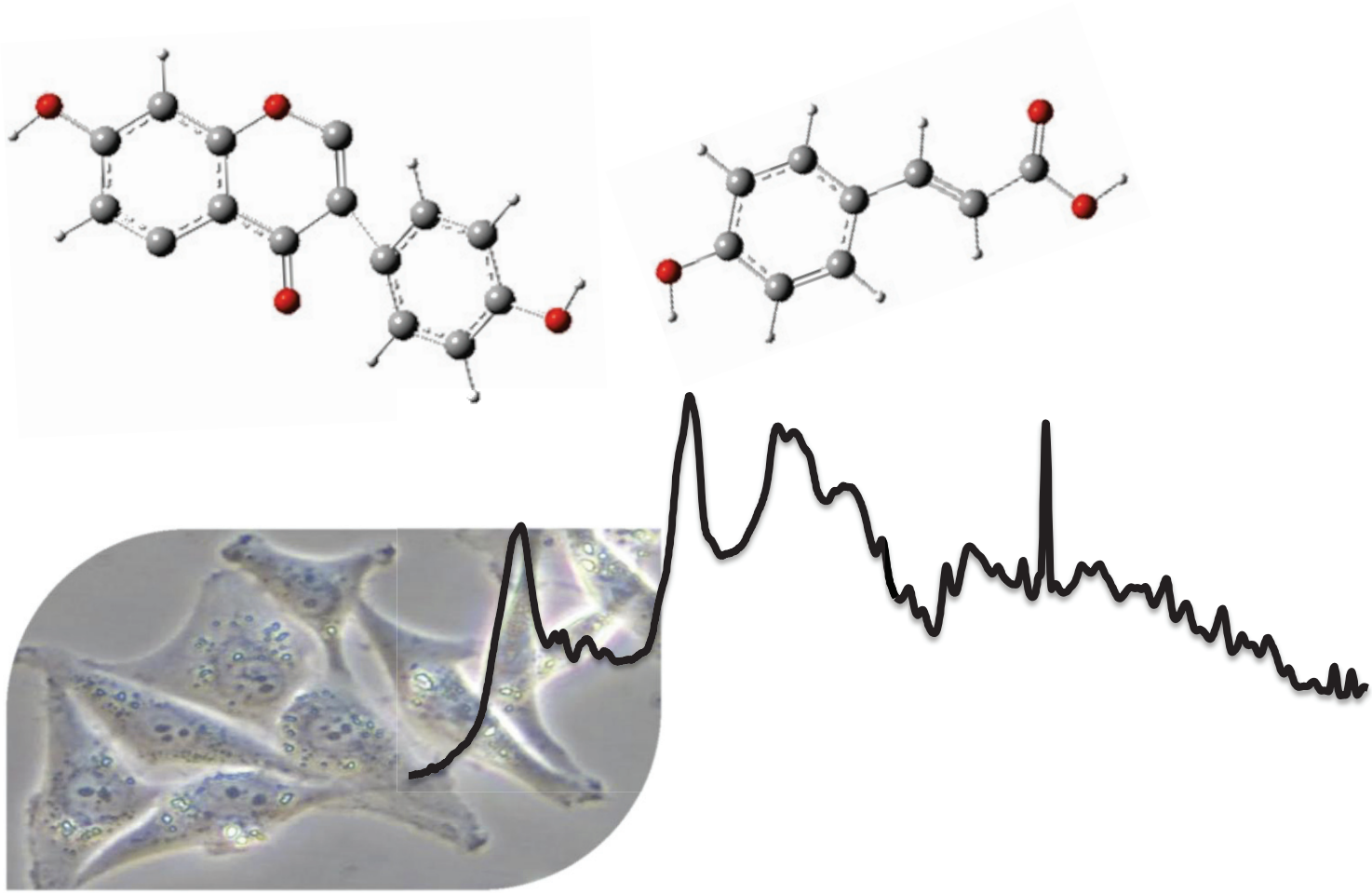
UNIVERSIDADE DE COIMBRA

Correlating Biological Activity and Chemical Fingerprint: Raman Microspectroscopy Studies in Human Breast Cancer Cells



Paula Sofia Coutinho Medeiros

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Correlating Biological Activity and Chemical Fingerprint: Raman Microspectroscopy Studies in Human Breast Cancer Cells

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquimica, realizada sob a orientação científica da Professora Doutora Maria Paula Marques (Universidade de Coimbra)

Paula Sofia Coutinho Medeiros

2015

Be the Best of Whatever You Are - (2009)

If you can't be a pine on the top of the hill,
Be a scrub in the valley, but be
The best little scrub by the side of the rill;
Be a bush if you can't be a tree.

If you can't be a bush be a bit of the grass,
And some highway happier make;
If you can't be a muskie then just be a bass
But the liveliest bass in the lake!

We can't all be captains, we've got to be crew,
There's something for all of us here,
There's big work to do, and there's lesser to do,
And the task you must do is the near.

If you can't be a highway then just be a trail,
If you can't be the sun be a star;
It isn't by size that you win or you fail
Be the best of whatever you are!

Douglas Malloch

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"Acredita quando te digo que o sol de Coimbra é diferente do sol do resto do mundo."

Abstract

Breast adenocarcinoma is one of the major causes of death among women worldwide (the second most lethal cancer type in Portugal) and can be caused by oxidative stress conditions. The development of chemopreventive agents against this type of neoplasia is therefore of the utmost importance, in order to reduce the risk of this type of oxidative stress-related diseases.

Phytochemicals (such as hydroxycinnamic acids and isoflavones) are an abundant source of antioxidants present in our daily diet known to significantly decrease the harmful effect due to oxidative species, and therefore being promising chemopreventive agents towards carcinogenesis.

The present work aims at assessing two dietary antioxidants – p-coumaric acid (p-Ca, a monohydroxylated phenolic acid) and daidzein (DA, an isoflavone) – as potential chemopreventive agents, using as biological models the human breast cancer cell lines MCF-7 (estrogen-dependent) and MDA-MB-231 (estrogen-independent). A multidisciplinary approach was followed, coupling biological assays to cutting-edge vibrational spectroscopy techniques, to evaluate (for distinct concentrations, at 48 h exposure time): (i) cell proliferation/viability, by the SRB and MTT colorimetric tests; (ii) antimetastatic ability, using a transwell invasion chamber; (iii) generation of ROS/RNS species, through a fluorimetric technique (2',7'-dichlorofluorescin diacetate probe); (iv) metabolic impact at the cellular level *via* Raman microspectroscopy of fixed cells. MicroRaman of cell cultures, in particular, allows an accurate and non-invasive probing of biospecimens, constituting an invaluable and innovative method for monitoring the effect of an external agent on the intracellular biochemical profile.

At both 50 and 100 μM, p-Ca was found to induce a significant increase in cell viability for MDA-MB-231, although the Raman data did not show a clear differentiation between the control and the treated cells. In contrast, at 7.5 and 15 μM p-Ca induced a slight viability decrease, the Raman spectra having reflected changes in the cellular chemical composition (mostly in lipids and proteins). For the MCF-7 cells, p-Ca did not show any significant effect (in the whole concentration range tested) through either biological assays or Raman microspectroscopy. The ROS/RNS measurements corroborated these conclusions, and evidenced a striking cell-selectivity for both

polyphenols studied.

Regarding daidzein, a 100 µM dosage led to a marked decrease in cell viability (*ca.* 50%) for the MDA-MB-231 line, the Raman data evidencing differences in the lipid and protein content. For the MCF-7 cells, viability was found to decrease considerably (30% to 40%) for 50 and 100 µM concentrations, which was corroborated by microRaman.

In conclusion, the results gathered for 50 and 100 µM p-Ca allow us to hypothesise on a pro-oxidant effect of the phenolic acid on this type of cancer cells (mainly for the highest concentration tested), the increased intracellular levels of ROS leading to an enhanced neoplastic proliferation (which is known to be associated with a need for ROS). At low concentrations, the compounds appear to affect mostly lipids which could be a promising chemoprotective strategy towards ROS-induced lipid damage associated to carcinogenesis. DA, in particular, was shown to be a promising chemoprotective agent against hormone-dependent breast adenocarcinoma. In addition, both dietary polyphenols can be envisaged as potential agents in adjuvant therapy for non-hormone dependent breast cancer.

Resumo

O cancro de mama é atualmente uma das principais causas de morte entre as mulheres em todo o mundo (e o segundo tipo de cancro mais mortal em Portugal), podendo ser provocado por condições de stresse oxidativo. O desenvolvimento de agentes quimiopreventivos contra este tipo de neoplasia é assim da maior relevância, com vista a reduzir a incidência desta patologia induzida por stresse oxidativo.

Os compostos fitoquímicos (como os ácidos hidroxicinâmicos e as isoflavonas) são uma importante fonte de antioxidantes presentes na dieta. São responsáveis por uma diminuição considerável dos efeitos nocivos de espécies oxidantes no nosso organismo, constituindo assim um grupo promissor de potenciais agentes quimiopreventivos face a processos carcinogénicos.

O objectivo deste trabalho é o estudo de dois antioxidantes da dieta – ácido p-coumárico (p-Ca, um ácido fenólico monohidroxilado) e daidzeína (DA, uma isoflavona) – como potenciais agentes quimiopreventivos, utilizando como modelo biológico as linhas celulares humanas da cancro de mama MCF-7 (dependente de estrogénio) e MDA-MB-231 (independente de estrogénio). Foi seguida uma estratégia multidisciplinar, que combinou ensaios biológicos com técnicas de ponta de espectroscopia vibracional, para avaliar (a distintas concentrações e para um tempo de exposição aos compostos de 48 h): (i) a proliferação celular/viabilidade, através dos testes colorimétricos de SRB e MTT; (ii) a capacidade antimetastática, usando uma câmara de invasão "transwell"; (iii) a formação de espécies ROS/RNS, mediante uma técnica fluorimétrica (com a sonda 2',7'-diclorofluoresceína diacetato); (iv) o impacto metabólico ao nível celular *via* microespectroscopia de Raman em células fixas. Em particular, a técnica de microRaman aplicada a culturas celulares permite uma monitorização rigorosa e não invasiva de amostras biológicas, constituindo uma ferramenta preciosa e inovadora para a avaliação do efeito de agentes externos no perfil bioquímico intracelular.

Para as concentrações de 50 e 100 µM, o p-Ca demonstra ter capacidade de aumentar significativamente a viabilidade celular para MDA-MB-231, porém os resultados de Raman não mostram nenhuma diferenciação entre o controlo e as células tratadas. Em contraste, as concentrações de 7.5 e 15 µM de p-Ca induzem uma pequena diminuição da viabilidade, porém os espectro de Raman exibem modificações a nível da composição

química (maioritariamenta lípidos e proteínas). Para as MCF-7, o p-Ca não demonstrou ter qualquer efeito significativo (em todos os intrevalos de concentrações) quer nos ensaios biológicos como para microscopia de Raman.

As medições de ROS/RNS concordam com estas conclusões, e evidenciam a seletividade demostrada pelos polifenóis em estudo.

Para a daidzeina, a concentração de 100 µM de daidzeina reduz a viabilidade celular (aproximadamente 50%) para a linha celular MDA-MB-231, os resultados de Raman evidenciam diferenças nos lípidos e no conteúdo proteíco. Para as MCF-7, a viabilidade é reduzida (30% e 40%) para 50 e 100 µM, confirmado por microRaman

Em conclusão, os resultados obtidos para 50 e 100 µM p-Ca permitem hipotetizar sobre o efeito pro-oxidante do ácido fenólico neste tipo de células (dando enfase às concentrações mais elevadas), o aumento dos níveis intracelulares de ROS levam ao aumento da proliferação neoplasica (que se sabe estar associada à necessidade de ROS).

Em concentrações mais baixas, os compostos demonstram interagir com os lípidos, podendo vir a ser uma promissora estratégia quimiopreventiva para carcinogéneses induzidas por alterações nos lípidos provocadas por ROS.

Abbreviations

ATCC	American Type Culture Collection
c.a.	Circa
CAT	Catalase
CCD	Charge-coupled device
DA	Daidzein
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMEM-HG	Dulbecco's Modified Eagle Medium - high glucose
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
e.g.	*Exempli gratia*, "for the sake of example"
EDTA	Ethylenediamine tetraacetic acid
EGF	Human epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptors
ER-	Hormone-independent breast cancer cell
ER+	Hormone-dependent breast cancer cell
FBS	Fetal bovine serum
Fd	Focal distance
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulphide
HCAs	Hydroxycinnamic acids
He-Ne laser	Helium–neon laser
Lasers	*Light Amplification by the Stimulated Emission of Radiation*
LDL	Low-density protein
MAPKs	Mitogen-activated protein kinase
MgF_2	Magnesium fluoride
MMP	Metalloproteinases family
MnSOD	Manganese superoxide dismutase
MTT	3-(4,5-dimethylthiazol-2-2yl)-2,5-diphenyl tetrazolium bromide
myc	Gene of transcription factor

NA	Numerical aperture
NOSs	Nitric oxide synthases
p53, p21, PTEN	Suppressor genes
PBS	Sodium phosphate buffer pH 7.4
PC	Principal Component
PCA	Principal Component Analysis
p-Ca	*para*-Coumaric acid
pH	potential of Hydrogen
PUFA	Polyunsaturated fatty acids
Ras, myc	Oncogenes
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSS	Reactive Sulphide Species
SD	Standart deviation
SERS	Surface-enhanced Raman Spectroscopy
SOD	Superoxide dismutase
SRB	Sulforhodamine B
UV	Ultraviolet
UV-B	Ultraviolet B
v/v	Volume/Volume
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
Δ	In plane deformation vibration of skeleton atoms
Γ	Out of plane deformation of skeleton atoms
δ	In plane deformation vibration of a X-H bond
γ	Out of plane deformation vibration
FT-Raman	Fourier transform Raman spectroscopy
YAG	yttrium-aluminum-garnet
vs	versus
ν	Stretching vibration of a X-H bond

Guideline

1. Biological Systems

1.1. Free Radicals, Oxidative Stress and Disease

1.1.1. Free Radical Species

In the 1950´s, free radicals (reactive oxygen, nitrogen and sulphur species – ROS, RNS and RSS) were identified in biological systems and were proposed to be involved in diverse pathological processes [1]. Previously, Denham Harman had proposed that free radicals might have an active role in the aging process [2]. Over the years, the interest and research efforts on free radicals' effect on biologic systems, including their role in oxidative-related disorders, has grown and is currently an active field of study with a recognized impact on human health.

A free radical is defined as a species capable of independent existence [3] that contains one or more unpaired electrons in the outer electron shell [3-8], which is responsible for a high reactivity [5]. Two free radicals can join their unpaired electrons to form a covalent bond [3] or can bind with electrons of neighbouring species giving rise to a chain reaction [6,9] and finally yielding a new free radical. This chain reaction can modify the structure and/or function of molecules, interfering with the normal biochemical processes within the cell. Therefore, organisms need an antioxidant defensive system [10] (Section 3).

There is a diversity of free radicals, namely oxygen and nitrogen reactive species (ROS and RNS, respectively, Table 1) [4,6,8,10]. The former include superoxide anion, hydroxyl, peroxyl, lipid peroxyl, alkoxyl radicals and hydrogen peroxide (which is not a free radical but is chemically very reactive, being involved in the production of other free radicals) [4,6,8,10,11]. RNS encompasses nitrogen dioxide [4,8] and nitric oxide [1,4,8,10], peroxynitrite ($ONOO^-$) and nitrite (NO_2^-) that are produced by a family of enzymes (nitric oxide synthases, NOSs [9,11] very important in a variety of intracellular signalling processes (*e.g.* neurotransmission) and in the relaxation of smooth muscle [10]. In addition, ROS and RNS comprise non-radical species that can also lead to free radical reactions in living organism [8].

Table 1 – Most relevant oxygen (ROS) and nitrogen (RNS) free radicals in living organisms.

Free radicals					
ROS	**Ref**	**Biological effect**	**RNS**	**Ref**	**Biological effect**
superoxide anion (O2•–)	[3,4,6,8,10, 11]	Lipid, proteins and DNA oxidation	**nitric oxide (NO•)**	[1,4,8,10]	Neurotransmitters and relaxation of smooth muscle
hydroxyl (•OH)	[3,4,6,8,10, 11]	Damage in specific targets (guanine, histidine, tryptophan and tyrosine)	**nitrogen dioxide (NO2•)**	[4,8,12]	
peroxyl (ROO•) including lipid peroxyl (LOO•)	[3,4,6,8,10, 11]	lipid peroxidation	**nitrite (NO_3•)**	[9,12]	
alkoxyl (RO•)	[3,4,6,8,10, 11]				
hydrogen peroxide (H_2O_2)	[4,6,8,10,11 ,13]	DNA damage			

Oxygen and nitrogen reactive species are produced in animals and humans under both physiologic and pathologic conditions [8,10], and have an important role in living organisms [5], their effect being strongly dependent on concentration. As an example, ROS induces cell growth and proliferation at nanomolecular levels [13,14], cause transient growth arrest and protective adaptive alterations in gene expression at micromolar levels and lead to oxidative stress at milimolar concentrations [13] having even been found to trigger signalling pathways which arrest cell proliferation at high dosages [14,15].

Although human life depends on oxygen for several crucial pathways, it is also a source of free radicals: in aerobic respiration, 80% of O_2 consumed [9] is used to generate energy in mitochondria [16], and 1-5% is transformed into superoxide and hydroxyl radicals [9,15,17]. Biological systems are therefore exposed to free radicals produced *via* endogenous and exogenous pathways [8,9,18,19].

1.1.1.1. Endogenous Sources of Free Radicals

Endogenous production of ROS is essential for regulating physiological functions in healthy cells (*e.g.* metabolic processes and cellular signalling, [5,6,8-10,16,20,21] (Figure 1). During inflammation, a cellular signal process, for instance, free radicals such as $O_2^{\bullet -}$ and H_2O_2 are generated by the immune system [6,8-10,15,19,22], when neutrophils and macrophages increase oxygen consumption). Phagocytes also produce ROS [21], in order to destroy invading pathogens and modulate the inflammatory response [19,22]. Numerous pathways can be activated in response to low levels of ROS, such as the epidermal growth factor receptor (EGFR) pathway [11]. Additionally, metabolic processes include some the activity of enzymes and metal ions (*e.g.* Cu^{2+}, Fe^{3+}) which also sources of free radicals in cells, as well as mental and body stress [21], conditions such as ischemia and an excessive physical activity are also endogenous sources of ROS [15].

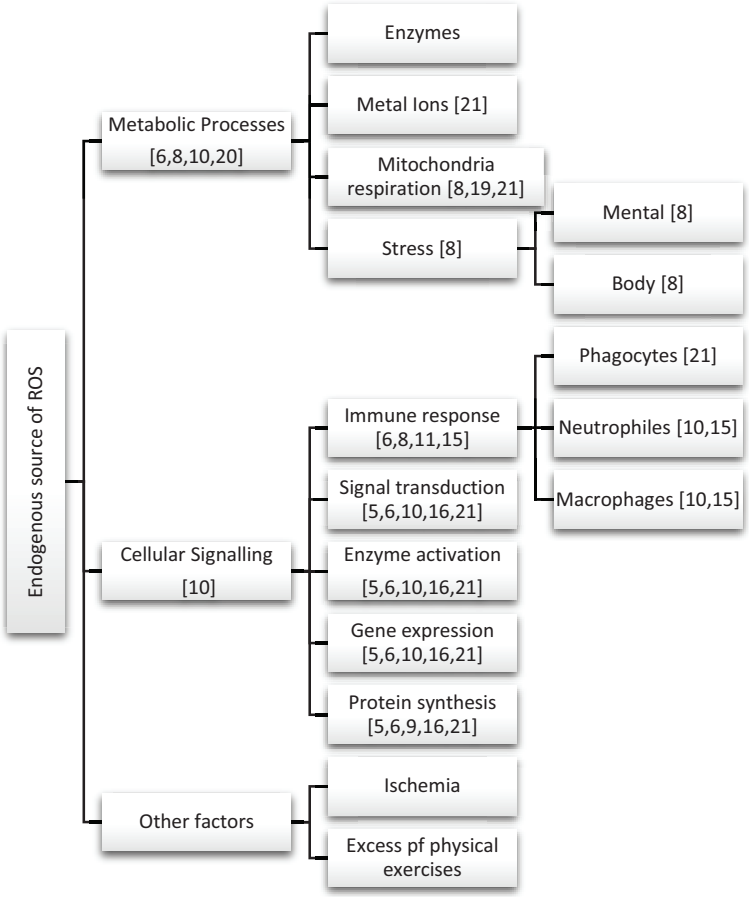

Figure 1 – Endogenous sources of ROS and RNS.

1.1.1.2 Exogenous Sources of Free Radicals

External sources of free radicals include xenobiotics [11,19], pollution (air and water pollutants), chemical solvents and external radiation (*e.g.* UV-B, [8,11,15,18,19,21,23]) (Figure 2). Exposure to these deleterious species is responsible for an increased level of free radicals, which may lead to oxidative stress related diseases [15]. Tobacco smoke is one of the main sources of free radicals, due to oxidative compounds present in smoke [11,15,21]. Furthermore, alcohol and drug consumption [8,11,18], wrong dietary habits or certain therapeutic schemes may also promote free radical production [15,19].

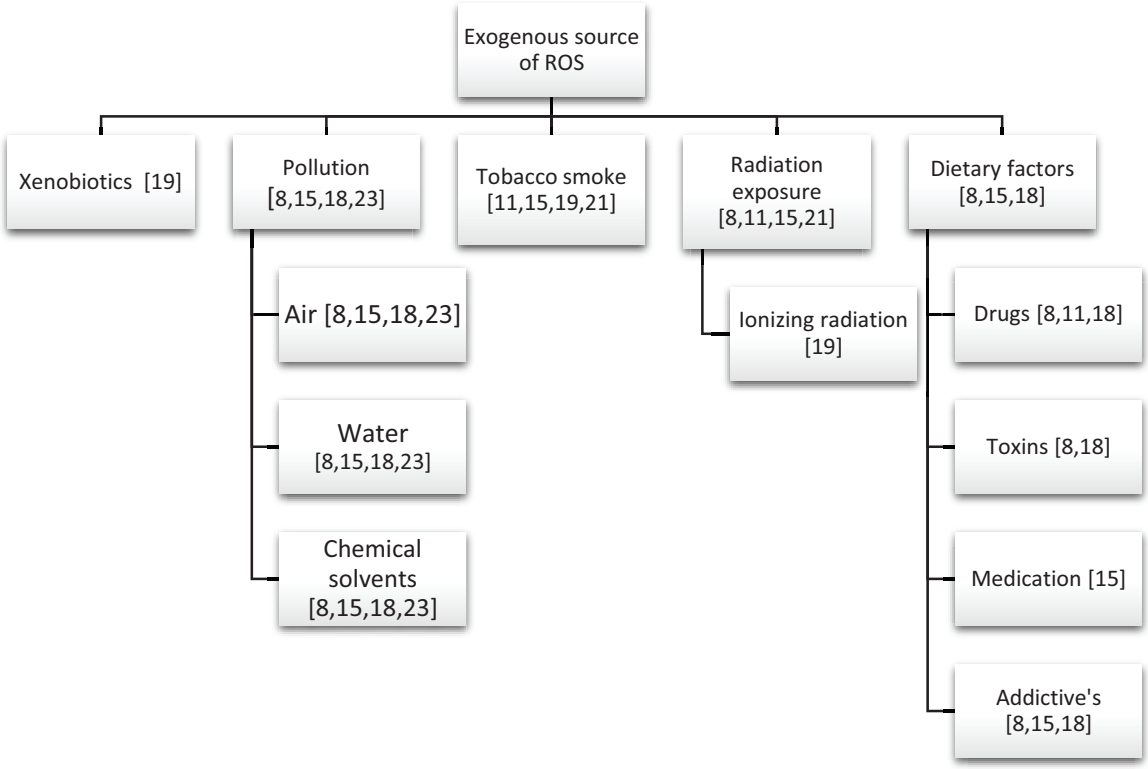

Figure 2 – Exogenous sources of ROS and RNS.

