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In vitro effects of bioactive extracts of local Italian cultivars: from molecular mechanisms to potential nutraceutical applications for consumers' well-being

Stefania Moccia

UNIVERSITÀ DEGLI STUDI DI SALERNO
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Dottorando

Dott. *Stefania Moccia*

Tutore

Chiar.ma Prof.
Rita Patrizia Aquino

Co-tutore:
Dott. *Gian Luigi Russo*

Coordinatore: Chiar.mo Prof. *Gianluca Sbardella*

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Abbreviations list

AChEIs: Cholinesterase inhibitors

ABTS: 2,2'-azinobis (3-etilbenzotiazolin-6-sulfonate)

AD: Alzheimer

AFC: 7-Amino-4-trifluoromethyl coumarin

AFR: Ascorbyl Free Radical

APL: Acute Promyelocytic Leukemia

AMPK: Adenosine 5′-Monophosphate–activated Protein Kinase

APP: Amyloid precursor protein

ATP: Adenosine Triphosphate

ATRA: All-trans retinoic acid

ASC: Ascorbate

BBB: Blood brain barrier

BSA: Bovine Serum Albumin

CAT: Catalase

CEN: Carotenoid extract nanoemulsion

CGA: Chlorogenic acids

CNS: Central nervous system

CVD: Cardiovascular diseases

DHA: Dehydroascorbate

DMSO: Dimethyl sulfoxide

DPPH: 1,1-diphenyl-2-picrilidrazilic

EFSA: European Food and Safety Authority

EGCG: Epigallocatechin-3-gallate

EGFR: Epidermal growth factor receptor

FIC: Ferricyanide

FOC: Ferrocyanide

FOSHU: Foods for Specific Health Use

FRAP: Ferric Reducing Antioxidant Power

FUFOSE: Functional Food Science in Europe

GAE: Gallic acid equivalent

G6PD: Glucose-6-Phosphate Dehydrogenase

GPx: Glutathione Peroxidase

GR: Glutathione Reductase

GSH: Glutathione

GSSG: Oxidized glutathione

IGFR: Insulin like growth factor receptor

ISPA-CNR: Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle Ricerche

LC3: Microtubule-associated protein Light Chain 3

LDL: Low-Density Lipoprotein

MAPK: Mitogen-activated protein kinase

MDA: Malondialdehyde

mTOR: Mammalian target of rapamycin

MUFA: Monounsaturated fatty acid

ND: Neurodegenerative diseases

NFκB: Nuclear factor

NO: Nitric Oxide

NREA: Research and education nutraceutical

OxLDL: Oxidized LDL

PASSCLAIM: Process for the Assessment of Scientific Support for Claims on Foods

RT-PCR: Real-time PCR

PD: Parkinson's disease

PI_3K: Phosphoinositide 3-kinase

PMRS: Plasma Membrane Redox System

PSA: Prostate antigen specific

PUFA: Polyunsaturated fatty acid

RBCs: Red Blood Cells

ROS: Reactive Oxygen Species

RW: Red wine

RWP: Red wine polymers

SnPE: *Sambucus nigra* polyphenolic extract

SOD: Superoxide Dismutase

ULK1: UNC-51-Like Kinase 1

Chapter I

Introduction

1.1 **Biological properties of phytochemicals**

Several hundred scientific studies focus on the beneficial effects for human health, of bioactive compounds, collectively called phytochemicals and usually present in the diet. These molecules are widely present in plant derived foods and beverages (fruits, vegetables and beverages, such as tea, wine, beer) and include a very heterogeneous group of natural substances, like vitamins (carotenoids) and polyphenols, phytoalexins, indoles and sulfur rich compounds [1, 2].

Phytochemicals are secondary metabolites that plants produce to protect themselves from other organisms and they regulate enzymes involved in cell metabolism and in mechanisms of defense against foreign agents such as radiations, viruses and parasites. Due to the variety of their physiological roles in plant tissues, phytochemicals have been associated to pleiotropic effects in animal cells [3, 4]. In fact, their physiological role on human body could be exerted by activating the detoxifying defense mechanisms, interacting with the metabolic pathway, by modulating the signaling cascades implicated in the cell growth, proliferation, differentiation and death [5] [6]. In particular, several signaling pathways are affected by phytochemicals, such as insulin like growth factor receptor (IGFR), Ras/Raf signaling, mitogen-activated protein kinase (MAPK/ERK), phosphoinositide 3-kinase (PI_3K), epidermal growth factor receptor (EGFR) and nuclear factor (NF-κB) [7]. Their beneficial properties on human health have been considered mainly in relation to their anti-inflammatory and antioxidant activity, since the inflammation and oxidative stress are the critical conditions in many human degenerative diseases such as cardiovascular disease (CVD), neurodegenerative disorders, aging and cancer, as reported by several epidemiological studies [8, 9].