1.1.2. Oxidative Stress-Induced Diseases

Living organisms have specific mechanisms for keeping low intracellular ROS/RNS levels [1], *e.g.* a strictly regulated equilibrium between radicals´ production and elimination processes, leading to a steady-state (homeostasis) [1,9], thus avoiding oxidative damage due to non-controlled ROS/RNS formation [9]. Under physiological conditions, it has been estimated that a human cell is exposed to *ca.* $1x10^4$ to $1x10^5$

oxidative lesions *per* day [9,10,19], that are dependent on a variety of factors, namely environment, pollution, metabolism, diet, lifestyle and age [13].

The endogenous antioxidant system counteracts the harmful effects of free radicals, aiming to maintain a homeostatic state. However, under some conditions the equilibrium between free radicals and antioxidant production is lost [6,10] and the cell´s antioxidant defences are unable to overcome the oxidative attack and fail to repair their consequent damage, leading to a physiologic condition known as oxidative stress [1,6,10,11,24]: a serious imbalance between free radical production and antioxidant defences (Figure 3) [22].

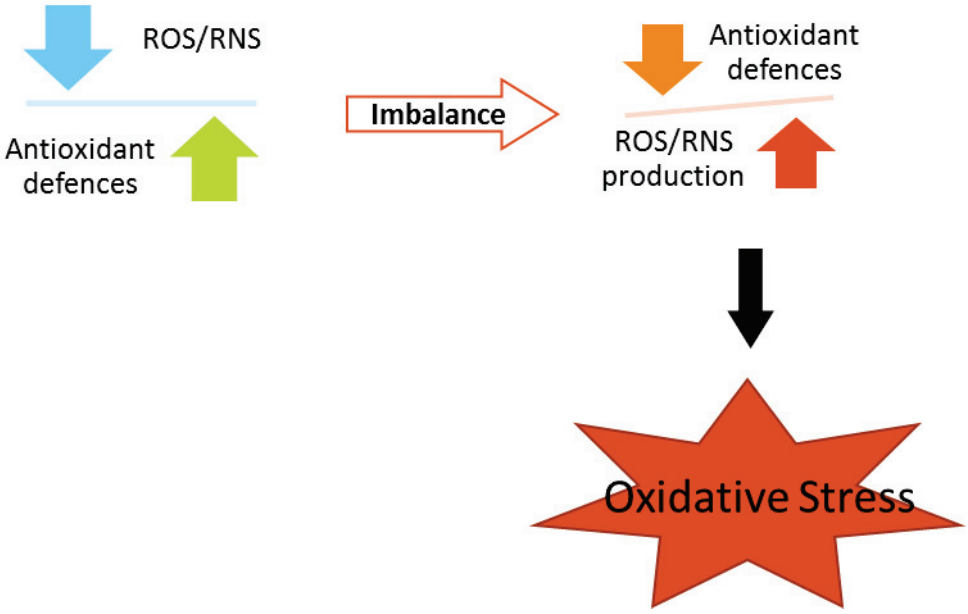

Figure 3 – Schematic representation of oxidative stress conditions.

This can be due to a variety of reasons: a) endogenous and exogenous overproduction of ROS [1,6,8,11]; b) insufficient antioxidant defence mechanisms [6,11,22], by depletion or/and decrease of antioxidant production [1,8,22]; c) a combination of these two factors. A similar condition may occur regarding reactive nitrogen species and is referred as nitrosative stress [8].

Oxidative (and/or nitrosative) stress accumulation over time contributes to the development of several severe diseases [25], such as cardiovascular and neurodegenerative disorders and cancer [24]. $NO^{\bullet}$ expression affects tumour development, may inhibit or promote, according to cell setting and concentration. Their promotion role is related to DNA damage and stimulation of vascular endothelial growth

factor (VEGF) which promotes angiogenesis, contributing to tumour growth and cell invasion [9].

Typically, the majority of ROS have a short half-life and cause damage only locally, but some of these species, namely H_2O_2, have a relatively long half-life and can diffuse through quite long distances within the organism causing DNA damage at distant sites from its origin [1]. The $^{\bullet}OH$, in turn, is a lower-energy singlet molecular oxygen and can produce a massive damage through particular targets (guanine, histidine, tryptophan, and tyrosine) reacting with both DNA and proteins [1,9,12] and being implicated in mutagenesis, carcinogenesis and aging processes [12,15]. In contrast, $O_2^{\bullet-}$ is completely unreactive towards biomolecules [1]. Additionally, RNS appear to contribute to cardiovascular disease, since peroxynitrite (generated by reaction of NO with $O_2^{\bullet-}$ in vascular endothelium), leads to proinflammatory cytokine-mediated myocardial dysfunction [26].

Hence, the presence of an excessive concentration of free radicals constitutes a serious risk for normal cellular function, especially when mediated by changes in biomolecules such as nucleic acids, proteins, polysaccharides and lipids, that are preferentially affected by oxygenated free radicals [1,6,9-11,24]. Table 2 comprises the main cellular targets of oxidative stress and the most significant physiologic consequences.

Oxidative DNA damage is one of the most severe consequences of oxidative stress conditions [19], since injured DNA has the potential to induce mutations [6,27]. Therefore, DNA damage needs to be properly addressed and repaired by endogenous mechanisms, prior to or during replication, with a view to prevent replication errors, genomic instability, cell death, or impairment of transcription and signalling pathways, all of which have been associated to carcinogenesis [19,28].

Free radicals can interfere directly with proteins, or can interact with sugars and lipids yielding products that then react with proteins [19]. Oxidative protein damage can induce several alterations, from fragmentation and/or aggregation to an enhanced susceptibility to proteolysis leading to oxidation and degradation [6,10]. This may impair protein function [6,10,16] and enzymatic activity [9]. Reactive oxygen/nitrogen species can also induce conformational changes in protein receptors, thus affecting cellular signalling mechanisms [19].

Table 2 – Main cellular targets of oxidative stress and physiological impact.

Target	Ref	Consequence	Ref
DNA	[21,29]	Mutation gene sequence; oxidized purines and pyrimidines; single- and double-strand breaks;	[6,19,28,29]
RNA	[19]	Modification of bases; ribose and strand break	[19]
Proteins	[13,21]	loss of sulfhydryl groups; amino acid modification;	
		Fragmentation and aggregation; susceptibility to proteolysis; degradation and structural alterations;	
		inhibition of normal cellular functions; cell death (apoptosis or necrosis)	[13]
Enzymes	[21]	inhibition of enzymatic activity	[13]
Lipids		Lipid peroxidation; alteration of membrane fluidity and permeability	[15]
Polyunsaturated fatty acids (PUFA)	[19]	unstable membrane structure; altered membrane fluidity and permeability; impaired signal transduction	

Lipids are the most susceptible biomolecules to ROS/RNS injury, membranes of cells and organelles being very prone to damage (especially those with polyunsaturated fatty acids (PUFA), [6,9,10,16] either *via* direct interaction with free radicals or by formation of peroxides and their breakdown products [21]. This results in unstable membrane structures and altered membrane fluidity and permeability [6,16], also affecting signal transduction [6].

Moreover, oxidative stress has been associated with neuronal death [30], through mitochondrial damage, when proteasome pathway are inhibited (oxidation and nitrate inactivation of proteasome subunits) - ubiquitin-proteasome system is needed to development and maintenance of neurons and have a role in axonal degeneration after nerve injury [12]-, or *via* dopamine oxidation.

Some studies have demonstrated that free radicals activate neoplastic transformation by diverse mechanisms [10]: inactivation of expression of oncogenes (*e.g.* Ras, myc, telomerase) and suppressor genes (p53, p21, PTEN) [10,11], or increased expression of proto-oncogenes [31]. These transformations can lead to the induction of cell damage [10], senescence or escape from apoptosis [11]. ROS is also associated with

hypoxia, which is a condition reported in almost every tumours, which is an oxygen diffusion limitation in primary tumours or their metastasis [14]. Hypoxia leads to an increase of mitochondrial ROS [14] causing oxidative stress which activate metalloproteinases (MMP) [31,32]- a family of collagenase that aids vessel growth [31]– which are associated with increasing tumour vessel supply promoting angiogenesis, cell invasion as well as metastasis [31,32].

In summary, oxidative damage plays a pivotal role in a wide variety of human diseases: besides cancer and cardiovascular or neurodegenerative disorders, it can cause rheumatoid arthritis, muscular dystrophy, diabetes, among others [1,16,17,24,32,33] (Table 3).

Table 3 – Main oxidative stress-induced human pathologies.

Diseases Oxidative Damage Related	Ref
Cancer	[1,16,17,22,25]
Diabetes mellitus type 2	[1,16,17,34]
Cardiovascular diseases	[1,16,17,34]
Neurodegenerative diseases	[1,12,16,17,25,34]
Parkinson's	[19]
Alzheimer's	[19,25]
Cataract formation	[1,16,17,34]
Aging	[1,3,16,17]
Rheumatoid arthritis	[1,16,17,34]
Acute respiratory distress syndrome	[1,16,17]
Asthma	[1,16,17]
Obesity	[1,16,17]
Hepatitis C	[19]

1.2. Breast Cancer

Cancer is becoming the major cause of morbidity and mortality worldwide [35], and is estimated that in the next decades it will affect every region of the world, closely following cardiovascular disorders [36]. Cancer results from a deregulated cell proliferation caused by DNA mutations, induced by a carcinogenesis process. This may be due to a variety of carcinogenic agents: chemical (drugs and chemical pollutants); biological (viruses), or physical (radiation) [35].

Among cancers, breast cancer is an important public health problem as it is the second most lethal cancer among women worldwide [37,38]: according to the World Health Organisation (WHO), it affects 28% of the women in Europe [39]. In Portugal, the woman death rate attributed to breast cancer has been growing steadily, 30.3% deaths in 2010 [40]. Despite being associated with women, breast cancer can also affected men. The major risks of developing breast cancer are age [35,36,41] , early menarche, delayed menopause, use of contraceptives, hormone therapy, genetic predisposition and obesity [35]. Women over 50 years old are the most affected, with a 79% incidence (in United Sates of America, 2006-2010) and metastasis remains a significant cause of mortality [42]. There are two models proposed for carcinogenesis regarding breast carcinoma: a) DNA mutation and b) induced by steroid hormones.

Epidemiological evidence has demonstrated a clear difference in breast cancer incidence between Eastern and Western countries [37,41,42], Asian women being the least affected [41,42] which can be related to diet. Additionally, a large number of epidemiologic studies support a diet rich in vegetables and fruits as an effective chemopreventive strategy towards this type of cancer [37].

1.2.1 Oxidative Stress-Induced Breast Cancer

Carcinogenesis is a multi-step process, comprising initiation, promotion and progression [19] (Figure 4).

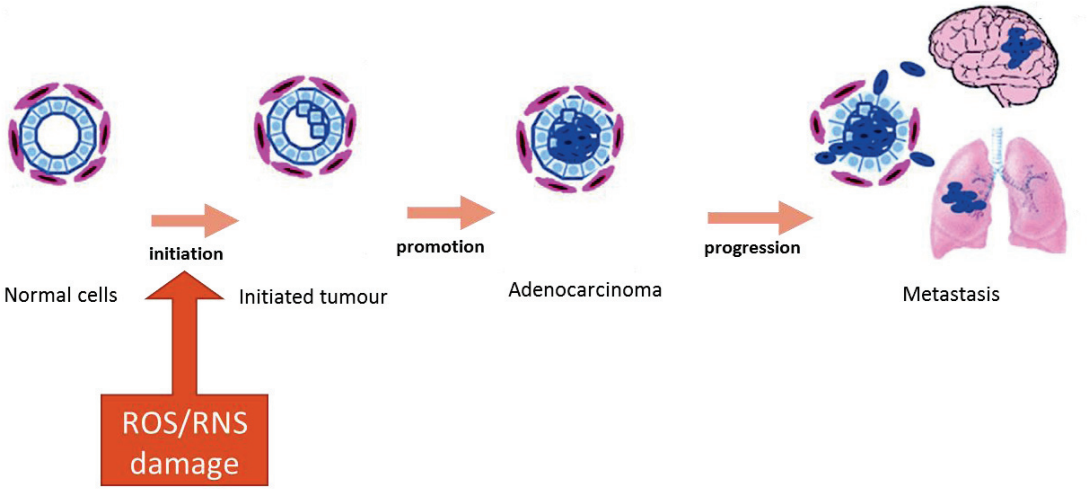

Figure 4 – Cancer progression. (Adapted from [43])

Initiation takes place when a normal cell undergoes DNA damage (due to ROS/RNS) and proceeds *via* DNA synthesis, resulting in the fixation of the mutation, thus yielding an initiated cell [19,44]. The promotion process involves modulation of gene expression resulting in an increased cell division and/or decreased apoptotic cell death, leading to a continuous cell proliferation that may induce subsequent mutations in these preneoplastic cells, resulting in a neoplasm [19]. Progression, the last step of the process, involves additional damage to the genome and, unlike the promotion step, is irreversible [19].

Epidemiologic data have shown that chronic oxidative stress is strongly related with carcinogenesis [10,11,19,29,38] , ROS/RNS species being present at higher concentrations in cancer cells (relative to non-neoplastic ones) [19,29,35]. Actually, the role of free radicals in breast cancer development may not be limited to early mutations, but probably affects all phases [31], including angiogenesis and metastasis [10,16,22,28]. Upregulated levels of free radicals in breast cancer that may cause DNA damage along with defective DNA repair mechanisms when compared to normal breast tissue [10,38].

Numerous factors and different pathways are responsible for an increased level of reactive oxygen species in breast tumour tissue [35], the most important of which will be described in this section. Telomere genes are highly susceptible to mutation in the

presence of free radicals [27] and it is apparent that suppressor genes such as *p53* are affected by oxidative stress [10,14,27,35]. This gene activates several pathways involved in programmed cell death [35], regulation of the expression of gene code antioxidant proteins and prevention of oncogenic transformation [10,14,35]. ROS-related *p53* mutations or *p53* loss have been detected in over 50% of human cancers and are associated with aggressive tumour growth [14]. *p53* is dose-regulated by free radicals [10,35]: under physiological conditions free radicals´ concentration is low and *p53* expression is moderated; when the level of free radicals increases *p53* is stimulated, promoting oxidative stress and the consequent DNA damage and cellular apoptotic death. Another effect of a high concentration of ROS/RNS is *p53* inactivation by direct DNA damage [10].

In addition, persistent inflammation proceeds a variety of cancers, including breast cancer, and involves an immune response (namely through macrophages and neutrophils) [35]. Sublethal oxidative stress promotes cell proliferation *in vitro* by stimulation of superoxide and hydrogen peroxide, which activates MAPKs (mitogen-activated protein kinase) pathways [31]. MAPKs play a role in cell growth and cancer under conditions of oxidative stress [35]. Since oxidative stress may induce apoptosis, some therapeutic schemes that lead to the production of ROS in carcinoma cells are used in the clinic: certain drugs (*e.g.* doxorubicin, mitomycin C, tamoxifen) [31,45], photodynamic therapy or radiotherapy. Regarding breast cancer, tamoxifen, a well-known antioestrogen agent, is known to induce oxidative stress *in vitro* [31,45].

1.3. Antioxidant Defences

Upon exposure to oxidants (or oxidative stress-inducing agents), mammalian cells express stress-induced genes, which encode the antioxidant defence mechanism to metabolize these reactive species into nonreactive ones [19] , thus reducing (or hindering) their harmful effect. Halliwell and Gutteridge defined antioxidant "as any substance that, when at low concentration compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate" [3,8,46]. Indeed, this is the first line of cellular defence against free radicals [9,47].

Antioxidant defences depend on both the endogenous [8,15,47] production of antioxidant compounds and the dietary intake of antioxidant food products (exogenous, mostly phytochemicals) [8,15,47]. These molecules counterbalance the deleterious effect of free radicals [8,46,47] by: a) catalysing a complex cascade of reactions that convert ROS/RNS into stable entities like water and O_2; b) through direct scavenging of the radical species [47], thus neutralizing them and maintaining the body´s redox balance [8,35]. An antioxidant compound is classified according to their biochemical mechanism of action [15,30] (

Table 4).

Table 4 – Main antioxidant defences in mammalian cells.

Antioxidant enzymes					
Antioxidant Species	**Forms present in body**	**Location**	**Source**	**Function**	**Ref**
Superoxide dismutase (SOD]			Endogenous production	catalyses the dismutation of $O_2^{\bullet -}$ to H_2O_2	[6,9,15,30, 46,47]
	SOD1	Mitochondria, cytoplasm	Endogenous production		[8,15,46]
	SOD2 (Manganese superoxide dismutase]	Mitochondria, cytoplasm, lysosome	Endogenous production		[8,15,46]
	SOD3	Extracellular	Endogenous production		[8,15]

Catalase (CAT]		Common in cell	Endogenous production		[6,8,9,15,30,46]
Glutathione peroxidase (GPx]		Cytoplasm	Endogenous production	Reduction of H_2O_2	[6,9,15,35,47]
	GPx1	Cytosol	Endogenous production		[15,48]
	GPx2	Intestinal epithelium	Endogenous production		[15,48]
	GPx3	Plasma	Endogenous production		[15,48]
	Gpx4		Endogenous production	Protection of membranes	[15,48]
	GPx8 (mammalian glutathione peroxidase]	Membrane protein of the endoplasmic reticulum	Endogenous production		[15,48]
Peroxiredoxin			Endogenous production		[15]

Chain-Breaking Antioxidants

Antioxidant	**Form(s] present in body**	**Biodistribution**	**Source**	**Function**	**Ref**
Glutathione (GSH]			Endogenous production		[6,9,15,35,47]
	Reductase form (Glutathione reductase] (GR]	Cytosol, nuclei and mitochondria	Endogenous production	sequestrates of ROS which are transformed and recycled [9]	[6,8,9]
	Oxidized form (Glutathione disulphide (GSSG]]		Endogenous production		[6,8,9]
Peroxidases		Cytosol, mithocondria	Endogenous production		[15,49]
Thioredoxin			Endogenous production		[15,35,47]
Uric acid			Endogenous production		[6,15,35]
Lipoic acid			Endogenous production		[6,8]

	reduced form (dihydrolipoic acid]		Endogenous production		[8]
Vitamin C (ascorbic acid]		cytosol, plasma and other body fluids	Dietary source	protection of endogenous DNA damage; lipid peroxidation; reduction of pro-oxidant effect of tocopherol radical [48]	[6,15,35,46]
Vitamin D			Dietary source		[47]
Vitamin E	family of fat-soluble compounds	low-density lipoproteins (LDL]	Dietary source		[6,35,46]
Beta-carotene	Vitamin A precursor		Dietary source		[46]
Vitamin A	fat-soluble compounds		Dietary source		[35,46]

1.3.1. Antioxidant enzymes

Antioxidant enzymes include catalase (CAT), peroxidases and superoxide dismutase (SOD) which is probably the most important one in the cell [15]. SOD catalyses the neutralization of the superoxide radical-induced damage [8,30,46] by converting it to hydrogen peroxide (H_2O_2) [8,15] and O_2 [8,30]. This reaction is the body's primary antioxidant defence, preventing further generation of free radicals [30]. Humans have three forms of superoxide dismutase: SOD1 is found in mitochondria as well as the cytosol, [8,15] and requires both copper and zinc for its function [15]; SOD2 only occurs in mitochondria [8,15]; SOD 3 is found in the extracellular media [8,15] and requires copper-zinc as cofactor [15].

Catalase is an enzyme widely distributed in cells that converts hydrogen peroxide into water and oxygen in cells [8,15,30], completely neutralizing the superoxide free radical [15,30]. CAT requires iron as a cofactor, which is attach to active site [15].

The glutathione peroxidases (GPx) comprise eight distinct enzymatic forms (GP1x to GP8x) [15], which prevent the formation of hydroxyl radicals from H_2O_2 and protect the cell against H_2O_2-induced oxidative injury [8]. GPx1 to GPx4 and GPx6 (selenium-containing enzymes) and GPx5, GPx7 and GPx8 (non-selenium) have an important role in detoxifying the cell from hydroperoxides. GPx1 can interact with hydrogen peroxide and soluble low-molecular-weight hydroperoxides, being involved in the prevention of cancer and neurodegenerative diseases. GPx3 is suggested to regulate the bioavailability of nitric oxide produced from platelets and vascular cells. GPx4, in turn, reduces hydroperoxides in membrane lipids and lipoproteins [50].

Peroxiredoxins and thioredoxins are also enzymes involved in the detoxification of hydrogen peroxides within eukaryotic cell [15,49]. The former degrades hydroperoxides into water and can act as a redox sensor (monitoring disruption of cellular redox homeostasis). Thioredoxins have important roles in several vital cellular processes such as cell cycle regulation, cell growth, gene expression and apoptosis.

1.3.2 Chain-Breaking Antioxidants

Chain-breaking antioxidants are molecules found in the blood and extracellular fluids [30], with the ability to neutralize free radicals by breaking the chain reaction initiated by them (Figure 5):

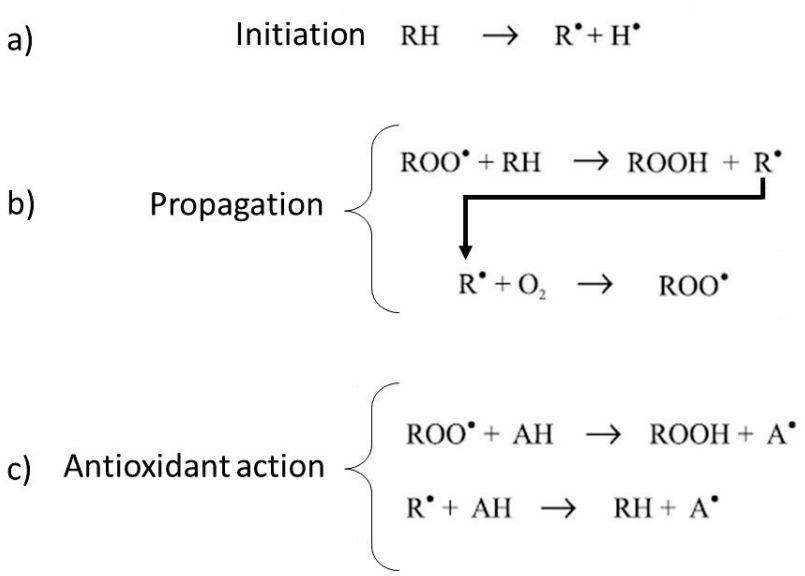

Figure 5 – Scheme of antioxidant activity against a free radical-initiated chain reaction. A• represents an inert antioxidant (with no capability to initiate a chain reaction).

Antioxidants can be either endogenous or exogenous [15] (

Table 4), and include both hydrophilic and lipophilic small-molecules [30]. Vitamin C is a water-soluble antioxidant capable of scavenging $O_2^{\bullet -}$, $OH^{\bullet}$ and $ROO^{\bullet}$ radicals, as well as H_2O_2, hypochlorous acid and singlet oxygen [46] (Figure 5). Uric acid is a lipophilic chain-breaking antioxidant that can coordinate oxidative Fe^{3+} ions, thus preventing the deleterious Fenton reaction.

Glutathione (GSH) is an essential endogenous antioxidant responsible for keeping the intracellular redox homeostasis of the cell [9,15]. In the reduced state (GSH, the most abundant form in the reducing intracellular medium), glutathione displays a high antioxidant ability by scavenging a variety of free radicals and oxidative ions [15]. GSH is capable of capturing hydroxyl radicals and singlet oxygen directly and can be used as a reductant of GPxs to detoxify hydrogen peroxide and lipid peroxides [8,48]. In the oxidized form (glutathione disulphide, GSSG], it is mostly present in the nucleus for maintaining the redox state of protein sulfhydryls that are essential for DNA expression and repair [8].

Lipoic acid is another relevant endogenous antioxidant, sometimes referred to as “universal antioxidant” since it is able to quench free radicals in both hydrophobic and hydrophilic domains, and also to chelate pro-oxidant cations [8]. Vitamin E, a dietary compound, is a potent, lipid-soluble antioxidant present in low-density lipoproteins [15,46]. Moreover, it is very effective at breaking the chain reaction leading to lipid peroxidation, through peroxyl radical scavenging. Carotenoids are another relevant group of natural antioxidants, able to neutralize peroxy radicals and singlet oxygen [15].

1.4. Dietary Polyphenols

Polyphenols are an abundant source of micronutrients [51] with recognised antioxidant activity [52]. Several studies have established a direct relationship between the intake of this type of compounds and prevention of some oxidative stress-induced diseases, such as neurodegenerative and cardiovascular disorders and some types of cancer [53-55]. These compounds are secondary metabolites of plants (phytochemicals) [51,53,55,56], and are therefore widespread in fruits, vegetables, cereals, vegetable oils, chocolate and beverages such as tea, coffee and wine [52,55,57,58]. Phytochemicals, even though not considered as essential nutrients, are known to display numerous biological functions, from cell growth to defence against pathogens or UV radiation in plants [51,53,56,59]. Furthermore, they constitute the active substances found in many medicinal plants, being responsible for modulating the activity of a wide range of enzymes and cell receptors [51].

Polyphenols can be classified into different groups, according to their chemical characteristics. Over 8000 distinct structures are known [33,55,57], mostly differing in the number of aromatic rings and their substituent groups (Figure 3) [33,52,53,55]: phenolic acids, flavonoids, stilbenes, coumarins and tannins.

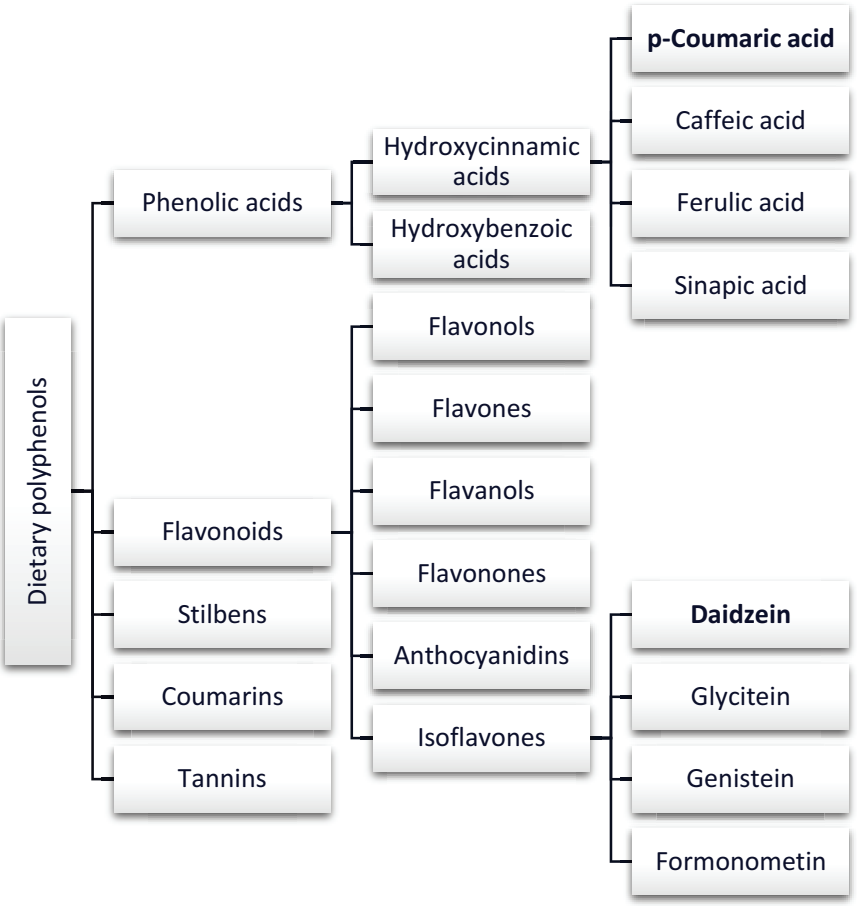

Figure 6 – Classification of dietary polyphenols. (Adapted from [51-53,60])

The most abundant polyphenols in the human diet are flavonoids [33,53], which account for about 2/3 of the total phenolic content, the remaining 1/3 being phenolic acids [53,56]. These can be grouped into two major subclasses: benzoic acid derivatives and cinnamic acid analogues (hydroxycinnamic acids, Figure 7) [33,51,52,55-57].

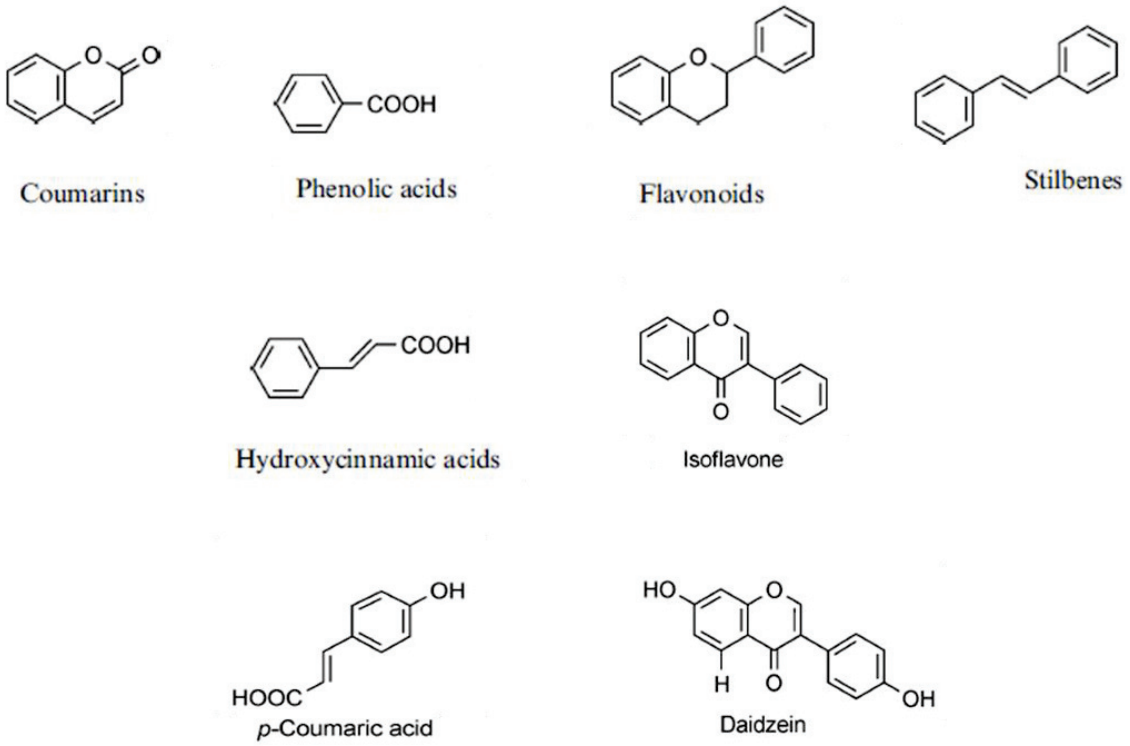

Figure 7 – Chemical structure dietary phytochemicals. (Adapted from [57])

The average intake of phenol acids in a normal diet was reported to be *ca.* 200 mg/day, but this value is strongly determined by dietary habits [56]. Phenolic acids and their derivatives have been described to possess both antioxidant and prooxidant abilities (depending on their concentration], as well as preventive properties towards cardiovascular diseases, neurodegenerative disorders, inflammatory processes and cancer [61-63].

Flavonoids comprise two C6 aromatic rings (A and B) and may be divided into 6 groups: flavonols, flavones, flavanols, flavonones, anthocyanidins and isoflavones (Figure 6) [51,53,55,57]. In the basic flavonoid structure the hydroxyl groups are usually at the 4',5' and 7' positions, and sugar substituents are common (yielding flavonoid conjugates abundant in green plants [57]. Extensive experimental evidence (mostly from epidemiologic studies) suggests that flavonoids display health-promoting properties related to their antioxidant capacity (through both chain-breaking and radical scavenging processes [24].

This type of compounds have been the subject of intense research in the last two decades, and numerous studies have suggested that a diet rich in fruits, vegetables and cereals can significantly reduce the risk of developing pathologies attributed to

deleterious oxidative processes, including cancer [31,53,55,62,64]. Hence, dietary habits have been highlighted as an effective chemopreventive strategy against this type of pathologies.

1.4.1 Hydroxycinnamic Acids

Hydroxycinnamic acids (HCAs) are hydroxyl derivatives of cinnamic acid (Figure 7). They are biosynthetised from phenylalanine and tyrosine, and contain an unsaturated side chain with either *cis* or *trans* conformation [60]. These compounds are commonly present in nature as conjugates, linked (through ester bonds) to polyamines, proteins or cell-wall structural components (*e.g.* cellulose or lignin) [53].

HCAs are more common that hydroxybenzoic acids and comprise p-coumaric (p-Ca), caffeic, ferulic and sinapic acids [51-53,57,60]. Numerous studies have shown that cinnamic and hydroxycinnamic acids display relevant biological properties, namely antiproliferative effect towards several types of cancers (*e.g.* colon, cervical, breast and lung carcinomas [56,62,65]) and a potential inhibitory effect in cancer metastasis [54].

1.4.1.1 *para*-Coumaric acid

trans-4-Hydroxycinnamic acid (*para*-coumaric acid, p-Ca) is the major HCA in citrus, pinnaple [34], spinach, grapes, apples, berries and tomatoes [66], being also present in beverages such as tea, coffee and wine [67-69]. It is one of the three geometric isomers of hydroxycoumaric acid (besides the orto- and meta-coumaric derivatives) [69]. In nature, p-CA is the most abundant of the three isomers, and is biosynthesised from cinnamic acid in the presence of the p-450-dependent enzyme 4-cinnamic acid hydroxylase [69]. Its bioavailability in humans is around 1.0 µmol/L [54]. Although the mechanism of action is not still fully understood at the molecular level, several studies have hypothesised that p-Ca´s antioxidant properties (and potential chemoprotective effect) are due to quenching of carcinogenic ROS and RNS within the cell [68,70].

Coumaric acid has been shown to inhibit cell cycle progression and to reduce cell viability and colony formation in colon cancer cells [54,66,71], as well as in SW 620, Hep G2, A549 and T-47D cells (IC_{50} equal to 87, 215, 412 and 474 µM, respectively [72]). A

study in cadmium chloride-induced renal toxicity in rats showed that p-Ca (100 mg *per* kg *per* b. wt) displays an effective protective effect [73].

1.4.2 Isoflavones

Isoflavones are a subgroup of flavonoids comprising a phenyl group at the C3 position and a specific orientation between rings B and C (Figure 7) [55,57,59]. They are found in leguminous vegetables, mainly soybean and soybean derivates [41,55,65], and are often referred to as phytoestrogens since they can mimic estrogen activity [37,41,51,65,74], the relative position of their hydroxyl groups at positions 7 and 4' being analogous to that of the estradiol molecule. These compounds have shown to prevent cardiovascular diseases and osteoporosis [65], and decrease cancer progression [41,65].

1.4.2.1 Daidzein

Daidzein (DA] is an isoflavone found in nuts, citrines and soybean products (*e.g.* beans, tofu, tempeh, soy milk, textured vegetable protein, flour and miso) [34,37,74]. DA displays structural and functional similarities to the endogenous hormone estrogen [74], and since some breast cancers are estrogen-dependent, this isoflavone may compete with natural estrogen reducing its availability and therefore inhibiting cancer cell growth [57]. Similarly to other isoflavones, DA has a high antioxidant activity [74], being able to induce neoplastic cell death in a variety of cancer types [37]. Dietary consumption of daidzein from soybean products has been verified to reduce prostate and breast cancers [57].

The health-beneficial properties of phytochemicals have led to an intense search for novel and more efficient antioxidants from natural sources, involving the Medicinal Chemistry and Nutritional Pharmacology fields. Establishment of new nutritional habits is sough, aiming at the prevention of cancer and cardiovascular disorders through a regular consumption of these dietary antioxidants [24].

1.5. Chemoprevention

In 1976, Michael Sporn reported a new approach against cancer, *chemoprevention* [36,75]. He defined this approach as the use of natural, synthetic or biological agents to revert, suppress or prevent the development of a variety of steps within the process of carcinogenesis (initiation, promotion and/or progression) [75-78].

Conventional cancer chemotherapy is associated to significant non-specific deleterious side effects [36,75], and in long-time treatments patients can develop drug resistance [36,59]. Additionally, cancer treatment is very expensive for the healthcare provider and to the community in general, which renders chemoprevention based on phytochemicals, usually inexpensive, readily applicable, acceptable and accessible [79], an attractive alternative aimed at avoiding or minimising cancer incidence. Actually, recent studies have shown that some cancers (such as breast and colorectal) could be prevented in 2/3 of the cases.

Epidemiologic studies establish that women from Asian countries display a much lower incidence of breast cancer than western women, this having been associated to dietary habits (*e.g.* higher consumption of soy products [75,80]. A similar scenario has recently been reported for prostate cancer [80]. Additionally, studies performed in different cell lines (breast [31] and colorectal cancer [81]), and animal models [77,80,82] as well as clinical trials [77,82], suggest a protective role of antioxidant dietary polyphenols against different types of neoplasias [77]. In fact, polyphenols have already been recognised as effective protective agents against cardiovascular diseases, decreasing mortality due to coronary heart distress and reducing the incidence of myocardial infarct [26,61].

Nowadays it is well-established that diets rich in antioxidant compounds from grains, fruits and vegetables are health-beneficial [25,75], and protect against the deleterious action of free radicals that may lead to cancer development [75] and other oxidative stress-induced disorders (*e.g.* neurodegenerative diseases).

In addition, natural products are chemopreventive agents of choice due to their minimal toxicity coupled to their favourable protective capacity [31,75,80] and nutritious properties [75].

Despite the efforts to establish the numerous advantages of chemopreventive strategies, controversy still remains among researchers regarding a widespread use of this type of antioxidants, mainly due to their concentration-dependent dual effect (antioxidant *versus* pro-oxidant) and the difficulty to accurately quantify their bioavailability in food products [77]. Over the years research in the nutritional, pharmacological and medicinal chemistry fields has aimed to find new antioxidants from natural sources, with particular emphasis on the prevention of cancer and cardiovascular disorders through dietary intervention, in view of the high morbidity of these disorders and the limited success of conventional chemotherapy [59].

Chemoprevention agents can be classified into three main groups: a) primary prevention, in high-risk healthy individuals; b) cancer prevention, in individuals which had developed pre-malignant lesions; c) continuous prevention, in cancer patients already treated (conventionally) for a primary tumour [83].

1.6. Structure-Activity Related Mechanisms of Action

The protective effects of compounds present in fruits and vegetables appear to be involved in the early rather than the later stage of carcinogenesis [75]. Experimental evidence suggests that antioxidants and anti-inflammatory compounds can be used to modify the redox environment of cancer cells, and consequently their behaviour and growing profile [75]. The anticarcinogenic activity of these dietary compounds is reflected in their cytoprotective effect towards healthy cells coupled to their cytotoxic effect against pre-neoplastic or neoplastic cells [77].

Despite the extensive knowledge on dietary polyphenols regarding their structure and antioxidant capacity, the mechanism of action underlying their chemopreventive activity has not yet been fully elucidated [84], having been generally assigned to: ROS/RNS scavenging [59,85,86], inhibition of oncogene activity and activation of tumour suppressor genes, modulation of hormone/growth factor activity, induction of terminal differentiation, activation of apoptosis, re-establishment of the immune response, and/or inhibition of angiogenesis.

Dietary chemopreventive agents can be classified according to their different mechanisms of action: (i) Blocking agents, that act immediately before or during carcinogenesis initiation, thus preventing the interaction between carcinogenic compounds (*e.g.* exogenous or endogenous ROS/RNS) and cellular targets, through different mechanisms including carcinogenic detoxification, modification of carcinogen uptake and metabolism, scavenging of ROS and other oxidative species, and enhanced DNA repair [83-85,87]. (ii) Suppressing agents, which exert their effect during initiation, promotion and progression of pre-neoplastic cells, *via* radical scavenging activity, or inhibition of xenobiotic metabolizing enzymes [83-85,87] . Phytochemicals were also shown to display anti-invasive and antimitotic properties thus interfering with cell migration and adhesion, but the exact molecular mechanism responsible for these properties remains unclear [77].

According to several reported studies, the beneficial effects of phenolic compounds rely on their structure and conformation through defined Structure-Activity Relationships (SARs), namely the number and location of the phenolic hydroxyl groups [59,64,80], the nature of the spacer chain between the ring and the carboxylic, ester or amide moieties,

and the length of the ester group in polyhydroxylated esters [62] (Figure 8). Furthermore, the biological activity of these compounds is determined by their rate of incorporation into the cells, which is directly related to their hydrophilic *vs* lipophilic properties [62,64].

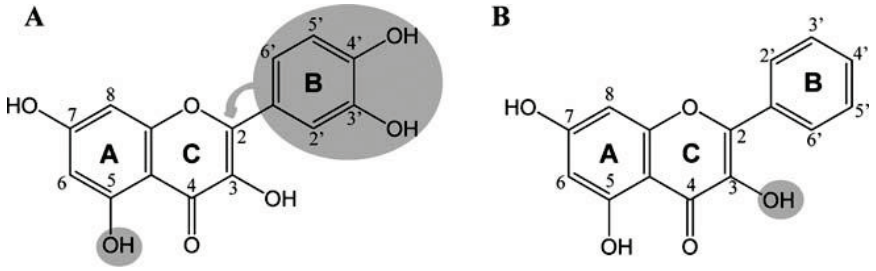

Figure 8 – Main structure–activity relationships (shadowed areas) established for isoflavones, in the presence (A) or absence (B) of a catechol group (from [59]).

1.6.1. Hydroxycinnamic acids

The antioxidant properties of hydroxycinnamic acids are intimately related to their characteristic chemical structure [54] (Figure 9).

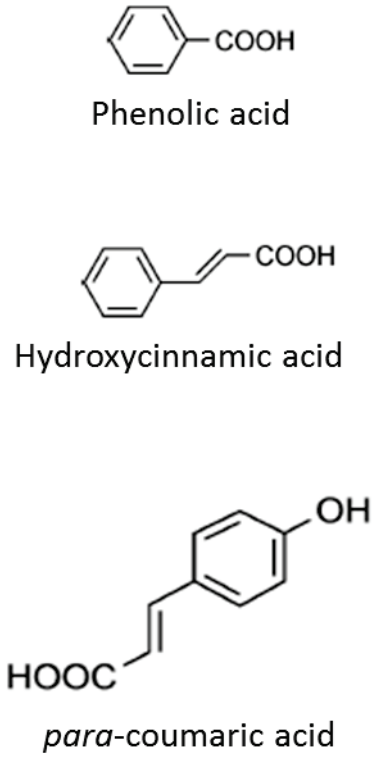

Figure 9 – Chemical structure of phenolic acids, hydroxycinnamic acid and p-coumaric acid. (Adapted from [57])

HCAs have been associated with growth-inhibition properties towards some human cancer cell lines [54,64], along with a reduced risk of cardiovascular disease and other oxidative stress-related disorders. HCAs have a strong radical-scavenging ability (OH-donation) related to the presence of –CH=CH-COOH groups [88] and are able to capture pro-oxidant metals, although other mechanisms are also suggested, namely triggering of gene induction/repression modulation of enzymatic activity, inhibition of cell proliferation and O_2 scavenging [54,89].

1.6.2. Isoflavones

The beneficial health effects of flavonoids (Figure 10) are described as being mainly due to their metal chelating ability, as well as to activation of antioxidant enzymes, reduction of free radicals and inhibition of oxidases [26], and is determined by the dissociation enthalpy of the O–H bonds present in the molecule [59].