1.1.1 Reduction of cardiovascular diseases risk

Atherosclerosis is the most common cause of CVD, characterized by the inflammation of the vessel wall and the damage of the endothelium, caused by the infiltration and accumulation of low-density lipoproteins (LDL), forming oxidized LDL (oxLDL) [10]. The accumulation of several cell types, including living and dead foam cells, endothelial cells and smooth muscle cells, is involved in this inflammatory process and produces different multifocal lesions and the formation atherosclerotic plaque. Among phytochemicals, polyphenols have been largely considered as agents able to promote health, including amelioration of CVD, where an increased intake of polyphenol-derived preparations have been shown to improve markers of cardiovascular risk (hypertension, endothelial dysfunction, arterial stiffness, dyslipidemia, inflammation, oxidative status and hyperglycemia) (**Figure 1.1**) [9].

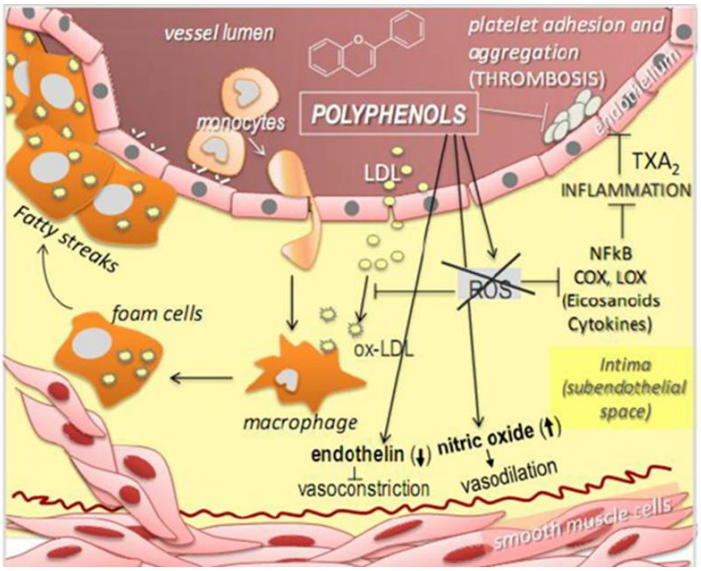


Figure 1.1. *Cardioprotective effects of polyphenols and molecular mechanisms by which polyphenolic compounds may counteract endothelial dysfunction [11].*

Several meta-analysis have shown that preparation based on cocoa and tea polyphenols improve blood pressure and endothelial functions (**Figure 1.1**). In particular, the increase of nitric oxide (NO) levels and NO-synthase activity, has been reported in dietary intervention studies where flavanol-enriched cocoa drinks were administered to volunteers. Furthermore, other studies have been carried out to evaluate the possible role of polyphenols in coffee, such as chlorogenic acids (CGA), on the blood pressure. As an example, dark roast coffee intake reduces the systolic blood pressure in overweight subjects. In a different meta-analysis, the reduction of blood pressure by soy isoflavones is measured in hypertensive subjects, but not in normotensive ones. Moreover, the consumption of a meal based on virgin olive oil with high content of phenolic compounds improves the endothelium-dependent microvascular vasodilation and decreases the oxidative stress during the first 4 h from the assumption in hypercholesterolemic subjects [9].