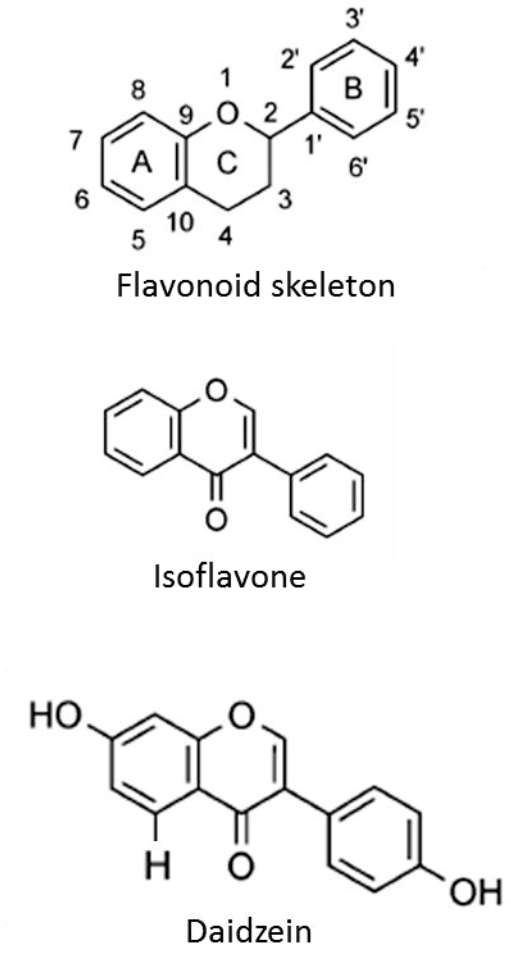

Figure 10 – Flavonoid skeleton, isoflavone general structure and daidzein (Adapted from [57]).

These mechanisms are strongly structure-dependent, namely on the presence of 2 or 3 hydroxy substituent groups in the B-ring, or of a 4-oxo function in the C-ring, especially when coupled to a 2,3-double bond in C-ring [59,80]. Also important are the additional presence of OH substituents in ring B at either 3′ or 5′ positions and at ring A in postion 7 [90].

1.7. The aims

The purpose of this research work is to assess the relationship between the biological activity – potentially chemopreventive – of two dietary phytochemicals (*p*-coumaric acid and daidzein) and their biochemical impact on human breast cancer cells, assessed by microRaman spectroscopy. A multidisciplinary approach was chosen, comprising:

(i) Evaluation of the biological activity of the dietary compounds regarding cell viability and proliferation, in human breast cancer cells (estrogen-dependent/MCF-7 and independent/MDA-MB-231), using the MTT and SRB colorimetric tests, respectively. Simultaneous screening in non-tumour human breast cells (MCF-12A).

(ii) Measurement of the ROS present in the intracellular medium, in the presence and absence of the phytochemicals, through a fluorimetric method.

(iii) Assessment of the effect of the polyphenols on the cell´s metastatic, in a transwell invasion chamber.

(iv) Probing of the compounds´ metabolic impact on the human breast cancer cells *via* Raman microspectroscopy of fixed cells.

2. Models

2.1 Growth-Inhibition and Cytotoxicity Assays

In order to evaluate cell proliferation (growth-inhibition ability), the sulforhodamine B (SRB) colorimetric assay was used, while the reduction of 3-(4,5-dimethylthiazol-2-2yl)-2,5-diphenyl tetrazolium bromide (MTT) was applied for determination of cellular viability (cytotoxic effect). p-coumaric acid and daidzein were tested at different concentrations and incubation times, for the human breast cancer cell lines MDA-MB-231 (estrogen-independent) and MCF-7 (estrogen-dependent), as well as for the non-cancerous human breast cells MCF-12A.

The experimental protocol followed to assess the compound´s cytotoxic and anti-proliferative activities is schematically represented in Figure 11.

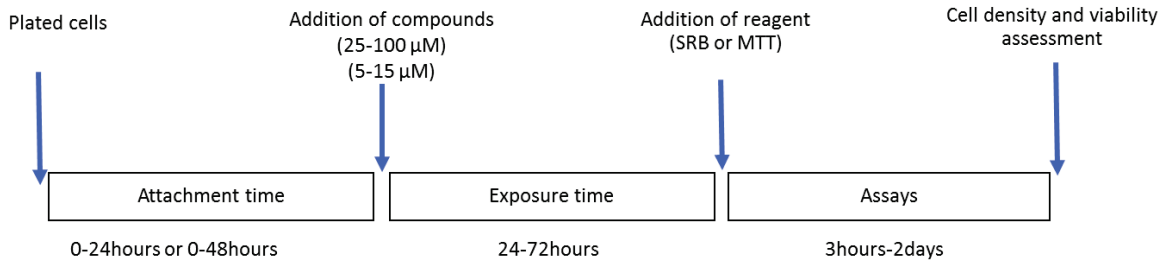

Figure 11 – Main experimental steps for the SRB and MTT assays.

For both methods, a 1 mL of a cellular suspension containing $5.79x10^4$ cells/mL, with a $3.0x10^4$cell/cm^2 density were transferred to 24-wells plates (area=1.93 cm^2). After 24 hours for MDA-MB-231 and MCF-7, and 48 hours for MCF-12A, the tested compounds were added (from stock solutions) in suitable volumes according to the desired final concentration: 5 to 10 µM for p-Ca and 25 to 100 µM for DA. The cell cultures were incubated at 37°C. After 24 hours of incubation, the cells were harvested and analysed (both in the controls and in the drug-treated cultures) every 24 hours, for a total of two days. The cell density and viability were determined by the SRB and MTT tests, respectively.

All assays were performed in triplicate (except for MCF12A which was carried out in duplicate), in three independent experiments. The results were compared with the control (untreated cells), considered for each independent experiment.

2.1.1. MTT Assay

The MTT test is based on the reduction of the tetrazolium salt by viable cells.

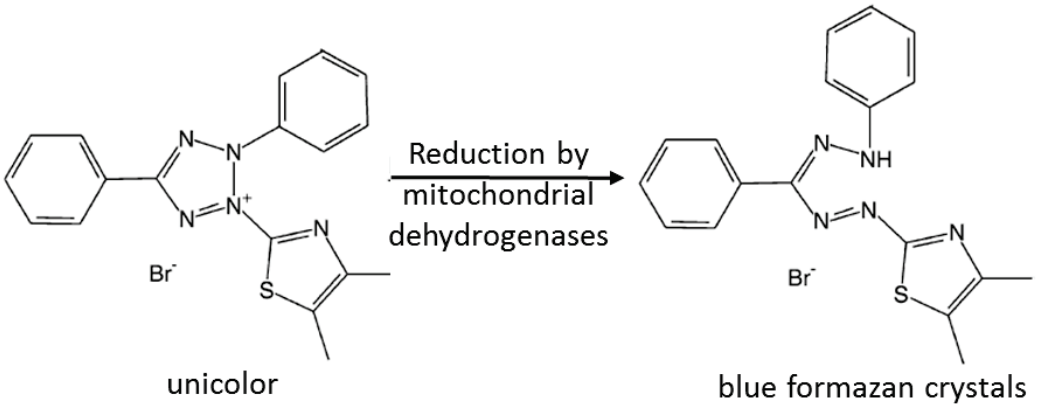

Figure 12 – Representation of MTT reduction to formazan crystals by mitochondrial dehydrogenases.

MTT is taken up *via* endocytosis by living cells and is reduced to blue formazan crystals (Figure 12) inside the cells by mitochondrial dehydrogenases [91]. This test allows to measure the enzymatic activity of viable cells [92], since only the metabolically active ones (*e.g.* viable) are able to reduce MTT. The amount of formazan formed is directly proportional to the cell´s viability [91] and can be measured spectrophotometrically (at a defined wavelength).

This method, however, presents some disadvantages: possible reduction of MTT by contaminants; the results depend on the cell´s metabolic state (*e.g.* mitochondrial dehydrogenases of apoptotic cells display an unchanged activity), which can lead to a underestimation of the effect of a compound. In view of these limitations, a second cytotoxicity assay is envisaged in a near future to complement the data already obtained.

2.1.2. Sulforhodamine B assay

The Sulforhodamine B (SRB) assay is a well-established colorimetric method for drug-toxicity screening in different types of cell lines (cancerous and non-cancerous, [93]).

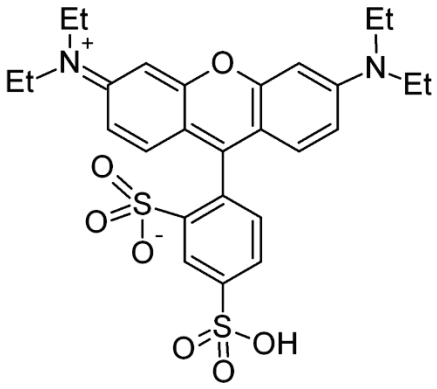

Figure 13 Chemical structure of Sulforhodamina B

Sulforhodamine B is a bright-pink aminoxanthene dye, comprising two sulfonic groups that can bind to basic amino acid residues of cellular proteins, under acidic conditions [93-95]. SRB dissociates in basic medium [93]. This colorimetric evaluation provides an estimate of the total protein mass, which is directly proportional to the cell density [93].

2.1.3. Transwell Migration Assay (Boyden Chamber Assay]

Cells, particularly cancer cells, have the ability to migrate and invade tissues that can be quite distant from the original tumour. The transwell migration assay allows to assess this migration for several cell types (*eg*. cancer cells, leukocytes). This method is based on two medium-containing cavities separated by a microporous membrane through which cell migration is monitored [96,97] (Figure 14).

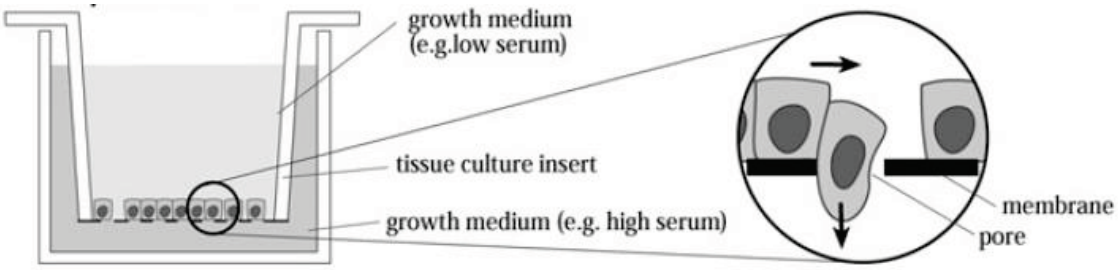

Figure 14 – Represention of the Transwell migration assay. (Adapted from [96])

Cells with the tested compound (and without FBS) were placed in the upper compartment of the chamber, and were allowed to migrate vertically through the membrane to the lower compartment, which was filled with FBS-supplemented culture medium (attachment medium) [96,97].

2.1.4. Evaluation of ROS/RNS by Fuorimetry

2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) is a non selective cell-permeable fluorogenic probe, which allows ROS and RNS detection [98]: DCFH-DA diffuses into the intracellular medium and is deacetylated by cellular esterases to non-fluorescent 2',7'-dichlorodihydrofluorescin (DCFH) which is quickly oxidized by the ROS/RNS present within the cell to the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF species) (Figure 15). The intensity of fluorescence measured is therefore proportional to the ROS level in the cytosol [98,99].

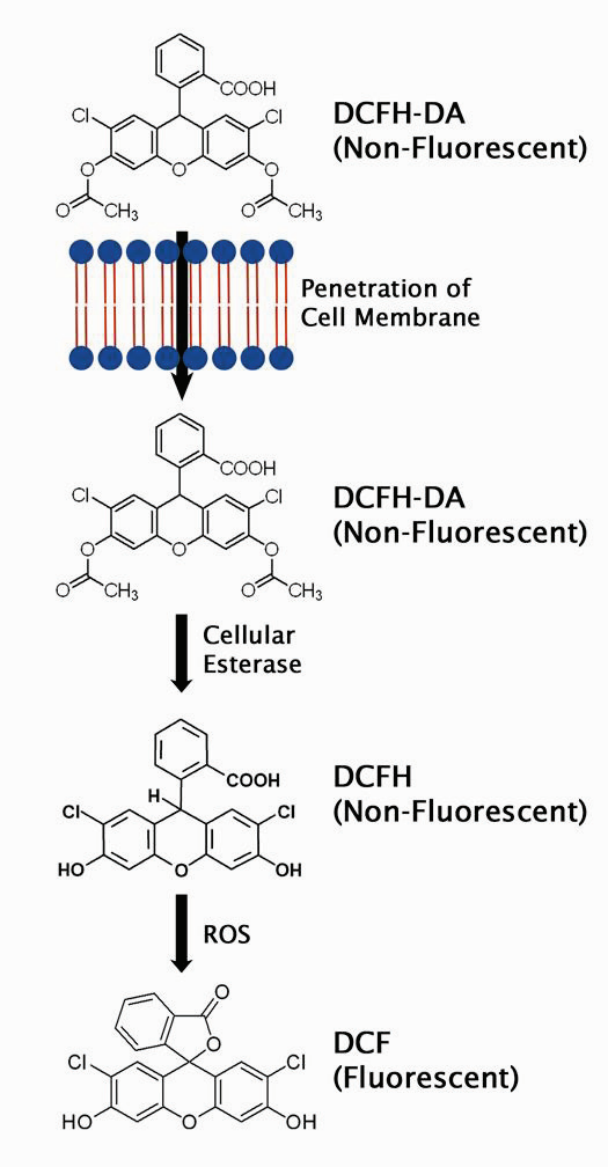

Figure 15 – Scheme of the ROS measurement assay based on the fluorogenic DCFH-DA probe. (From [99])

2.2. Raman Spectroscopy

In 1928, the Indian physicist, C.V. Raman, observed, in a home-made spectrometer, the phenomenon of inelastic scattering of light that became known as the *Raman effect* [100,101]. On account of this discovery, he was awarded the Nobel Prize in Physics in 1930 [100,102].

The *Raman effect* is therefore based on the interaction between an electromagnetic radiation and the molecules from the analysed sample [103], through to an inelastic process (involving energy transfer) [104,105], the shifts in energy of the scattered radiation from the incident frequency being a direct measure of the vibrational modes of the molecule [102,106]. This is a very weak effect, the Raman signals having a quite low intensity (*ca.* 5 to 9 orders of magnitude lower than the elastic Rayleigh band). Raman used the filtered sunlight as the incident radiation, and he needed several hours of irradiation to record a spectrum even for large volumes of sample [107].

In the 1960´s the Raman technique experienced a significant progress, due to the development of Lasers (*Light Amplification by the Stimulated Emission of Radiation*), by C.H. Townes and A.L. Schawlow, in 1958 [108]. In 1960, T.H. Maiman observed for the first time the lasing action [102] and verified that these monochromatic laser sources provide significant brightness and intensity to partially overcome the low efficiency of the Raman scattering process [102]. The Raman effect couples an induced polarization of the scattering molecules with the molecular vibrational modes, no ‘‘absorption’’ process being involved [102]. In a Raman spectra, that detects scattered light, the intensity of the signals depends on the change in molecular polarizability during the corresponding vibrational transitions [106].

When a molecule is irradiated with photons of frequency υ_0 and energy $h\upsilon_0$ an elastic process occurs, the diffused photons having the same energy as the incident ones [102] (*e.g.* no changes occurring in the incident photon frequency) - Rayleigh scattering. However, another (much weaker) process also takes place, the diffused photon having a different frequency from the incident one (either gain or loss - $\upsilon_0 \pm \Delta\upsilon$) due to the molecular vibrations within the sample [102,104,105] – Raman scattering. Two regions are then defined: the Stokes spectra interval, for $\upsilon_0 = \upsilon_0 - \Delta\upsilon$, and the anti-Stokes region for $\upsilon_0 = \upsilon_0 + \Delta\upsilon$ (Figure 16).

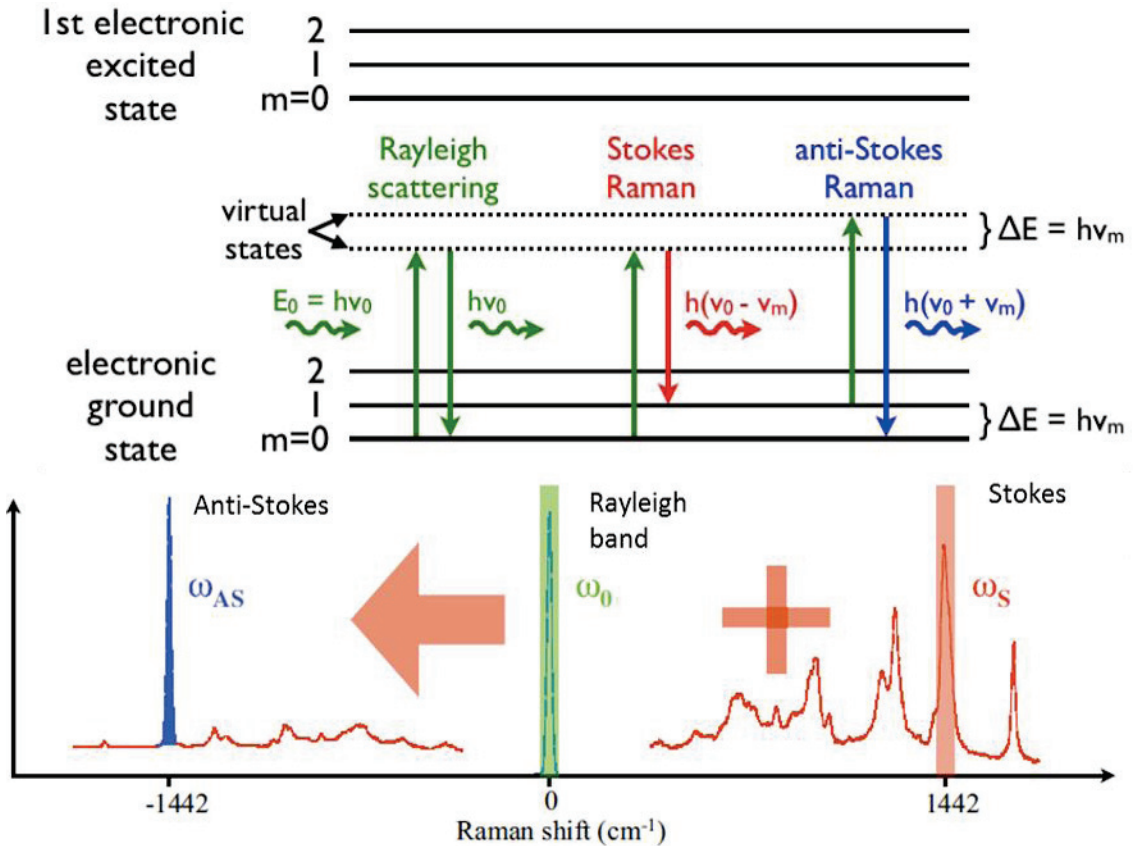

Figure 16 – Electronic-vibronic energy level diagrama, showing the Stokes and anti-Stokes Raman scattering and the Rayleigh elastic band. (Adapted from [106])

Hence, Raman spectroscopy measures the fundamental vibrations of a sample, which depend on the number of atoms (N) in the molecule (3N-5 for linear molecule and 3N-6 for a non-linear one), on its geometry/conformation and on the environmental conditions. The main vibrational modes are symmetric or asymmetric stretchings, deformations and torsions (Figure 17).

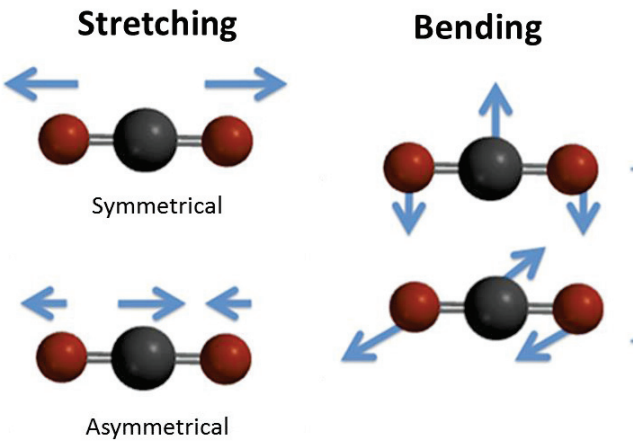

Figure 17 – Main vibrational modes of symmetric or asymmetric stretching, bending or deformation modes. (Adapted from [109])

The main components of a Raman spectrometer remain the same as those built by Sir Raman, but the performance of modern apparatus has greatly improved thanks to the development of high-quality lasers, modern charge-coupled device (CCD) detectors [101,102] and state-of-the-art computers with very high storage capacity. Nowadays, it is possible to obtain a good quality Raman spectrum from an extremely small amount of sample (μg or μl) in just a few seconds [101,107].

The interest in this technique has increased over the years, due to its numerous advantages and varied fields of application: it is non-invasive, non-destructive, can provide detailed spatial information and yield chemical images without the need of dyes or probes and with minimal sample preparation. Moreover, it is very suitable for the study of biological specimens, since water hardly interferes in the spectra [103,106,110]. Other significant advantage is the accurate information about cellular components [103,111], since this technique is extremely sensitive to the chemical composition of the sample (the Raman spectra represents an biochemical fingerprint of a molecule) [102,103,112,113]. Therefore, it allows to distinguish the smallest changes in a sample and can thus be used to monitor the composition, physical state and structure/conformation of a compound [102,105].

For biological samples, in particular, it is possible to achieve sub-cellular resolution and identify organelles and biomolecules, as well to obtain information about the chemical composition of cells under different conditions (*e.g.* pH, temperature, ionic strength, presence of drugs or xenobiotics) [100,101,105].

Nevertheless, the Raman effect is extremely week and can be masked by the sample´s autofluorescence [101,113]. In order to overcome these drawbacks, some techniques were developed, namely: (i) resonance Raman, involving the irradiation of the sample with a wavelength equal (or near) to the compound´s absorption maximum in the Visible region [100], allowing a magnification of the vibrational modes close to the chromophore moiety of the molecule; (ii) SERS (Surface-enhanced Raman Spectroscopy), by adding a metal colloid (*e.g.* of Au, Ag, Cu) to the sample, that will adsorb to it leading to an enhancement of the Raman bands from the oscillators that are closest to the metal surface [114].

Additionally, care should be taken regarding the use of high laser intensities (which would also increase the Raman bands to a certain degree), since this may cause local heating in the sample and possible damage. Also, it is possibly to minimize fluorescence by varying the wavelength of the exciting radiation (that should be different from the Visible absorption maximum of the sample) [101,115,116]. The choice of the most suitable excitation source is therefore very important, mainly when analysing biological specimens. At present, there is a wide range of lasers available with varied wavelengths (from the near-IR to the UV) namely 514, 532, 632, 647, 785 and 1064 nm [100,101]. Less energetic red laser sources such as He-Ne (633 nm) or diode lasers (785 nm) are also very helpful and widely applied in the study of biological samples [102,103,106,115].

In the last decade Raman spectroscopy has become a powerful and well-established analytical technique [100,103,104] , more and more applied for the study of cells and tissues [117-124]. Additionally, the technique has been shown to be of invaluable effectiveness in drug development [125,126], medical diagnosis (*e.g.* optical biopsies as opposed to conventional tissue removal for histological analysis) [100,127-131] and even for use in the operating theatre during surgical resection of tumours with a view to accurately distinguish cancerous from healthy tissue. Raman spectroscopy is also applied in areas such as microbiology [132-136], nanomedicine [61,112,137], forensic science [100,138,139], and archeometry and cultural heritage [140-144].

2.2.1. Raman microspectroscopy

The birth of Raman microspectroscopy dates from the early 1970´s, when Delhaye and Dhamelincourt, in the University of Lille (France), combined optical microscopy with Raman light scattering [115,116,145]. The further implementation (fifteen years later) of confocal Raman configurations by Puppels and coworkers [146-148] allowed to obtain data at a very high spatial resolution (cellular and sub-cellular), with a high signal-to-noise ratio and low underlying spectral backgrounds [145]. A Raman microspectrometer is configured with an optical microscope, a NIR laser and a CCD detector that promote the signal improvement, this combination reducing the acquisition time and the fluorescence interference [113] (Figure 18).

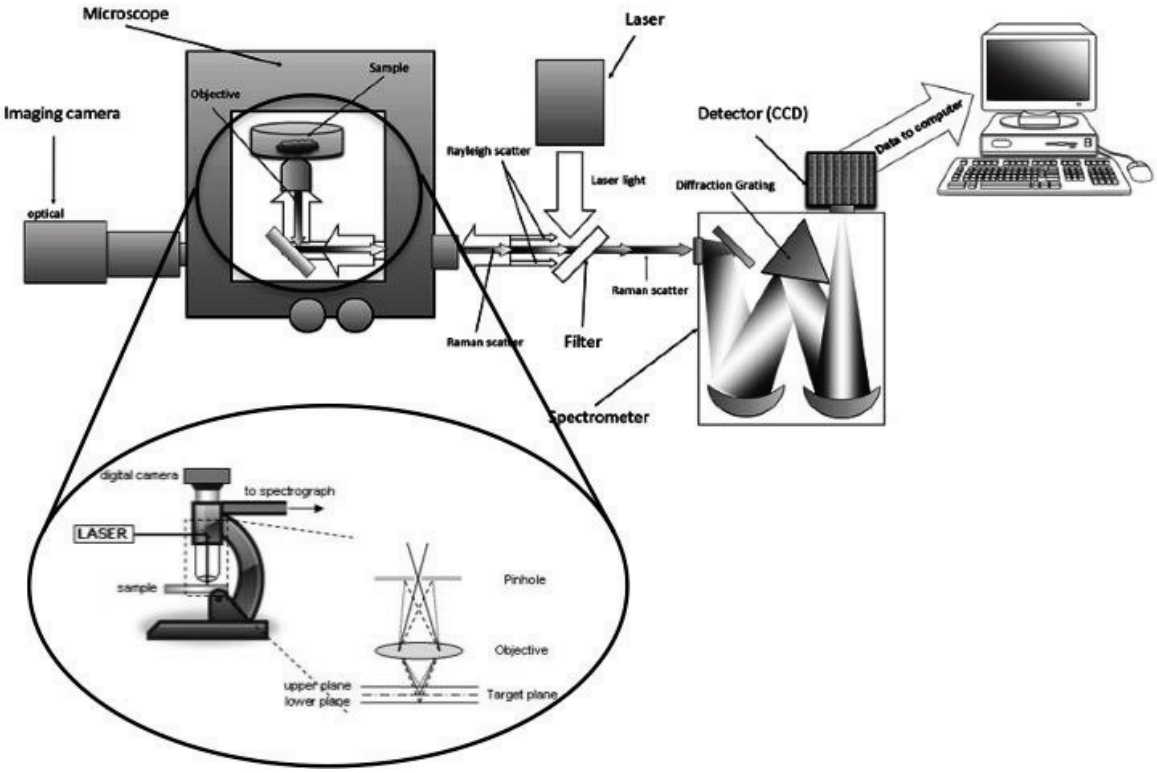

Figure 18 – Representation of a Raman microspectrometer. (Adapted from [103])

Raman microspectroscopy allows to analyse extremely small amounts of sample, even for inhomogeneous biospecimens, and yields detailed information on tissues and cell structure and composition with excellent quality and resolution [101]. Single cell analysis is also possible, yielding relevant data for understanding cellular process as well as the impact of exogenous compounds (such as chemoprevetive agents or drugs) on the cell´s biochemical profile. Actually, microRaman analysis is a label-free technique that

allows the direct observation of macromolecules inside a living cell without disturbing the system, and gives spatially resolved biochemical information [102,115,117,149-151]. For cells, the so-called spectral signature can be obtained, comprising bands corresponding to the different cellular components: nucleic acids, proteins, lipids, carbohydrates and other metabolites (Table 5). Furthermore, Raman microspectroscopy can be applied to either live or fixed cells, preserving cell integrity and function (provided the laser frequency and power are carefully chosen).

Table 5 – Principal vibrational modes assignments.

Assignment	wavenumber (cm^{-1})	Vibrational modes
DNA and RNA		
Adenine	729 1220-1284 1342 1420-1480 1578	
Cytosine	782 1245-1275	$\nu(C=C)_{sym\ breathing}$
Guanine	667 1320 1342 1420-1480 1578	
Thymine	667 746 1220-1284	
Uracil	1135 1235 1395	
Phosphates	788 811 828 1060-1095	$\nu(O\text{-}P\text{-}O)_{sym}$ $\nu(O\text{-}P\text{-}O)_{RNA}$ $\nu(O\text{-}P\text{-}O)_{asym}$ (B-DNA) $\nu(PO_2^-)$
Carbon skeleton	914-925 1420-1480	$\nu(C\text{-}C)$ $\nu(C\text{-}O)_{Ribose\text{-}Phosphate}$ $\delta(CH_2)$, $\delta(CH)$
Proteins		
Phenylalanine	621 1005 1033 1209 1607	twist (C-C) $\nu(C=C)_{sym\ breathing}$ $\delta(C\text{-}H)_{ip}$ $\nu(C\text{-}C_6H_5)$ $\nu(C=C)$
Tyrosine	645 828, 854 1176	$\nu(C\text{-}C)$ $\nu(C=C)_{sym\ breathing}$ $\delta(C\text{-}H)$

	1617	ν(C=C)
Tryptophan	875-880 1553	H bonds/indol ring ν(C=C)
Amide I	 1650-1660 1670-1680	ν(C=O) α-helix β-sheet
Amide III	 1250 1264 1267	ν(C=O) + δ(CNH) β-sheet Random α-helix
	937 980 1106-1120 1158 1384 1420-1480	ν(C-C) α-helix ν(C-C) β-sheet ν(C-N) ν(C-C), ν(C-N) δ(CH_3) δ(CH_2/CH)
Lipids		
	717 891, 908 1080 1060-1095 1259 1301 1384 1420-1480 1650-1660 1736	ν($CN^+(CH_3)_3$) δ(CH_2) ν(PO_2^-)$_{sym}$ ν(C-C) ν(PO_2^-)$_{asym}$ twist CH_2 δ(CH_3) δ(CH_2), δ(CH) ν(C=C) ν(C=O)
Polysaccharides		
	877 1025,1047,1155 1060-1095 1384 1420-1480	C-O-H $_{ring}$ ν(C-O)$_{glycogen}$ ν(C-O), ν(C-C) δ(CH_3) δ(CH_2/CH)
Glycose	937	C-O-H

3. Experimental

3.1. Reagents, Materials and Equipment

3.1.1. Materials and Equipment

The compounds and reagents used along this project are described in Table 6.

Table 6 – List of compounds and reagents used along the present work.

Product	Laboratory
Fetal bovin serum (FBS)	Invitrogen (Barcelona, Espanha)
Penicillin-Streptomycin Antibody (10 units/ml Pen and 10 µg/ml Strep)	
Tris (hydroxymethyl) aminomethane or Tris	Pational Diagnostic (Hessle Hull, United Kingdom)
Acetic acid, glacial	Sigma-Aldrich (Sintra, Portugal)
3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium (MTT ≥97.5%)	
2',7'-Dichlorofluorescin diacetate (DCFH-DA ≥97%)	
Dimethyl sulfoxide (DMSO ≥99.9 %)	
p-coumaric acid (p-CA ≥98,0%)	
Sodium bicarbonate (NaHCO3)	
Sodium phosphate buffer pH 7.4 (PBS)	
Sulforhodamine B (SRB)	
Trypan Blue Solution (0.4%)	
Trypsin	
Multiwell plates (24, 48 and 96 plates) and culture flasks T 75 cm2	Orange Scientific (Braine- l'Alleud, Bélgica)
Daidzein (97%)	Alfa Aesar (Lancashire, United Kingdom)

The equipment used in this project is described in Table 7.

Table 7 – List of equipment used with mark and model

Equipment	Mark	Model
Analytical balance	Mettler	Toledo AB54
Centrifuge with cooling	MPW	MPW-350R
Culture chamber with vertical laminar flow (flow rate : 1050m3/h)	BioWizard	BioWizard - 100
Incubator	Sheldon Mfg. Inc	3517-2 Waterjacket CO2

PH meter	Sargent-Welch	IP
Shaker " Vortex "	IKA	MS2 Minishaker
Visible/UV spectrophotometer (with Gen5 software)	BioTek	µQuant MQX200
Water purification apparatus Milli -Q	TKA	GenPure

3.1.2. Solutions

The compounds under study were solubilized in DMSO (due to low water solubility), in the concentration range 5 to 100 µM. The final concentration of DMSO in the culture medium did not exceed 0.1% (*v/v*). A 0.01% DMSO solution was always considered as a control.

All solutions were prepared and filtered before use, and were stored in the dark to avoid photodegradation of the phenolic compounds. Trypan blue was used in 0.04% (*v/v*) solution in PBS. MTT (3-(4,5-dimethylthiazol-2-2yl)-2,5-diphenyl tetrazolium bromide) was prepared in PBS solution, in a concentration of 0.5mg/ml.

Initially, high concentrations of *para*-coumaric acid were tested (100 to 1600 µM) but in these conditions p-Ca showed to be cytotoxic, most probably due to the pro-oxidant effect known for these phenolic acids at very high dosages, that can interfere with relevant secondary metabolic pathways.

3.2. Biological System

3.2.1. Cell culture

The human cell lines choosen for this work were MCF-7, MDA-MB-231 and MCF-12A (Figure 19). MCF-7 has estrogen (ER+) and EGF receptors and is uninvasive, whereas MDA-MB-231 is a more aggressive, hormone-independent (ER-) breast cancer [152]. MCF-12A is non-tumour immortalised cell line of breast tissue.

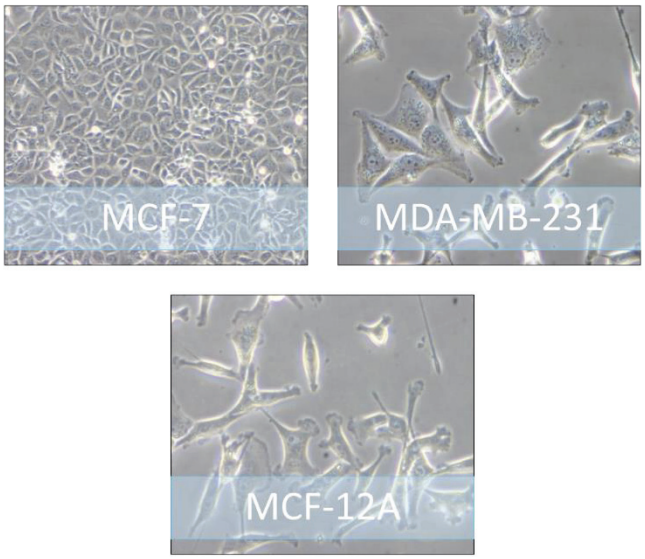

Figure 19 – MCF-7 (breast cancer cell line estrogen dependent), MDA-MB-231 (breast cancer cell line hormone-independent] and MCF-12A (non-tumour immortalized cell line of breast tissue) cell lines. Images obtained in a microscopy with 100x magnification (MCF-7) and 1000x magnification (MDA-MB-231 and MCF-12A).

The MDA-MB-231 and MCF-7 cells were cultured in monolayer, in Dulbecco's modified Eagle's high glucose (4500mg/L) medium (DMEM-HG), supplemented with10% (*v/v*] heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. MCF-12A]was also cultured in monolayer, in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, supplemented with hydrocortisone, human epidermal growth factor (EGF), bovine insulin and 5% heat-inactivated horse serum with 1% penicillin-streptomycin. All cells line were incubated at 37ºC under 85% relativity humidity and 5% CO_2. The FBS and horse serum were previously subject to thermal inactivation at 56ºC, for 30 minutes (to inactivate the complement and reduce the cytotoxicity caused by immunoglobulin's).

The cell subcultures were prepared in 75 cm^2 flasks, according to their characteristics of growth. The cell lines were subcultured twice a week, using a dissociation medium containing 0.05% trypsin-EDTA. All experiences were performed in cell cultures with less than 50 passages.

The tested compounds were added during the cells´ exponential grow phase.

3.3. Methods

3.3.1. MTT assay

The MTT solution (0.5 mg/ml) was prepared in PBS and filtered for sterilization and removal of insoluble residues. To determine cellular viability (for the different cell lines MDA-MB-231, MCF-7 and MCF-12A), the medium was removed and the cells were washed with 500 µl of PBS. MTT solution (250 µL) was then added, the plates were incubated at 37ºC for 3 hours (always light protected) and the MTT solution was discarded. The newly formed formazan crystals were dissolved in DMSO, yielding a purple colour solution, for which the absorbance was read (in each well) at 570 nm (in a multiwell plate reader). Cellular viability was determined according to the equation:

$$\% \text{ Cellular viability} = \frac{A_{sample}}{A_{control} - A_{blank}} \times 100 \qquad (1)$$

where A_{sample} is the absorption of solutions exposed to the compound tested (after subtracted of the absorption of the blank (DMSO)); $A_{control}$ represents the absorption of the control samples (upon blank subtraction).

The results are presented as a percentage relative to the control (always taken as 100%).

3.3.2. SRB assay

In order to perform this assay, incubation with the phytochemical (either p-CA or DA) was ended every 24 hours during two days, the cells were washed with 500 µl of PBS and 500 µl of water Mili-Q, after they were fixed with 1%-acetic acid in methanol and frozen overnight. Upon thawing and drying, 250 µl SRB in 1%-acetic acid was added to each well, followed by incubation at 37ºC for 1h, after which the cells were then carefully washed with 1%-acetic acid and dried. 1 ml of 10mM-Tris (ph 10) was then added to dissolve the protein-bound SRB, and absorbance was read at 540nm (in a multiwell plate spectrophotometer).

The percentage of proliferation or cellular growth was calculated by:

$$\% \text{ Cell grow} = \frac{A_{\text{sample}} - A_{0\text{ day}}}{A_{\text{control}} - A_{0\text{ day}}} \times 100 \qquad (2)$$

where: A_{sample} represents the absorption of solutions exposed to the tested compound, after subtraction of the absorption for the blank (Tris 10mM); $A_{0\text{ day}}$ is the absorption of the control at t=0 h (corresponding to the cellular density without the compound), after blank subtraction.

The results are shown as a percentage relative to the control (always taken as 100%).

3.3.3. Transwell Migration Assay (Boyden Chamber Assay]

This assay was carried out in plates of 24 wells containing the inserts, each of which was carefully coated with 0.1 ml-Matrigel diluted in a 0.01M-Tris (pH 8.0)/0.7%-NaCl solution (coating solution) (this procedure was performed on ice). The plate was then incubated at 37ºC for 2 hours, after which 0.5 ml of cell suspension and compounds to be tested are added (at a $5x10^4$ cells/ml density) into the insert and 0.75 ml of attachment medium was add to the chamber. Invasion chambers are then incubated at defined time points. Upon these periods inserts are scrub to remove the non-migrating cells, and the migrated cells are fixed with 4%-formalin and stained with 0.1%-crystal violet. In order to quantify the invading cells, microscopic images were captured in different points and the cells were counted (in triplicate). The percentage of migrating cells were calculated according to Equation 3:

$$\% \text{ Migration} = \frac{\text{mean number of cells insert with study compound}}{\text{mean number of cells insert control}} \times 100 \qquad (3)$$

The results are represented as a percentage of the control (taken as 100%).

3.3.4 ROS/RNS Measurement

In order to perform this assay, incubation with the phytochemicals (either p-CA or DA) was ended after 48 hours, the cells were washed twice with PBS (2x500 µl), and 150 µl of DCFH-DA (prepared in FBS-free culture medium) were added, after which the cells were incubated for 30 minutes. After removing solution, the cells were washed with PBS, trypsinized, resuspended with PBS and centrifuged. Upon discarding the supernatant the cells were ressuspended in PBS (250 µl) and fluorescence was measured 533nm (emission) with an excitation radiation at 480 nm. The percentage of free radical oxygen and nitrogen species presented was calculated according to:

$$\% \text{ ROS/RNS} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \qquad (4)$$

where A_{sample} is the absorption of the samples exposed to the compound tested, and $A_{control}$ represents the absorption of the control samples.

The results are presented as a percentage relative to the control (always taken as 100%).

3.3.5. Raman Microspectroscopy

Preparation of the Cell Samples

The cells (MDA-MB-231 and MCF-7) were transfected at a $3.0x10^4$cell/cm^2 density, in 12-well plates, each well containing the optical substrate (MgF_2 disks). After 24 hours to allow cell attachment to the substrate, p-Ca and DA were added, according with the desired final concentrations (5 to 10 µM and 25 to 100 µM). Upon a 48 hour incubation (at 37°C in a 5% CO_2 atmosphere), the cells were washed twice with 1ml PBS, fixed with a 4% formalin solution for 20 minutes, and washed again (2x) with 1ml H_2O. These samples were kept in the fridge (*ca.* 5 ^oC, to avoid fungal contamination) with PBS prior to the Raman analysis. Apart from the treated-cell samples, a control (without the compounds) was prepared.

Spectra Acquisition

The Raman experiments were performed at the Department of Physical-Chemistry of the University of Malaga (Spain).

The spectra were obtained at room temperature, in a Renishaw Reflex inVia Raman microspectrometer coupled to a RenCam CCD (Charge Coupled Device) detector. The 785 nm line from a diode laser was used as the exciting radiation, yielding approximately 55 mW (solid p-Ca and DA) and 0.11 mW (cells) at the sample position. A spectral window of 0 to 2000cm^{-1} was used for the pure phenolic compounds, while for the cells the spectra were recorded between 500 and 1800 cm^{-1}. In both cases, a 100x magnification was used (Leica lens, NA=0.9, fd=0.27mm), and the data was recorded with an integration time of 10 s and 5 scans.

Mapping experiments were also carried out, although these results are not presented in this work.

3.3.6. Statistical Analysis

All experiments were performed in triplicate. The results are expressed as the mean±SEM. Statistical treatment was performed using the One-Way ANOVA analysis of variance, followed by post hoc test Turkey's and Dunnett's multi-comparison tests for the SRB and MTT results, respectively. The differences were considered significant for $^{*}p<0,05$, $^{**}p<0,01$, and $^{***}p<0,001$.

The MicroRaman spectra were analyzed with the programs OriginPro 8 and MathLab R 2014b. Interpretation of these results was carried out upon multivariate analysis (unsupervised PCA), after baseline correction, spectral mean vector normalisation and smoothing (Savitzky–Golay, 11 points, first polynomial), followed by detraining (2^{nd} or 4^{th} polynomial [153]).

4. Results and Discussion

4.1. Evaluation of the Metabolic Impact of the Dietary Polyphenols in Human Breast Cancer Cell Lines

Breast cancer is the second most lethal cancer among women, which justifies the growing research in this field over the years, particularly regarding new agents, natural or synthetic, with chemopreventive and antitumour properties. Cytotoxicity screening of these compounds, in particular, gives important information for selecting the most promising ones for further studies, while Raman spectroscopy experiments provide knowledge on their impact on the cellular components by yielding the chemical profile of a suitable cell model in the absence and presence of the investigated agents.

The present study focuses on the effect of a hydroxycinnamic acid (*para*-Coumaric acid) and an isoflavone (daidzein) on a human breast cancer cell line. Previous studies have suggested that these compounds have a significant role in reducing several types of cancer, namely SW620, HT 29 (colon cancer cells), Caco-2 (colonic cell line) and also T-47D (epithelial ductal breast cancer) [42,65,66,72,154,155]. However, for polyphenols the majority of the reported studies refer to plant extracts and thus do not evaluate the effect of p-Ca alone [156-158], its impact on cells being unknown to this date.

4.1.1. Structural Features of p-Coumaric Acid and Daidzein

Despite the very similar structures of *p*-coumaric (p-Ca) and caffeic (CA) acids, that share a common polyphenolic structure, their Raman spectra reflects the different ring substitution pattern – CA being a dihydroxylated compound as compared to the monohydroxylated p-Ca (Figure 20).