Other studies focus on the role of red wine polyphenols in the CVD protection [12]. In particular, the consumption of wine, especially red wine, could be linked to the so-called "French paradox", which refers to the apparent contradiction existing between the low incidence of CVD in the French consumers despite the high consumption of saturated fats [13]. To this regard, several studies have reported a correlation between moderate wine consumption and a reduced risk of CVD, such as reduction of blood pressure, serum glucose, hypertension, LDL, hyperglycemia and progression of atherosclerosis, probably related to the presence of bioactive compounds in this beverage [14]. In fact, it has been shown that a moderate consumption of red wine (15–30 g/day of ethanol, about 130–250 ml of wine/day) provides an appropriate amount of bioactive compounds (a glass of red wine contains approximately 200 mg of polyphenols) able to induce beneficial effects on human health [14]. An intervention study was conducted in healthy volunteers who received beverages such as red wine, white wine, beer and whiskey and

has shown beneficial effects of red wine on endothelial function after 1-4 h after ingestion, probably due to its higher content of total polyphenols respect to other beverages [15, 16]. The reduction of platelet aggregation, which increases the risk of coronary thrombosis and myocardial infarction, and the reduction of the oxidative stress have been related to the assumption of grape and wine polyphenols, showing a positive effect on blood lipids and inflammation [12].

Among polyphenols, anthocyanins could play an important role in the CVD protection, by modulating the endothelial dysfunction and reducing platelet aggregability [17]. In fact, several anthocyanin-purified extracts from red wine, blueberry, bilberry and elderberry have shown the reduction of the inflammatory process, the endothelial dysfunction and vasoconstriction, through the NO production, reduction of blood pressure, oxLDL and serum malondialdehyde (MDA) [18].

1.1.2 Protection from neurodegenerative damage

Neurodegeneration is a progressive functional alteration of neuronal systems, resulting in a state of dementia which is considered one of the most common psychiatric disorders of the elderly. It is an age-related irreversible condition, resulting in a progressive decline of cognitive functions (**Figure 1.2**) [19]. Neurodegenerative diseases (ND), such as Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS) induced a deterioration of neurons or the myelin sheath, resulting in a reduction of sensory transmission and movements [20]. The two pathological hallmarks of AD are senile plaques and neurofibrillary tangles (**Figure 1.2**) [21]. Senile plaques are made of insoluble Amyloid-β (Aβ) peptides that result from sequential cleavage of the amyloid precursor protein (APP) by β-cleaving amyloid precursor protein enzyme (BACE) and γ-secretase. Neurofibrillary tangles are

made of aggregates of hyper-phosphorylated tau protein that becomes dysfunctional causing the microtubules collapse and blocking neurotransmitters and neuronal signaling [22]. Once these abnormal proteins, Aβ and hyperphosphorylated tau, accumulated in the brain, induce the apoptotic pathway in neuronal cells promoting the progression of the pathological process in an exponential way.