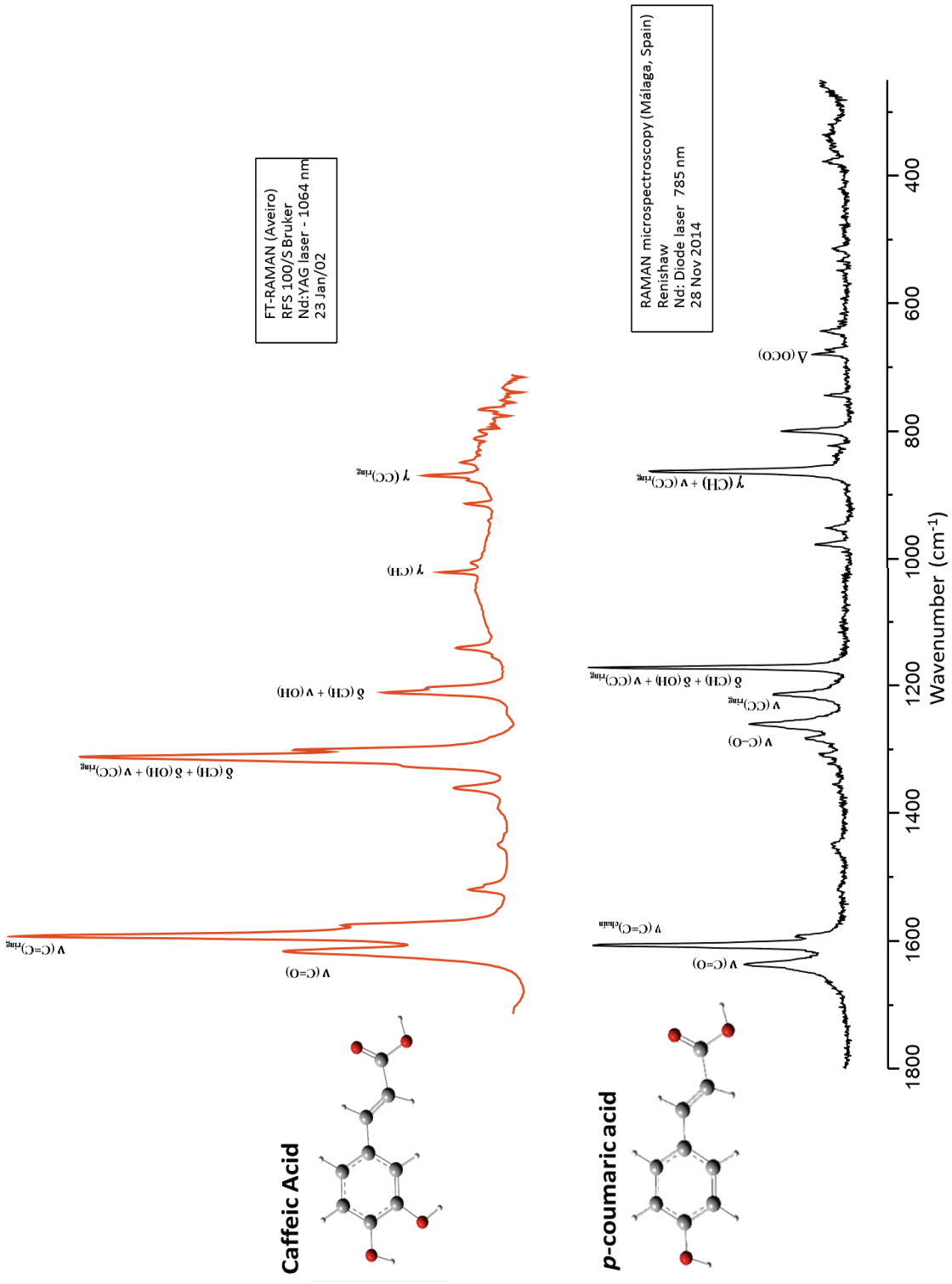

Figure 20 – Raman microspectroscopy and FTIR experimental spectra and optimised structures for caffeic (from [159]) and *para*-coumaric acids.

In this type of systems, any structural change can lead to a significant variation in activity and cell selectivity (even leading to inefficiency). The number and position of the OH groups in the aromatic ring, for instance, are determinant of antioxidant activity, as well as the double bond in the ring pendant arm [160]. Compounds with a double bond in the carboxylic acid moiety were shown to have an increased chemopreventive effect at low concentrations, as compared to their saturated analogues. Furthermore, the biological activity of this kind of systems is strongly concentration-dependent: the existing studies evidence a preventive effect for low concentrations, while suggesting a reverse outcome (pro-oxidant] for higher dosages.

The health-beneficial properties of isoflavones rely in the distinct substitution patterns of their chromone core. Although the chromone moiety seems to have an essential role for biological activity, other structural parameters are determinant such as the nature, number and position of the different substituent groups in rings A, B and C [59] (Figure 21).

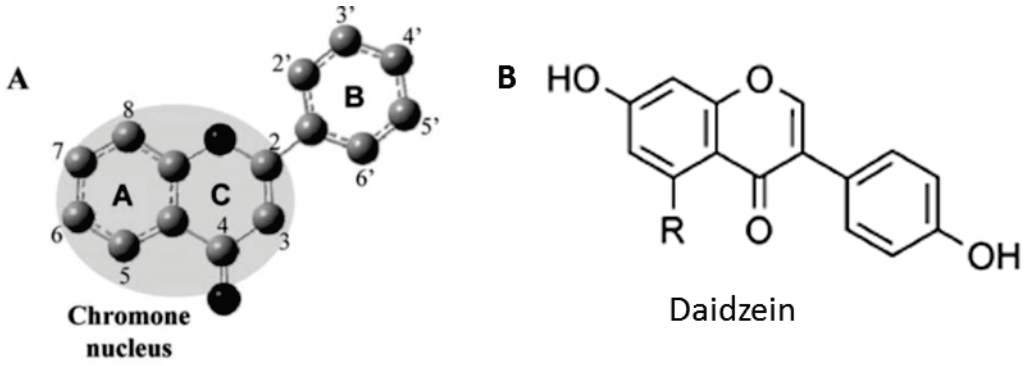

Figure 21 – A: Basic chromone structures (the atom numbering and ring labeling is included). B: Daidzein compound in study (Adapted from [59])

Additionally, these affect water solubility which is an important parameter in the use of isoflavones as chemopreventive agents *in vivo* [90]. Furthermore, the capacity of these compounds to conjugate with proteins, enzymes and diverse biological receptors within a biological matrix (cell or tissue) is strongly dependent on their three-dimensional conformation and electronic distribution. In general, the areas of highest electronegativity within an isoflavone are at the 5-hydroxyl and 7-hydroxyl positions of the A-ring, as well as at 3´ and 4´ hydroxylation sites on the B- ring (**Erro! A origem da referência não foi encontrada.**) [59].

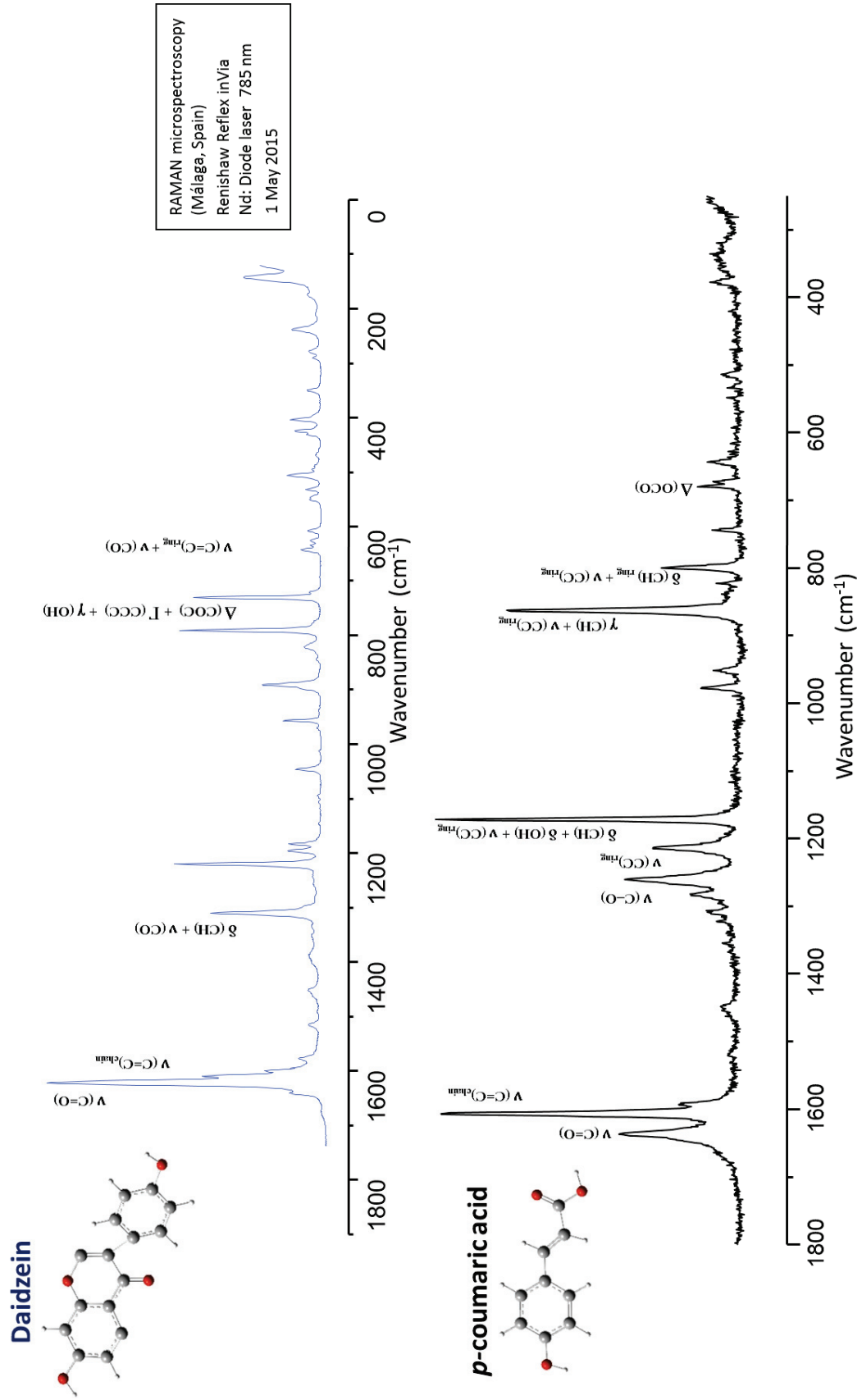

Figure 22 – Raman experimental spectra and optimised strutures for daidzein and p-coumaric acid (from [161]).

4.1.2. Evaluation of the Cytotoxic and Cell Growth-Inhibition Activities of p-Coumaric Acid and Daidzein

Cytotoxicity evaluation is a conventional method used in research and development of new compounds aimed as chemopreventive and/or antineoplastic agents.

The cytotoxicity effects of 4-*trans*-hydroxicinnamic acid (p-Ca) and 4',7-dihydroxyisoflavone (daidzein) were evaluated towards human breast cancer cells, both estrogen independent (MDA-MB-231) and estrogen dependent (MCF-7), using the MTT reduction assay (describe in 3.3.1. section) for viability measurement, and the SRB colorimetric test for cell density quantification. Comparison with a human breast non-neoplastic immortal cell line (MCF12A) was also carried out.

4.1.2.1. *p*-Coumaric Acid

p-Ca was tested at low (5 to 15 μM) and high (25 to 100 μM) concentrations, for incubation times of 24, 48 and 72 h (Figure 23). The data for 72 h is not presented, as it showed not to be relevant since it is identical to the data gathered at 24 hours. For the MCF-7 cells, at both 24 and 48 hours, the higher concentrations of p-coumaric acid seem to induce a slightly protective effect (potentiating cell growth and viability), although this effect was not found to be statistically significant. For the MDA-MB-231 cell line, this protective effect was more marked, leading to an increase in cell viability at 48 hours of 87% at 50 μM and 102% at 100 μM.

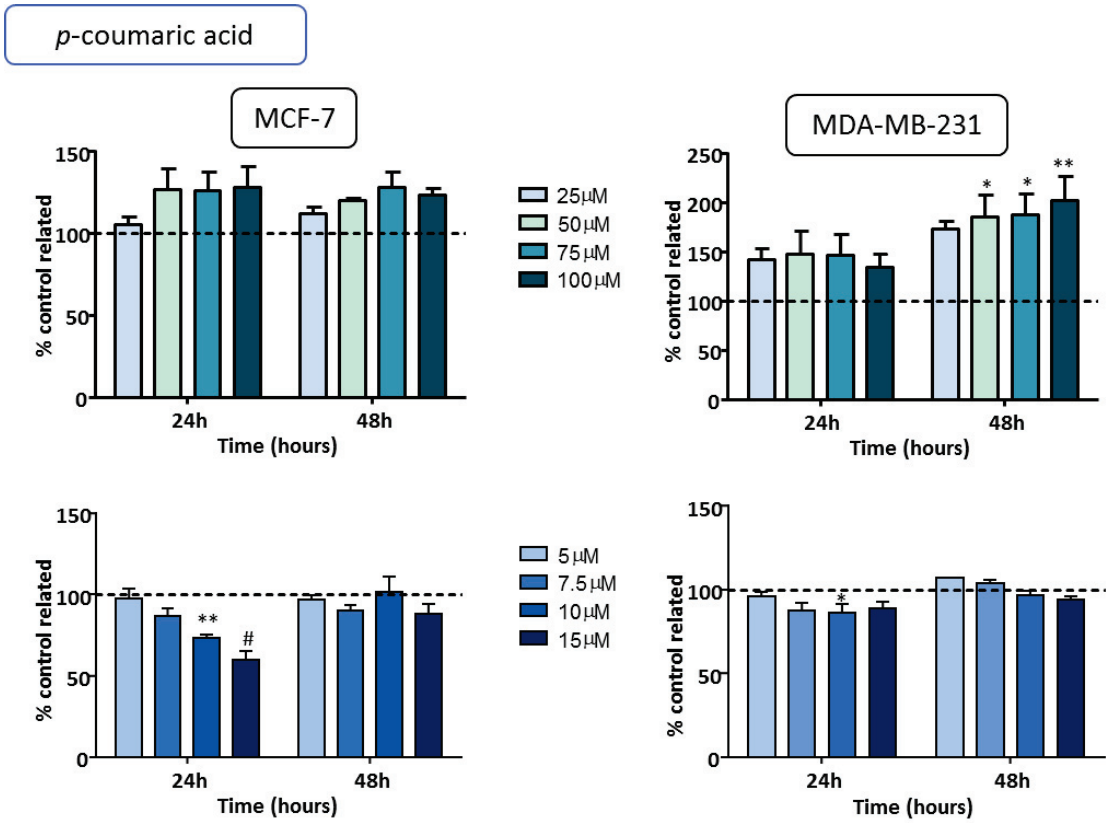

Figure 23 – Cytotoxic effect of p-coumaric acid against breast cancer cells (MCF-7 and MDA-MB-231), at 24 and 48 hours incubation times. The results, obtained by the MTT assay (3.3.1. section), are presented as a percentage of the DMSO control (cell treated with the DMSO vehicle) considered as 100% (horizontal dashed line). The data are the mean ±SEM of values obtained for 3 independent experiments initiated and treated in parallel (n= 3). Cells were plated at a density of 3.0x10^4 cell/cm^2. The results were statistically analysed in GraphPad Prism 6.00, applying the One-way ANOVA algorithm with Dunnett's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control cultures.

The MTT assay evaluates cell viability by the mitochondria ability to reduce formazan (as described in section 3.3.1.), although this method does not directly measure mitochondrial damage. Since oxidative stress conditions can affect mitochondria, it is possible that at the higher concentrations tested p-Ca may lead to an increase in ROS levels, which can affect mitochondria desidrogenases rendering them dysfunctional or allowing the cell to escape from apoptosis. Posadino and collegues (2013) [162] suggested that p-Ca can have different effects depending on its concentration: an antioxidant effect at low concentrations and a cytotoxic one at higher dosages. Based on the knowledge that the ROS level is higher in cancer relative to healthy cells [163], the present results suggest that p-Ca can have a pro-oxidant effect on breast adenocarcinoma cells, increasing ROS

levels and promoting an enhanced cell viability. This is in accordance with reported work [84,162], that unveiled a relationship between the presence of an unsaturated pendant chain in polyphenolic acids and a cell viability increase, namely for p-Ca at high concentrations.

Regarding the lower concentrations (5 to 15 µM), p-coumaric acid was found to exert different effects towards the MCF-7 cells, depending on the incubation period: a cytotoxic effect was observed at 24 hours, particularly for 15 µM with a 50% viability reduction, while at 48 hours no significant differences were detected relative to the control. For the MDA-MB-231 cell line, in turn, p-Ca displayed a protective capacity at 48 hours only for 5 and 7.5 µM concentrations.

In both concentration ranges, p-Ca seems to be cell line selective, since the effects on MCF-7 are less pronounced (or even inexistent) compared to those on the estrogen-independent cell line MDA-MB-231. This selectivity regarding the cell model has been previously observed for other types of compounds [164,165].

The data obtained from the SRB colorimetric (Figure 24) test did not reveal statistically significant differences relative to the MTT results, for all concentrations tested and the two cell lines under study. However, a decrease in the intracellular protein density was measured for MCF-7 at 48 hours incubation with p-Ca, from 7.5 to 15 µM and 75 and 100 µM (Figure 24). Furthermore, a blocking of protein synthesis appears to occur for the lowest concentration range

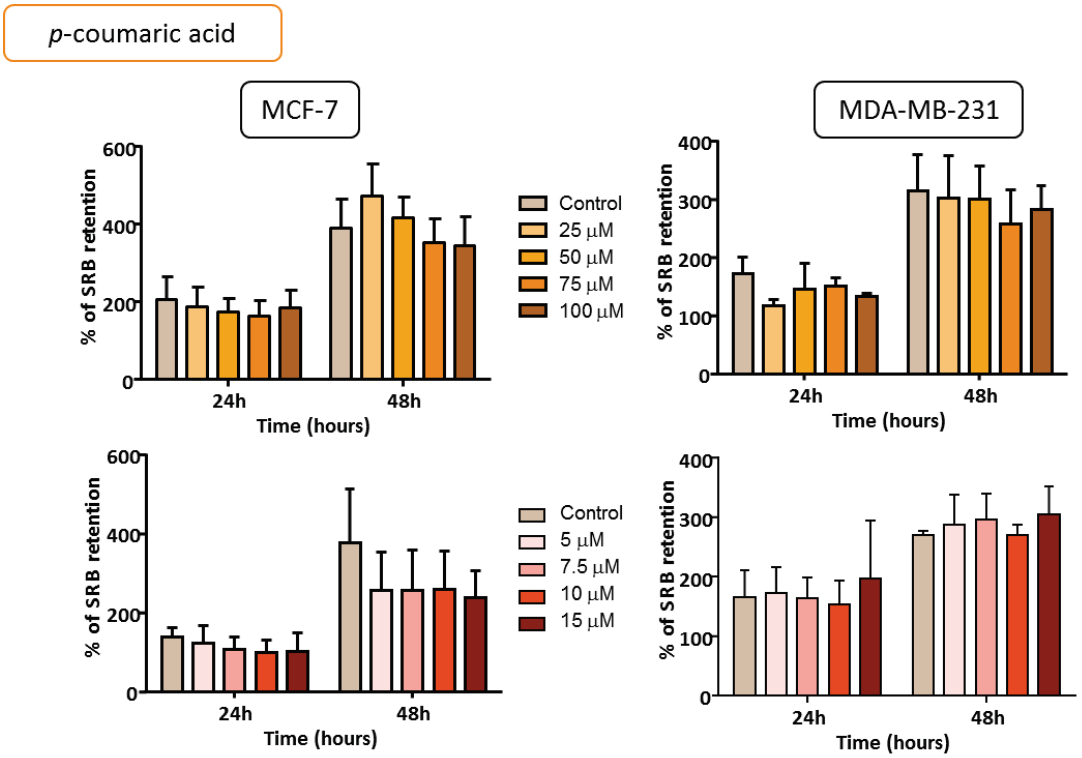

Figure 24 – Cytotoxic effect of p-coumaric acid against breast cancer cells (MCF-7 and MDA-MB-231), at 24 and 48 hours incubation times. The results, obtained by the SRB assay (3.3.2. section], are presented as a percentage of the DMSO control (cell treated with the DMSO vehicle) considered as 100% (horizontal dashed line]. The data are the mean ±SEM of values obtained for 3 independent experiments initiated and treated in parallel (n= 3). Cells were plated at a density of $3.0x10^4$ cell/cm^2. The results were statistically analysed in GraphPad Prism 6.00, applying the One-way ANOVA algorithm with Turkey's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control cultures.

Comparing the SRB and MTT data, it can be hypothesised that p-Ca improves cell viability without interfering with the cellular protein density in MDA-MB-231 cells, while in MCF-7 the protein density stabilises for the low concentration range, suggesting that the phenolic acid may interfere with protein synthesis.

4.1.2.2. Daidzein

Daidzein was found to exert a cytotoxic effect in both breast cancer cell lines (MCF-7 and MDA-MB-231) for incubation periods well below 24 hours (Figure 25): while for MDA-MB-231 the viability is affected only at 75 and 100 µM (*ca.* 50%), a DA-induced viability decrease was detected for the MCF-7 cells at concentrations 50, 75 and 100 µM (with a 42% viability reduction for the highest concentration).

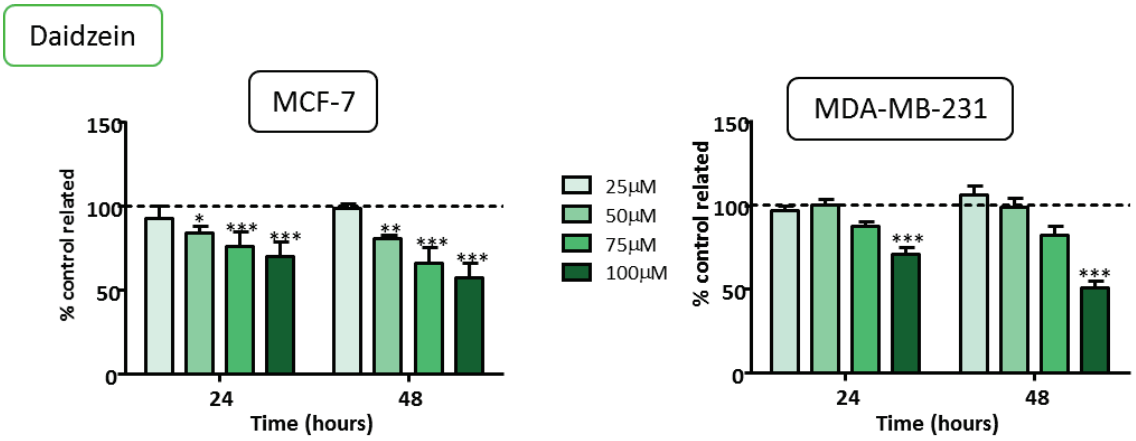

Figure 25 – Cytotoxic effect of daidzein against breast cancer cells (MCF-7 and MDA-MB-231), at 24 and 48 hours incubation times. The results, obtained by the MTT assay (3.3.1. section), are presented as a percentage of the DMSO control (cell treated with the DMSO vehicle) considered as 100% (horizontal dashed line). The data are the mean ±SEM of values obtained for 3 independent experiments initiated and treated in parallel (n= 3). Cells were plated at a density of $3.0x10^4$ cell/cm^2. The results were statistically analysed in GraphPad Prism 6.00, applying the One-way ANOVA algorithm with Dunnett's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control cultures.

These results, reflecting a more significant effect towards the estrogen-dependent MCF-7 cell line versus the estrogen-independent MDA-MB-231 were foreseen, since the isoflavone daidzein displays a structure similar to estrogen (Figure 26) (thus being expected to interact effectively with estrogen receptors therefore inhibiting estrogen synthesis and gene expression [59,65,155].

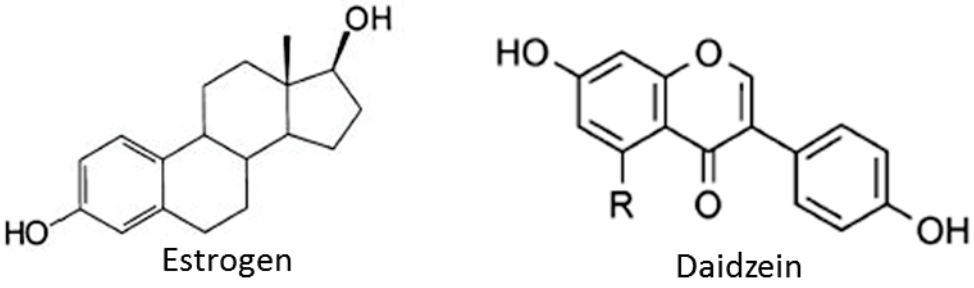

Figure 26 – Chemical struture of estrogen and daidzein.