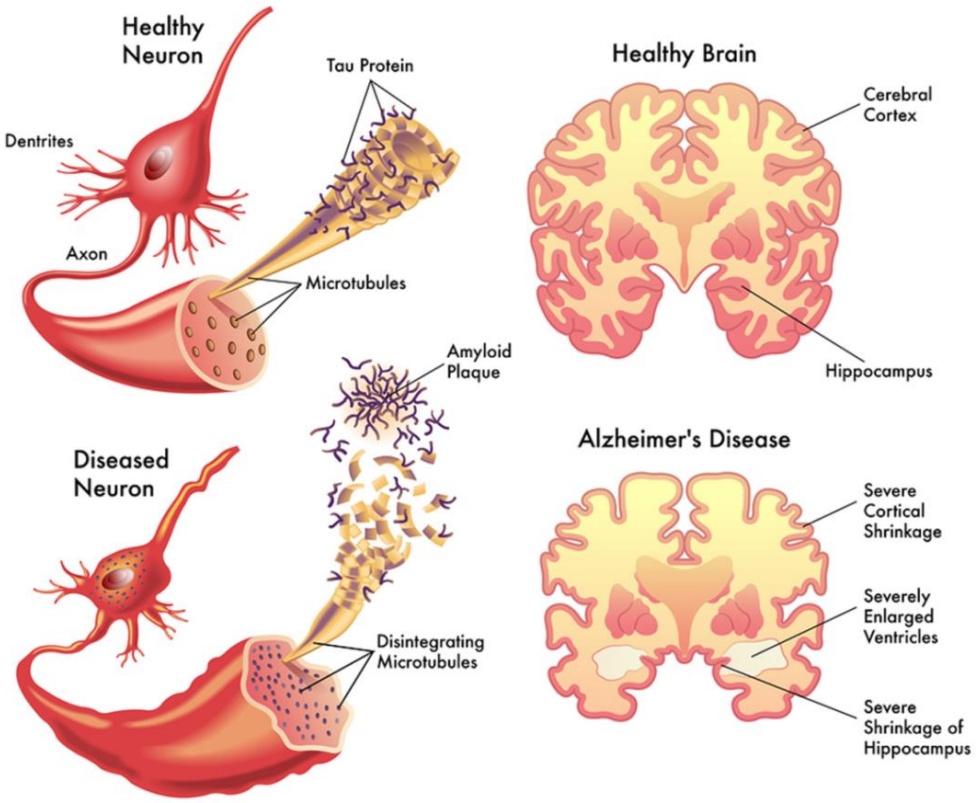


Figure 1.2. *Pathological hallmarks of Alzheimer's disease (http://www.brightfocus.org/).*

Another key event in AD pathophysiology is the neuroinflammation of Central Nervous System (CNS). The neuronal injury is induced by an uncontrolled

activation of microglial cells, the primary immune cells in the CNS, which could lead to an excessive production of various factors such as NO, pro-inflammatory cytokines (IL-1β, TNF-α), reactive oxygen species (ROS) and glutamate [23]. Therefore, the inflammatory response, resulting in oxidative stress, induces neuronal damage and death. Current drugs for the neurodegeneration therapy are cholinesterase inhibitors (AChEIs), employed in the mild stages of disease. The AChEIs maintain the cerebral availability of acetylcholine, the neurotransmitter involved in the cognitive functions, such as memory [24]. However, these are only "symptomatic" drugs, aimed to reduce the clinical symptoms of the disease, but they are not able to cure or prevent ND occurrence and the progressive decline of cognitive functions. The research is now directed towards the identification of new pharmacological strategies for the treatment of neurodegenerative ageing-related disorders.

The mechanisms involved in microglia and neuroinflammation or oxidative stress represent the promising targets of future ND treatment. Recently, a great number of natural plant extracts have been tested for their therapeutic properties in neuroprotection [4] [25-27]. Data present in literature highlight the role of polyphenols as promising sources in the prevention or treatment of neurodegenerative conditions, reporting how polyphenols could slow the disease progression and interfere with some mechanisms involved in the development of neurodegenerative processes.

In vivo studies have described the improvement of several aspects of memory and learning after assumption of dietary polyphenols. In particular, epidemiological data demonstrate that the assumption of green and black tea, rich in polyphenols, protects the brain from aging and reduces the dementia, which occurs in AD and PD [26]. In parallel, considering the potential properties of the single bioactive compounds present in these beverages and other foods, several preclinical studies have been carried out in order to evaluate the neuroprotective effects of curcumin, resveratrol and

epigallocatechin-3-gallate (EGCG), highlighting their antioxidant, anti-inflammatory, anti-amyloidogenic and anti-apoptotic activities [19]. More in details, the potential therapeutic utility of polyphenols in dementia has been associated with different mechanisms, such as the ability to interfere with cellular signaling, proliferation, apoptosis, redox balance and differentiation, all events which are involved in the progression of the disease [28] (**Figure 1.3**) [4].

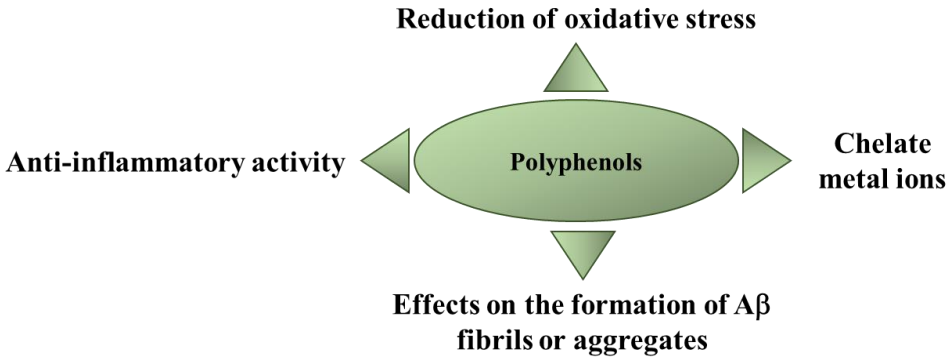


Figure 1.3. *Potential biological processes targeted by polyphenols in neurodegenerative diseases [4].*