In MDA-MB-231 cells, which lack estrogen receptors, DA may interfere with other metabolic pathways, probably oxidative-related ones, and exert an antioxidant effect.

The SRB results (Figure 27) for DA did not show any significant changes in protein density for either of the two tested cell lines. Nevertheless, a very slight decrease

measured for 50 and 100 µM dosages may suggest that the isoflavone is somewhat interfering with protein synthesis (as previously observed for p-Ca).

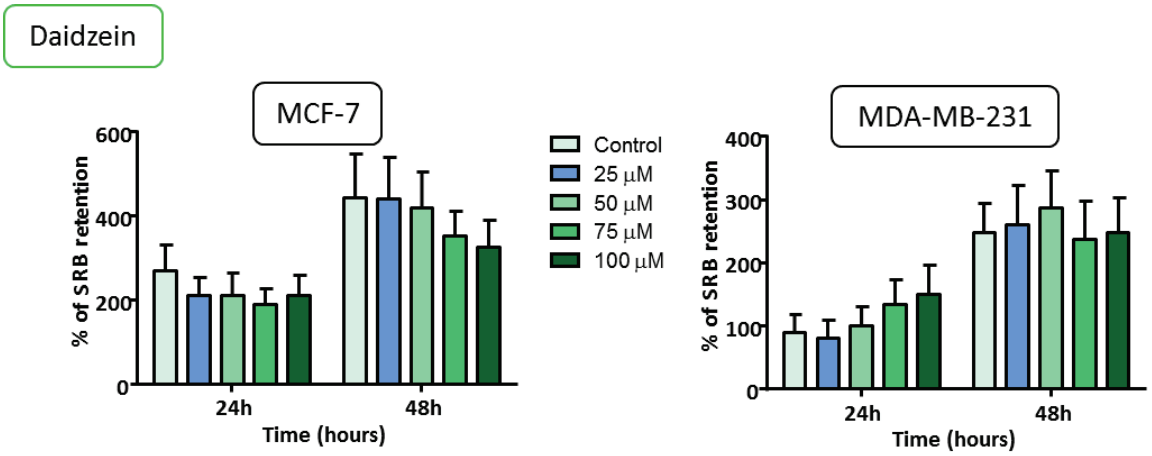

Figure 27 – Cytotoxic effect of daidzein against breast cancer cells (MCF-7 and MDA-MB-231), at 24 and 48 hours incubation times. The results, obtained by the SRB assay (3.3.2. section), are presented as a percentage of the DMSO control (cell treated with the DMSO vehicle) considered as 100% (horizontal dashed line). The data are the mean ±SEM of values obtained for 3 independent experiments initiated and treated in parallel (n= 3). Cells were plated at a density of $3.0x10^4$ cell/cm^2. The results were statistically analysed in GraphPad Prism 6.00, applying the One-way ANOVA algorithm with Turkey's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control cultures.

Both the MTT and SRB results clearly show that DA affects cell viability and may interfere with the cellular protein content. Hence, it is suggested that the biological effect exerted by these dietary compounds is cell- and concentration-dependent (in accordance with the literature).

Assays were also carried out for the non-tumour human breast cell line (MCF-12A), for chosen concentrations of the phytochemical compounds (according to the previous data on the neoplastic cells): 7.5 and 15 µM for p-Ca, and 50 and 100 µM for DA. A protective effect was verified (by the MTT assay) for DA-50 µM at 24 h, as well as for p-Ca-7.5 µM at 48 hours (Figure 28) (higher concentrations was not study for this cell line, although, it was considered to do in future).

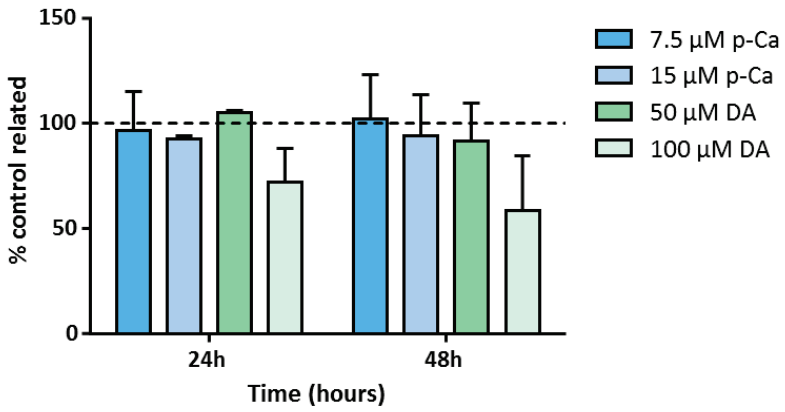

Figure 28 – Cytotoxic effect of p-coumaric acid and daidzein against non-tumour breast cells (MCF-12A), at 24 and 48 hours incubation times. The results, obtained by the MTT assay (3.3.1. section), are presented as a percentage of the DMSO control (cell treated with the DMSO vehicle) considered as 100% (horizontal dashed line). The data are the mean ±SEM of values obtained for 2 independent experiments initiated and treated in parallel (n= 2). Cells were plated at a density of $3.0x10^4$ cell/cm^2. The results were statistically analysed in GraphPad Prism 6.00, applying the One-way ANOVA algorithm with Dunnett's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 vs control cultures

The SRB results obtained for these non-neoplastic cells confirmed the MTT data, no statistically significant differences relative to the control having been found (Figure 29). No cytotoxic effect against healthy cells was observed for these compounds, in the investigated concentration range. For some of the conditions tested, a chemoprotective activity can be inferred. This is a relevant result, attending to the daily consumption of these compounds through the diet.

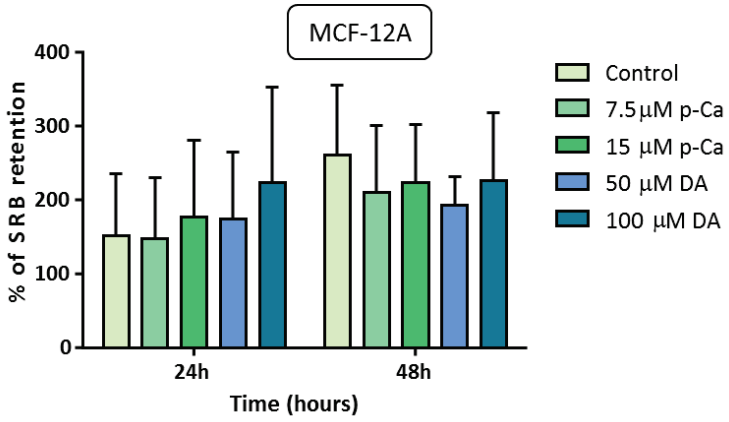

Figure 29 – Cytotoxic effect of p-coumaric acid and daidzein against non-tumour breast cells (MCF-12A), at 24 and 48 hours incubation times. The results, obtained by the SRB assay (3.3.2. section), are presented as a percentage of the DMSO control (cell treated with the DMSO vehicle) considered as 100% (horizontal dashed line). The data are the mean ±SEM of values obtained

for 2 independent experiments initiated and treated in parallel (n= 2). Cells were plated at a density of $3.0x10^4$ cell/cm^2. The results were statistically analysed in GraphPad Prism 6.00, applying the One-way ANOVA algorithm with Turkey's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control cultures.

4.1.3. Assessment of Cell Migration Inhibition

Transwell migration experiments (Boyden Chamber Assay) were performed, allowing to evaluate the cells´ migration and invasiveness abilities (in a biological matrix, to tissues other than the one containing the primary tumour) as a function of concentration, for a 72 h incubation period with either p-Ca or DA. This assay was only considered for the MDA-MB-231 cell line, as MCF-7 does not have an invasive potential. The results thus obtained evidence that DA at 100 µM reduces cell migration by 40% at, while p-Ca has 20% and 30% efficiencies at 7.5 µM and 15 µM, respectively (Figure 30).

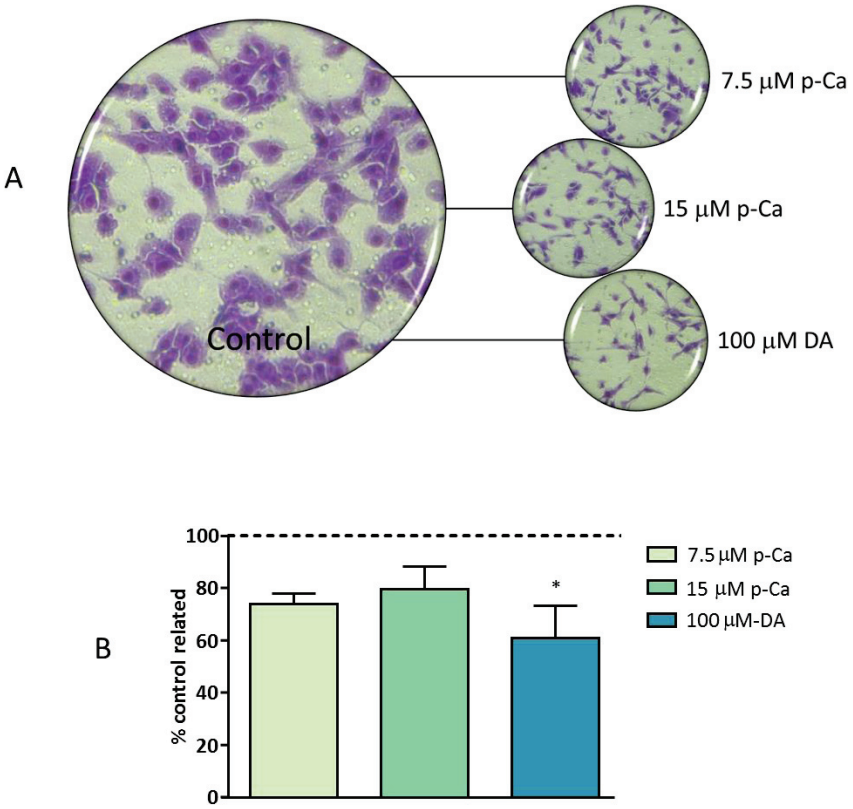

Figure 30 – Inhibition of cell migration by p-coumaric acid and daidzein, in the human invasive breast cancer cells MDA-MB-231, at 72 hours, by Transwell Migration Assay (Boyden Chamber Assay) (in section 3.3.3.). The results are present in percentage related with control cultures (cell treated with the compound vehicle, DMSO), considered 100% (horizontal dashed line). Every cultures were plated with $3.0x10^4$ cell/cm^2 density. The results for each condition, the values present are the mean ± SEM of values obtain for five different culture (triples) initiated and treated in parallel (n= 5). The data were statistic treat in GraphPad Prism 6.00, applying One-way ANOVA with Dunnett's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 vs control cultures.

Matrix metalloproteinases (MMPs) are zinc-dependent proteins associated to cell migration and metastasis formation, which are activated by an increase in the ROS level [31,32]. p-coumaric acid, with well recognised antioxidant properties, is proposed to act against cell migration through scavenging of oxidative species, thus inhibiting activation of MMPs. In fact, p-Ca was found to have an inhibitory effect in ROS-induced endothelial cell migration and angiogenesis [69]. Daidzein, in turn, was found along this work to affect cell viability, which can have consequences in cell migration. Moreover, DA is known to downregulate MMP-2 [42], which can also lead to a reduced cell migration.

4.1.4. ROS/RNS Measurement

Evaluation of the ROS/RNS levels present in the intracellular medium was performed for both the MCF-7 and MDA-MB-231 cells, after 48 hours exposure to the tested compounds.

The preliminary results currently obtained show clear differences among these cells lines, for both p-Ca and DA: MCF-7 displays an ROS/RNS increase for p-Ca at 7.5, 15 and 50 μM and DA at 100 μM, while for MDA-MB-231 the amount of free radicals only increases significantly for p-Ca-50 μM (Figure 31).

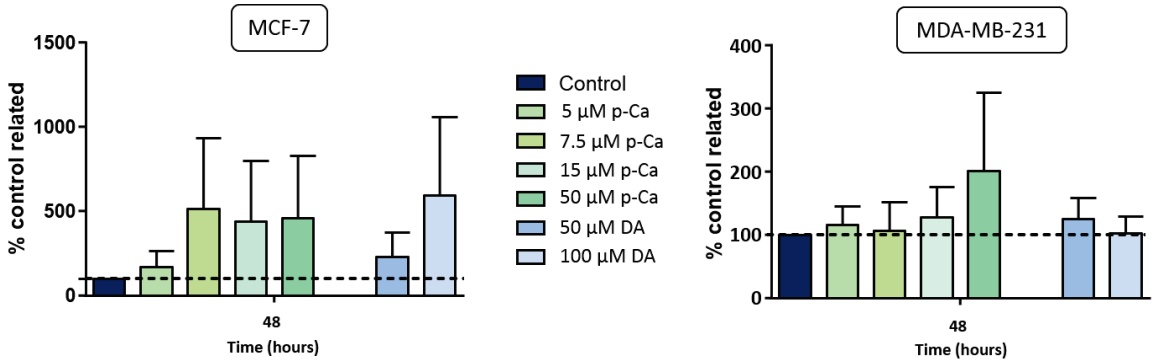

Figure 31 – ROS/RNS measurement in the MCF-7 and MDA-MB-231 cell lines ($3.0x10^4$ cell/cm^2), after incubation with p-coumaric acid and daidzein, using the fluorigenic probe DCFH-DA (see section 3.3.4.). The results are represented as a percentage relative to the control (untreated cells) considered as 100% (horizontal dashed line), and are the mean±SEM of the values obtained for three independent measurements, carried out in triplicate (n= 3). The data were statistically treated in GraphPad Prism 6.00, applying One-way ANOVA with Dunnett's test for multi-comparisons: *p < 0.05, **p < 0.01, ***p < 0.001 *vs* control cultures.

Overall, the MCF-7 samples containing p-Ca displayed much higher ROS/RNS levels than the estrogen-independent cells MDA-MB-231 incubated with the same phenol. Thus, p-coumaric seems to behave as a very cell-selective compound, with a pro-oxidant effect in the concentration range 7.5-50 μM for MCF-7, and only for high dosages (*ca.* 100 μM) for MDA-MB-231.

Daidzein evidences a similar cell-selective effect, ROS/RNS levels clearly increasing with isoflavone concentration for the estrogen-dependent line (MCF-7), while for MDA-MB-231 they are virtually unchanged by exposure to this compound [98]

4.1.5. Metabolic Impact of p-Coumaric Acid and Daidzein Evaluated by Raman Microspectroscopy

Raman microspectroscopy experiments were carried out, in order to assess the metabolic impact of the dietary polyphenols p-coumaric acid and daidzein in human breast cancer cell lines (MDA-MB-231 and MCF-7). Particular conditions were chosen, in the light of the previous assays (mainly MTT results), regarding concentration and incubation times: 50 and 100 μM, for 48 hours incubations.

Interpretation of the data was carried out after unsupervised Principal Component Analysis (PCA) of the spectra (vector normalised and mean centred).

Knowing that the Raman spectrum of a cell represents its chemical fingerprint, particular attention was paid to the bands from the main cellular components: Protein – Amide I at 1660 cm^{-1} and Amide III at 1250 cm^{-1}; Lipids – CH/CH_2 deformation modes around 1450 cm^{-1}; DNA and RNA – phosphate stretching at 1172 cm^{-1}, backbone modes at 950 to 810 cm^{-1}, and purine and pyrimidine residues at 800 to 650 cm^{-1}; phenylalanine at 1000 cm^{-1}.

4.1.5.1. *p*-coumaric acid

For MDA-MB-231, assessment of the spectra of p-Ca-treated cells against the control did not yielded a clear differentiation between either concentration or cell type (Figure 31), suggesting that this compound does not interfere significantly with particular cellular components. Actually, the results obtained by the MTT and SRB assays showed that this phenolic acid, at high concentrations (50 and 100 μM), increases cell viability without affecting the cell´s protein content. This behaviour can be related with a pro-oxidant effect in the cancer cells.

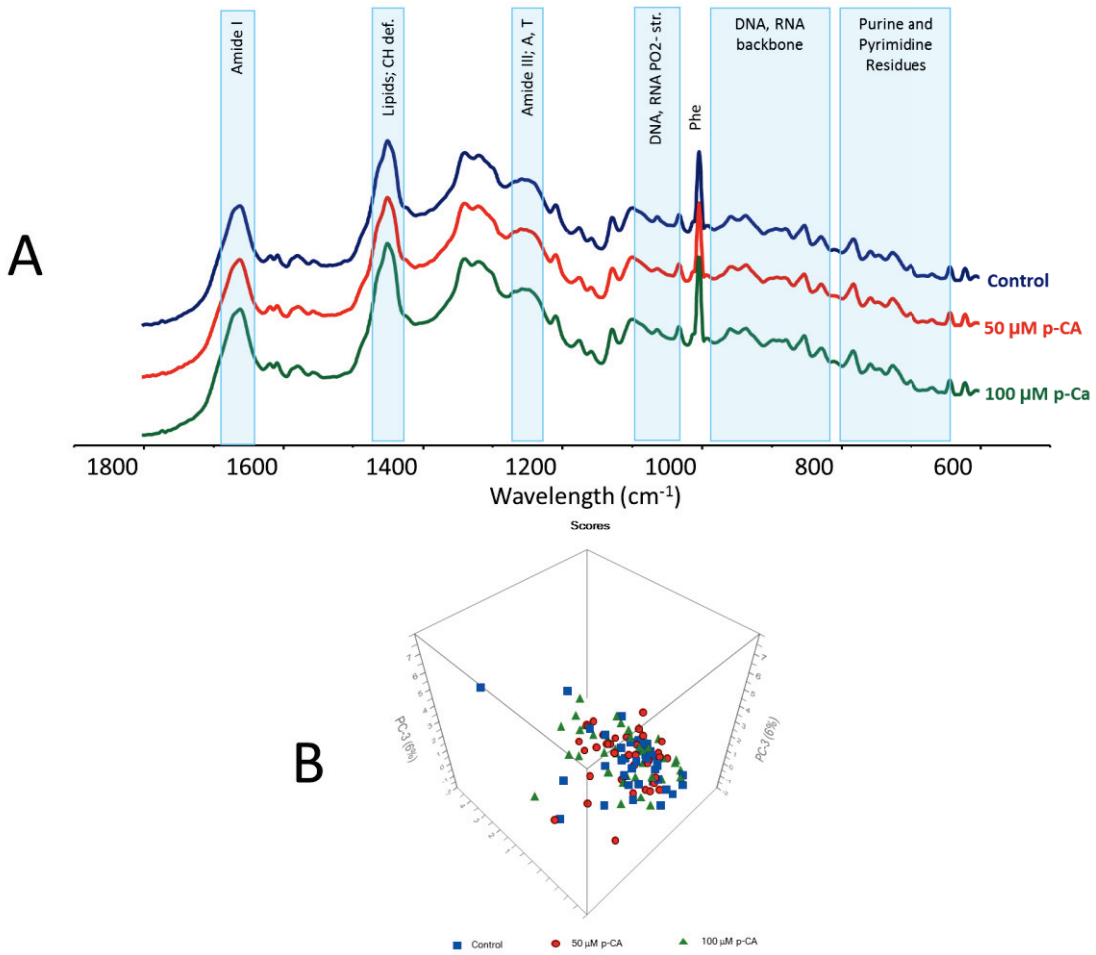

Figure 32 – Average Raman spectra (A) and PCA score plots (B) for p-Ca-treated MDA-MB-231 cells *vs* control (50 and 100 µM, 48 hours exposure).

The Raman data and corresponding PCA analysis for the MDA-MB-231 cells incubated (for 48 hours) with p-Ca-7.5 and 15 µM are comprised in Figure 33, and reflect a quite different behaviour. The PC1 score plot carries enough variance to distinguish the sample group from the control, while the PC2 score plot differentiates between p-Ca concentrations (Figure 33 (C)). A close PC1 inspection reveals a more significant impact on proteins (Amides I and III at 1660 and 1250 cm^{-1}), DNA/RNA (phosphate stretch at 1170 cm^{-1}) and phenylalanine (1000 cm^{-1}). In turn, PC2 exposes interference on lipids (1450 cm^{-1}) and DNA/RNA (1170 and 750-700 cm^{-1}) (Figure 33 (C)).

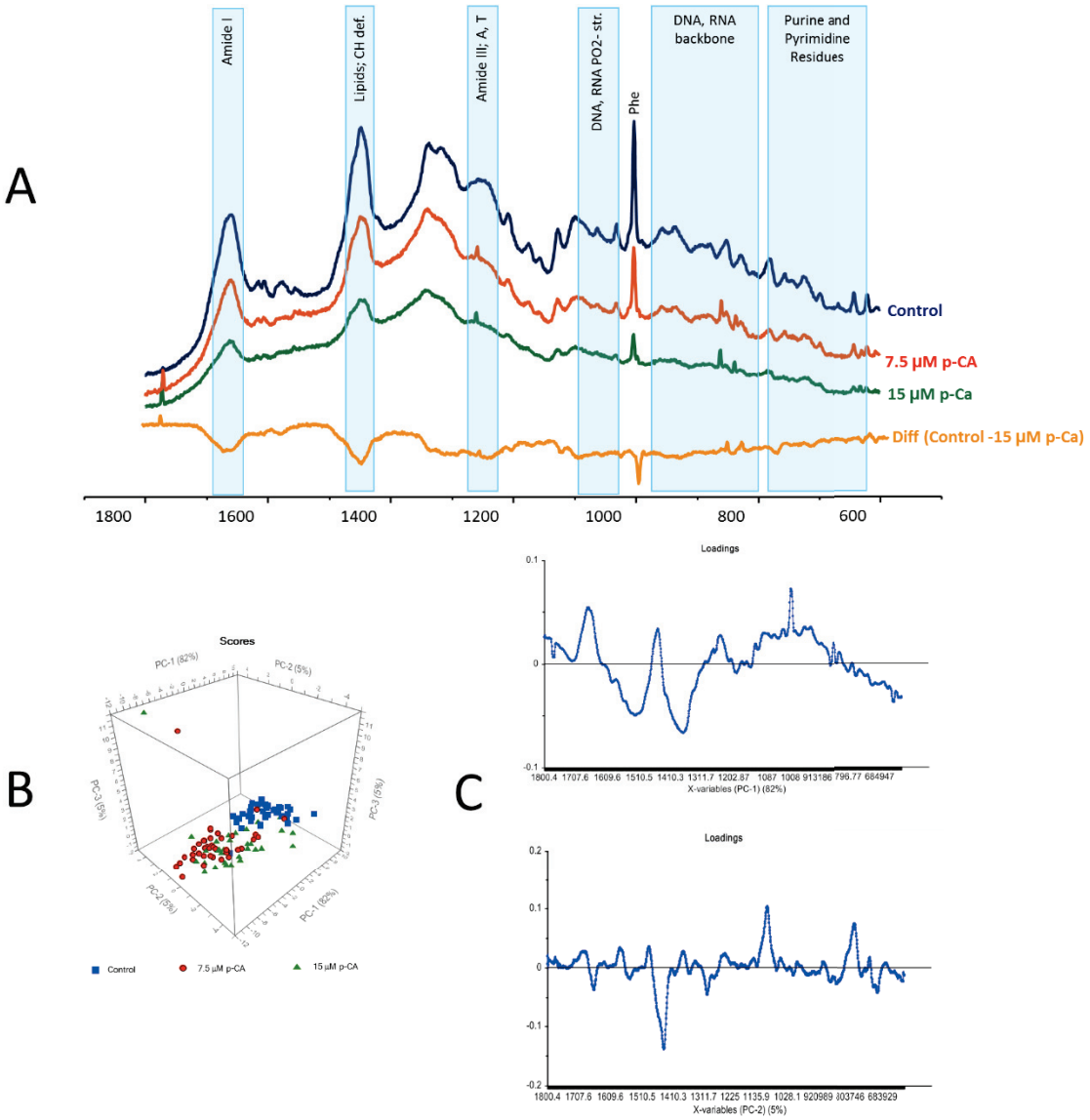

Figure 33 – Average Raman and Difference spectra (A), and PCA score (B) and loading (C) plots for p-Ca-treated MDA-MB-231 cells *vs* control (7.5 and 15 µM, 48 hours exposure).