In such a contest, the well-described anti-inflammatory activity of polyphenols have suggested their potential use against neuroinflammation-associated AD. In particular, they could modulate the expression of pro-inflammatory genes, such as cyclooxygenase, lipoxygenase, NO synthases and several important cytokines, mainly acting through NF-κB and MAPK [29]. In addition, the antioxidant activity of polyphenols could result in neuroprotection, since the oxidative stress plays an important role in pathogenesis of neurodegeneration [4] [30]. Furthermore, the potential neuroprotective activity of polyphenols is also related to a decrease of damage induced by neurotoxic agents, through the reduction of amyloid plaque formation, involved in AD etiology (**Figure 1.3**).

In fact, some *in vitro* studies have reported the neuroprotective effect of quercetin, curcumin, EGCG and resveratrol, resulting in a reduction of Aβ fibrils formation [31]. Another proposed mechanism to explain the neuroprotective potential effect of polyphenols is the chelating activity of metal ions, which accumulate in specific brain regions, inducing the oxidative stress. These compounds form complexes with transition divalent metal ions ($Fe^{2+,}$ Cu^{2+} and Zn^{2+}) and prevent the formation of iron induced free radicals by Fenton chemistry [32]. Despite these encouraging data, important limitations must be considered, such as the low bioavailability of polyphenols relative to their bio-transformation and conjugation occurring during the uptake in the gastrointestinal tract, liver and finally in the peripheral tissue cells. In fact, after oral intake, in the small intestine and liver, polyphenols are substrates for phase I enzymes (hydrolysis and oxidase) and Phase II (conjugation and detoxification) and are conjugated to methyl and sulfate groups and glucuronic acid [33]. Further transformations occur in the colon by the intestinal microflora enzymes with the formation of phenolic acids which undergo further metabolism in the liver. These chemical changes induce modifications in polyphenol structures and may result in the alteration of their biological effects. For these reasons, new formulation strategies are need in order to modify their bioavailability, allowing an improvement of their vehiculation in order to cross the blood brain barrier (BBB). The BBB is a highly selective semipermeable membrane barrier, formed by endothelial cells connected by tight junctions, which separate the circulating blood from the brain extracellular fluid in the CNS. The BBB limits the passage of macromolecules and small polar molecules from the circulation to the brain tissue [34]. The crossing capacity depends on the lipophilic nature of molecules and on the interactions with specific efflux transporters expressed in the BBB, such as P-glycoprotein. To investigate the trans-membrane transport across the BBB of polyphenols and their metabolites, specific *in vitro* models

have been selected, through cell cultures, highlighting that the metabolites have a better transport efficiency compared to native flavonoids [35]. For example, *in vivo* studies have found the accumulation of flavonoids in the brain hippocampus and at the level of the cerebral cortex, after the supplementation of blueberries in older animals [36]. These results have provided the first indication that polyphenols can cross the BBB, highlighting their potential function as neuromodulatory agents.

1.1.3 Chemopreventive effect

Phytochemicals also attracted scientists' interests since the demonstration that their biological targets in mammalian cells were the same involved in inflammatory processes and oncogenic transformation, such as the alterations of cell cycle control, apoptosis evasion, angiogenesis and metastases [3]. The transformation from normal to malignant cells involves the somatic mutations, induced by chemical or physical carcinogenic agents, with the activation of proto-oncogenes and inactivation of tumor suppressor genes, resulting in malignant phenotypic changes. After this irreversible initiation phase, which lasts a few days, the promotion phase occurs. The final stages of carcinogenic process is the progression (1-5 years) of tumor growth and the metastasis formation (**Figure 1.4**) *[1, 2, 37]*. A large number of epidemiological studies have suggested that a daily intake of phytochemicals, including vitamins, carotenoids and polyphenols, could reduce the incidence of several types of cancers [38]. These compounds act interfering with one or more stages of the carcinogenesis process, as potential chemopreventive agents. A complete definition of chemoprevention was given by Dr. Sporn and includes *the use of natural or pharmacological agents to suppress, arrest or reverse carcinogenesis at its early stages* (**Figure 1.4**) [2].

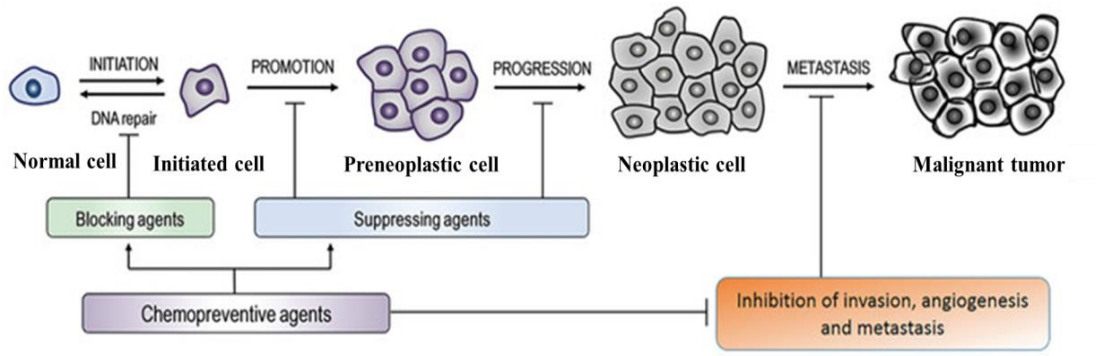


Figure 1.4. *Carcinogenesis phases: initiation, promotion, progression, and metastasis [37].*

Chemoprevention includes three areas: 1. primary prevention in healthy subjects with high risk, for example in smoking population or positive biomarkers for specific cancer; 2. cancer prevention in subjects with a premalignant lesions, which may progress to invasive cancer; 3. prevention of secondary forms of cancers in patients who have already developed a primary cancer [6]. The final goal of all these different aspects of chemoprevention is the attainment of clinical evidences for cancer reduction. Chemopreventive agents can be divided into two main groups: cancer-blocking and cancer-suppressing agents (**Figure 1.4**) [39].

The ability of cancer-blocking agents to interact with the initiation step can occurs through the detoxification of carcinogenic substances, modifying their uptake and metabolism, scavenging ROS, enhancing DNA repair. The cancer-suppressing agents block the cancer promotion and progression after the formation of pre-neoplastic cells and interact with cell cycle regulation, signal transduction, transcriptional regulation, and apoptosis [1, 39].

Growing evidences suggest that not only individual genetic background are implicated in cancer development, but also a heathy life style and correct diet [40]. The concept of "chemoprevention" was associated to the use of natural substances, able to interfere with the development or the progression of

neoplastic processes [6]. These compounds include vitamins, carotenoids, flavonoids and organosulfur compounds (**Table 1.1**).

Table 1.1. *Examples of dietary chemopreventive compounds [39].*

Class of chemicals	Nutrients	Chemopreventive mechanisms
Vitamins	Vitamin D, Folic acid, Vitamin A, E, ascorbic acid	IGF, apoptosis induction, autophagy, antioxidant activity, inhibition of angiogenesis
Carotenoids	Lutein, β-carotene	NF-κB inhibition, MAPK suppression, modulation of IGF-1.
Flavonoids	Genistein, rutin, quercetin, silymarin, catechins	Apoptosis induction, anti-inflammatory effect, MAPK suppression, cell cycle arrest, NF-κB suppression
Organosulfur compounds	Allyl mercaptan, allyl methyl trisulfide	Cell cycle arrest, apoptosis induction, anti-angiogenetic activity, oncogenic Ras inhibition

For example, several studies have demonstrated that natural molecules, such as genistein, rutin, silymarin, EGCG, are potential negative modulators of inflammatory processes and oncogenic transformation by inhibiting proliferation, inducing apoptosis, cell cycle arrest and downregulating the signals transduction pathways regulated by PI_3K, NF-κB (**Table 1.1**) [41].