These microRaman results evidence an interference of p-Ca preferentially with proteins, lipids and DNA/RNA. Since the MTT results previously discussed for this compound (section 4.1.2.1.) unveiled a chemoprotective effect (at low concentrations, below 15 µM), it is presently suggested that p-coumaric acid interacts with lipids within the cell, stabilising membranes and shielding against cell damage, in agreement with published studies on this phenol´s capacity to reduce lipid peroxidation and avoid altered membrane fluidity and permeability [166-168]. The compounds nitrosamine inhibitory and ROS scavenging abilities [169-171] may also add to this chemoprotective capacity.

Additionally, the effects on DNA, RNA and phenylalanine are related to changes in

cell proliferation [101] and protein synthesis, in accordance with previous works showing that p-Ca can delay cell proliferation by downregulating genes associated with the cell cycle [66,172].

In turn, the cytotoxic effect of p-Ca at 15 µM, determined by the MTT assay as a small one, appears to be significant since the PCA analysis of the microRaman data yiels a clear differentiation from the lower concentration and the control (Figure 33). Actually, for 15 µM p-Ca can have an opposite effect to the one detected for 7.5 µM, destabilising lipid structure and therefore changing membrane fluidity and permeability, which was found for another HCAs [64].

Furthermore, p-Ca´s impact on proteins (particularly Amide I band) can be related to its effect on cell migration (section 4.1.3.) which is associated to activation of particular proteins.

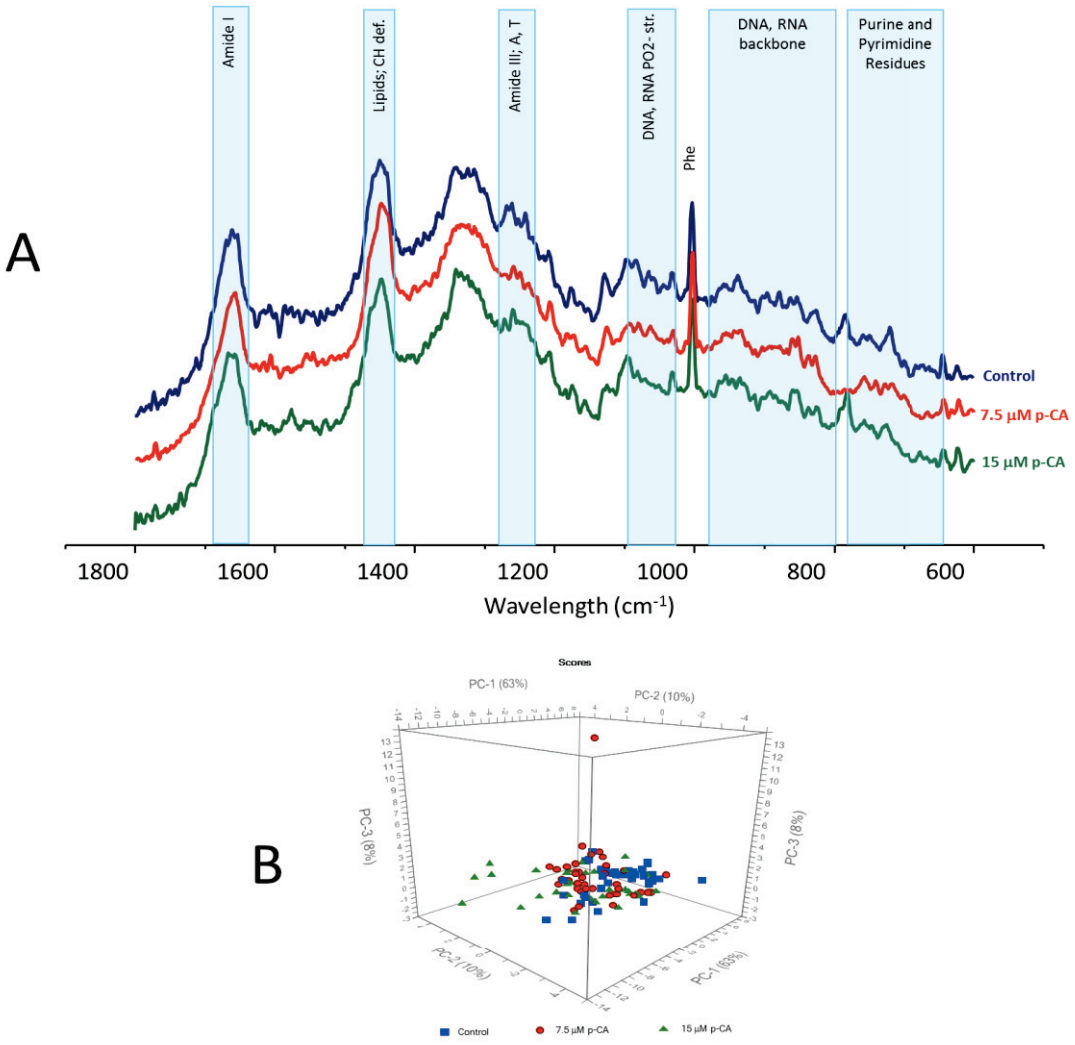

Figure 34 – Average Raman spectra (A) and PCA score plots (B) for p-Ca-treated MCF-7 cells *vs* control (7.5 and 15 µM, 48 hours exposure).

Figure 34 comprises the microRaman spectra of MCF-7 cells upon exposure (48 hours) to p-Ca-7.5 and 15 μM. PCA analysis of the data did not contain sufficient variance to discriminate between the different samples. Since the activity of phenolic acids is closely related to their hydrophylic *vs* hydrophobic properties, it is possible that p-Ca could not enter the MCF-7 cells in sufficient amounts, thus justifying the negligible effect currently observed in the Raman spectra (in agreement with the MTT and SRB results). In fact, the cellular uptake of p-Ca can be related to the glycolipids present in cell membrane, and since MDA-MB-231 and MCF-7 cell lines have different glycolipid composition (*e.g.* neutral glycolipids and gangliosides [152] this fact may be responsible for the cell selectivity reflected in the experimental results (MDA-MB-231 *vs* MCF-7).

4.1.5.2. Daidzein

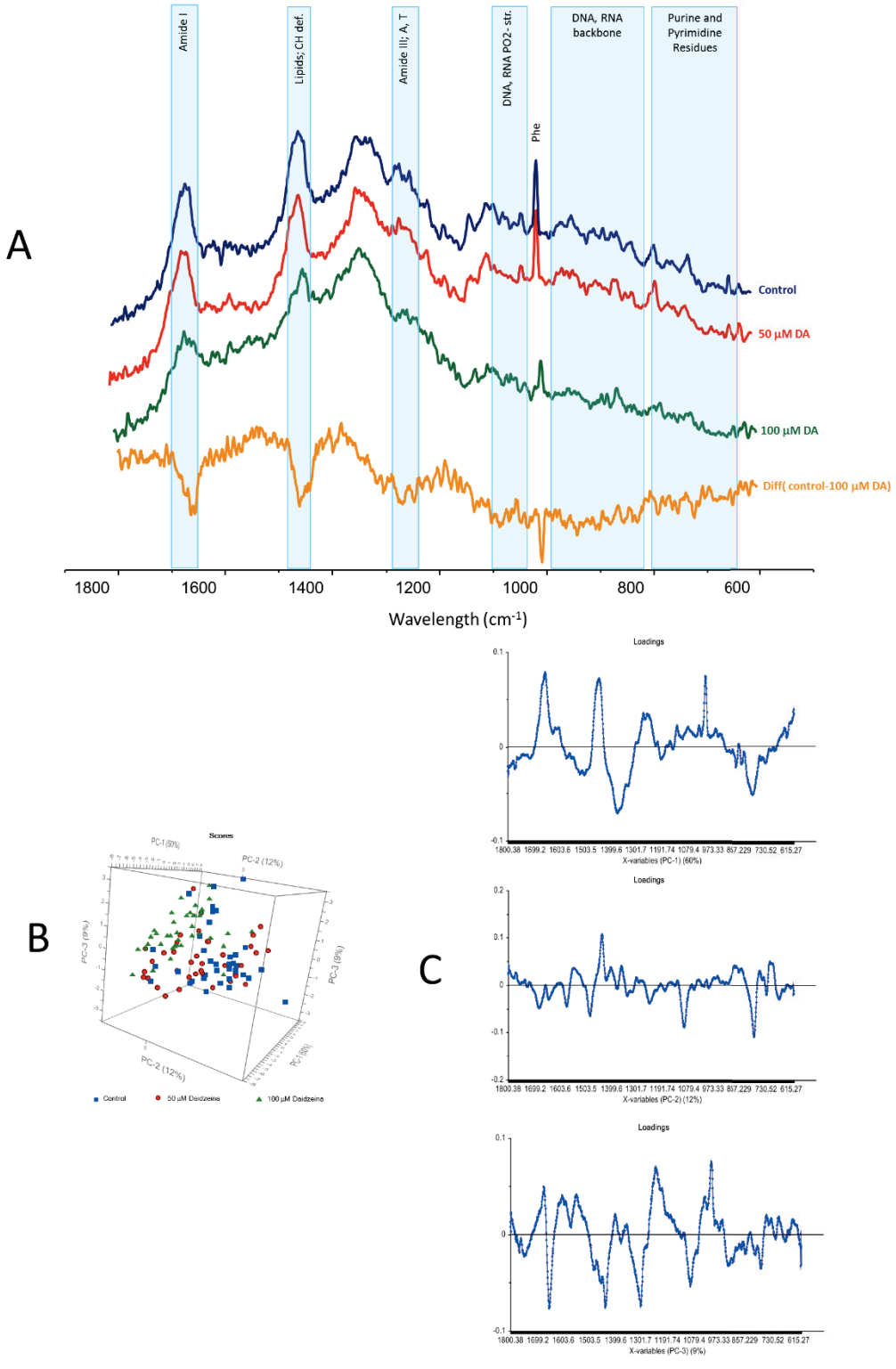

Figure 35 – Average Raman spectra (A), and PCA score (B) and loading (C) plots for DA-treated MCF-7 cells *vs* control (50 and 100 µM, 48 hours exposure).

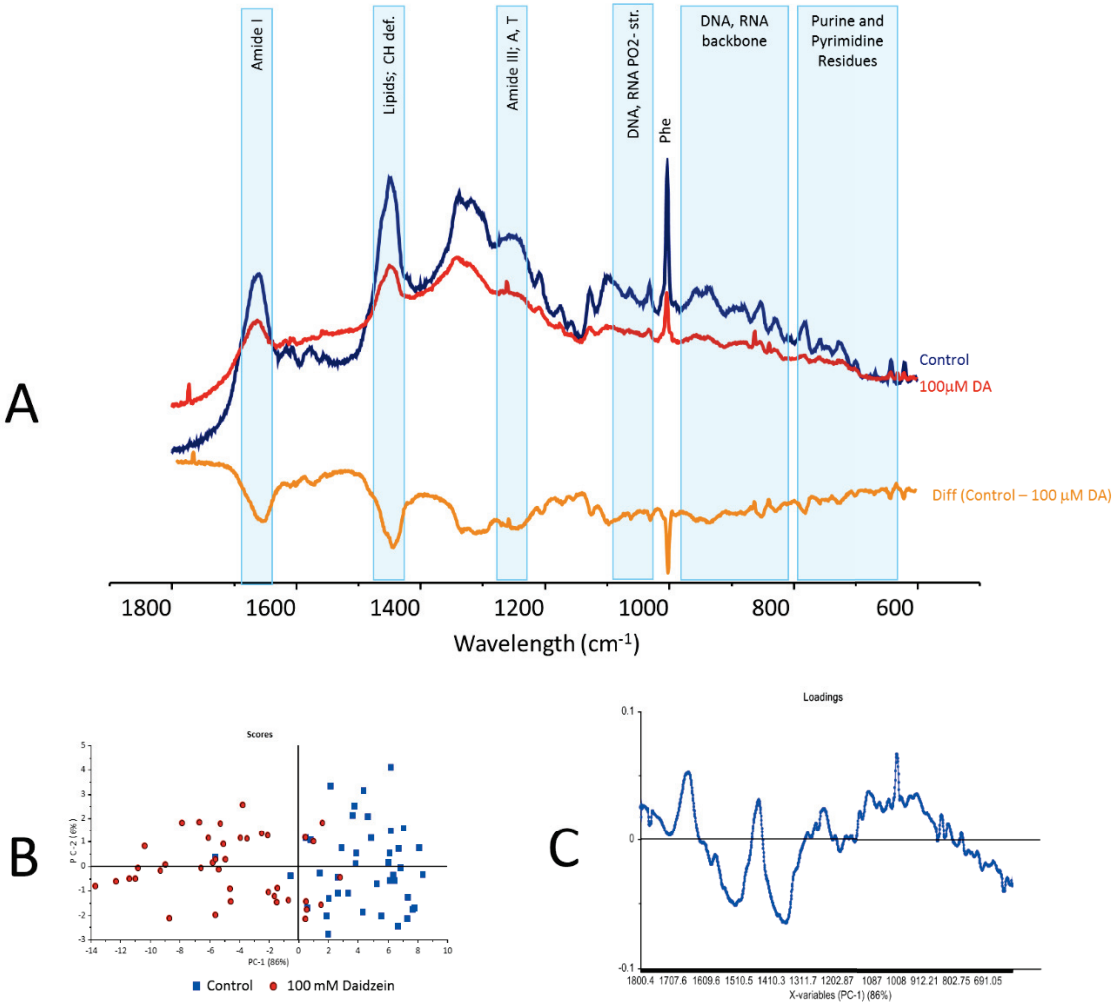

Figure 36 – Average Raman spectra (A), and PCA score (B) and loading (C) plots for DA-treated MDA-MB-231 cells *vs* control (100 µM, 48 hours exposure).

For the estrogen-independent cell line (MDA-MB-231), the spectra from the control and DA-treated cells are clearly different, and the PCA analysis allows a differentiation of the samples (control and 100 µM) (Figure 36). The loading scores reflect the isoflavone´s interference with proteins (Amide I band at 1660 cm^{-1}), DNA/RNA backbone (1170 cm^{-1}) and phenylalanine (1000 cm^{-1}), which are cellular components associated with cell proliferation [101] and protein synthesis (corroborating the decreased cell viability and density obtained by the MTT and SRB assays). In view of previous studies in other cell lines, evidencing a DA-induced apoptosis *via* a mitochondrial pathway [173], a similar mechanism is presently suggested for the impact of daidzein on MDA-MB-231 cells.

In turn, for MCF-7 differentiation of the data is only possible for the highest concentration (100 µM) (Figure 35). Since MCF-7 is an estrogen-dependent cell line the

main mechanism of action of daidzein and similar compounds can be the regulation of estrogen receptors. A reported study on DA-treated MCF-7 cells unveiled an induced apoptosis by cell cycle arrest in the G1 and G2/M phases, coupled to an accumulation of cells in sub-G0 phase [174]. Additionally, DA may have a role in manganese superoxide dismutase (MnSOD) overexpression leading to a decreased cell proliferation and an enhanced stress resistance [78], which are associated with ERα (estrogen receptor superfamily) that regulate cell growth and differentiation [155].

The mechanism of action of daidzein is therefore different in both cell lines tested (either estrogen-dependent or independent). Since DA is a powerful antioxidant, it can interfere with increased ROS/RNS cell levels leading to mitochondrial dysfunction. Also, the visible effect of DA on the phenylalanine band for the MDA-MB-231 cells (PC1 loading plot, Figure 36) may be a consequence of affected amino acid synthesis, which leads to reduced cell-viability and migration ability (in agreement with migration assays, section 4.1.3.)

5. Conclusions

The dietary phytochemicals currently investigated showed to have a significant chemopreventive activity (and even, under certain conditions, an antitumour effect), by interacting in different ways with the human breast cell lines tested (both non-neoplastic and neoplastic/estrogen-dependent and independent). p-coumaric acid appears to affect mostly the lipids, and since these are the most susceptible cellular components to undergo oxidation [21] this phenolic acid (at low concentrations, 5-7.5 μM) may be a promising chemoprotective agent towards ROS-induced lipid (and membrane) damage. Daidzein, in turn, will be a more powerful chemoprotective agent in hormone-dependent cells as compared to hormone-independent ones.

The ROS/RNS quantification assay currently performed corroborate these conclusions, and show a marked cell selectivity for the two tested phytochemicals.

The high sensitivity of the Raman microspectroscopy technique (coupled to a dedicated multivariate analysis of the data) allowed to unveil the impact of the compounds under study at the cellular level, by monitoring the biochemical profile of the cells in their presence (as a function of concentration), the lipid and protein components being the most affected.

Overall, there is a clear discrimination between both concentration ranges (7.5/15 μM and 50/100 μM) regarding the compounds´ effect on the breast cancer cells Figure 37.

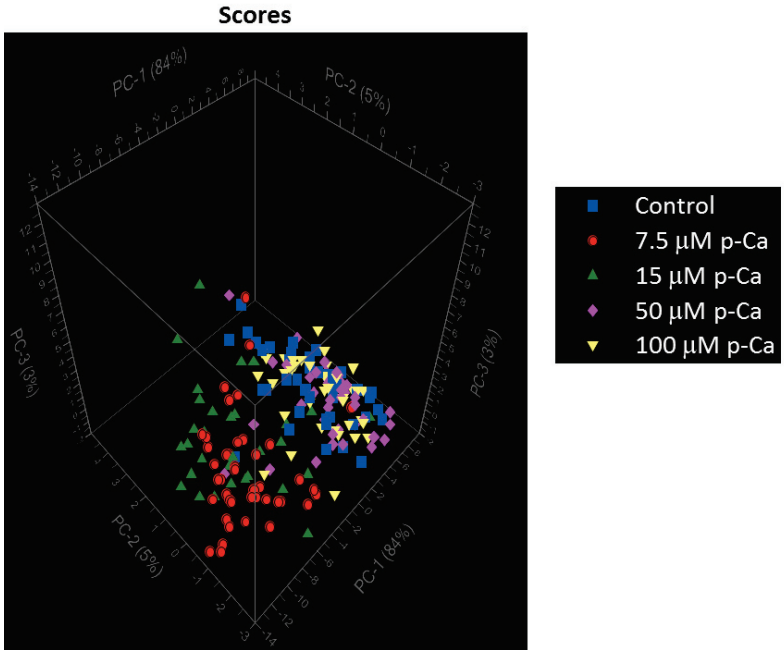

Figure 37 – PCA score plots for p-Ca-treated MDA-MB-231 cells vs control (7.5, 15, 50 and 100 µM, 48 hours exposure).

Also, it is evident from the results that for the highest concentration tested (100 µM) both phytochemicals behave differently as compared to the lower dosages, which should reflect a different mechanism of action still to be clarified by further studies.

Phytochemical compounds are the subject of an increasing research effort towards the development of natural-based chemopreventive agents, as well as of alternatives to chemically designed antineoplastic agents either as constituents of chemotherapeutic drug combinations or as adjuvants. Indeed, the costs associated with the extraction and purification of phytochemicals from their natural sources (fruits and vegetables) might well be lower than those involving the rational design of new chemical entities aimed as chemopreventives and/or drugs [175]. Additionally, the use of p-Ca and DA in adjuvant therapy (mostly when coupled to a suitable delivery system) may help to reduce deleterious side effects and acquired resistance, leading to an enhanced patient compliance and a brighter chemotherapy outcome.

6. Future Perspectives

The work presently reported should be extended to other dietary polyphenolic compounds as well as to different cell lines (namely distinct types of human cancers, preferably those with a poor prognosis). Currently, sufficient data has been collected to allow further evaluation of the use of phytochemicals as adjuvants, in combination with conventional chemotherapeutic drugs (such as Tamoxifen or Docetaxel in the case of breast cancer). Additionally, assessing the effect of combinations of p-Ca and DA, at different concetrations and incubation times, could yield interesting results, mainly if a synergetic effect regarding chemoprevetive potency can be unveiled.

Establishment of oxidative stress conditions followed by measurement of the intracellular ROS levels, in the absence and presence of the phytochemical agents (both in sole administration and in combination), would also yield important clues as to their preventive efficiency towards oxidative-induced carcinogenesis.

Another relevant experiment would be the *in vitro* wound-healing assay, to complement the cell migration data already gathered for the isolated p-Ca and DA. Coupled to anti-angiogenic evaluation tests, this would allow to attain a complete picture of the phenolic agents´ effect on cell invasiveness and metastatic ability.

Studies in 3D co-cultures are also a natural continuation of the present work, allowing to extend the conventional 2D biological model to a more realistic one, closer to the *in vivo* tissue morphology and function.

Regarding state-of-the-art microvibrational techniques (particularly Raman), optimisation of the experimental conditions for the specific models under study is essential for a full use of the extraordinary potential of such methods in probing the impact of exogenous agents on the cellular environment. Surface-Enhanced Raman Spectroscopy (SERS) can also be of use in this type of study, particularly for monitoring specific organelles within the cell. Finally, it would be ideal to be able to take profit of the brilliance of the synchrotron radiation in microFTIR measurements (complementary to the microRaman ones).

7. References

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