Currently, one of the major challenge is to demonstrate, by means of randomized controlled trial study design, that the positive effects of phytochemicals reported *in vitro* and in preclinical models are confirmed in human subjects [6]. Significant differences among these models are related to

the metabolic processes that these substances undergo after oral administration, moreover their low bioavailability could reduce the amount circulating in the bloodstream and their effectiveness. It should be considered that concentrations applied in *in vitro* studies (usually tens of micromolars) are not reachable *in vivo* after vegetable and fruit assumption (usually below 1 μM), relating to the scarce bioavailability and bio-transformation of these molecules after oral intake. In fact, despite the encouraging results and the abundance of *in vitro* studies which clarify the molecular mechanisms triggered by phytochemicals to inhibit cell growth and induce apoptosis in cancer cells, only few of them enter clinical trials. Phase I clinical trials have been completed only for few of these compounds and **Table 1.2** reports meta-analysis, cohort studies and case-control studies of different phytochemicals.

However, there are important examples of large clinical trials which failed, such as the β-Carotene and Retinol Efficacy Trial (CARET) aimed to test the combination of 30 mg β-carotene and 25,000 IU retinyl palmitate (vitamin A) daily in 18314 men and women at high risk of developing lung cancer. This study was stopped 21 months early because of clear evidence of no benefits and of possible harm. In fact, there were 28% more lung cancers and 17% more deaths in the active intervention group [42].

One generally accepted property of natural compounds refer to their functional pleiotropic effect. In fact, they interfere with multiple intracellular targets, affecting different cell signaling processes usually modified in cancer cells, with limited toxicity on normal cells.

Targeting simultaneously multiple pathways may help to kill cancer cells and slow drug resistance onset.

Table 1.2. *Reduced risk of cancer by phytochemicals associated with different scientific approaches [6].*

Phytochemicals	Cancer type (risk reduction)	Scientific evidences
Lycopene, α and β carotene	Prostate, Breast, Lung	Strong +++,++,+,*
Daidzein, genistein, glycitein	Breast, Colon, Prostate, Lung	Strong +++,++,+,*
Sulforaphane, benzyl isothiocyanate	Breast, Colorectal, Renal, Gastric, Bladder	Strong +++,++,+,*
Epigallocatechin gallate (EGCG)	Colon, Brest, Head, Neck, Lung, Prostate	Limited *,+
Resveratrol	Breast, Colon, Prostate, Liver, Pancreatic	Limited *,+
Curcumin	Breast, Colorectal, Prostate, Lung, Pancreatic	Limited *, +

*+++meta-analysis studies, ++cohort studies, +case-control studies, *animals or in vitro studies.*

Thus considering the pleiotropy of phytochemicals, they could be employed in association with classical chemotherapeutic drugs. In this case, through the synergism with cytotoxic drugs, a double positive effect can be expected, increasing their efficacy and lowering the toxic side effects on normal cells and also the combined treatment can delay resistance onset [1, 3]. Therefore, the association of several molecules might be effective in cancer prevention respect to single compounds, supporting the concept of "combined chemoprevention", i.e. the synergistic effect of low doses of chemopreventive

agents with different mechanisms of action, resulting in increased efficacy and low toxicity [3].

1.2 Chemical structure and classification of phytochemicals

Phytochemicals are secondary metabolites of plants, essential for the basic functions, such as growth, reproduction, defense against pathogens and protection from UV radiation [43]. Polyphenolic and carotenoid compounds represent two of the major classes whose structure and classification will be further described in the next paragraphs since they have been employed in the present project.

1.2.1 Polyphenols

It has been estimated that more than 8000 polyphenolic compounds in vegetable kingdom have been isolated and described [44]. Polyphenols are widespread constituents of foods (fruits, vegetables, cereals, olive, legumes, chocolate, etc.) and beverages (tea, coffee, beer, wine, etc.), partially responsible for their overall organoleptic properties. They have a common chemical structure, with the aromatic ring and associated one or more hydroxyl groups and they are classified into (**Figure 1.5**) [45]:

- **Phenolic acids**, which are divided into benzoic acid (hydroxybenzoic acid, gallic acid) and cinnamic acid (coumaric acid, ferulic acid, caffeic acid) derivatives;
- **Flavonoids**, which have two aromatic rings and an oxygenated heterocycle, and can be divided into 6 subclasses depending on the type of heterocycle: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (monomeric form: catechins; polymeric

form: proanthocyanidins). The flavonols are the most abundant, and quercetin is the main representative;

- **Stilbenes**, which are formed by an ethylene substituted with two benzenes and resveratrol is the most important compound and is found mostly in red wine;
- **Lignans**, which are constituted by two units of phenylpropane, such as pinoresinol.

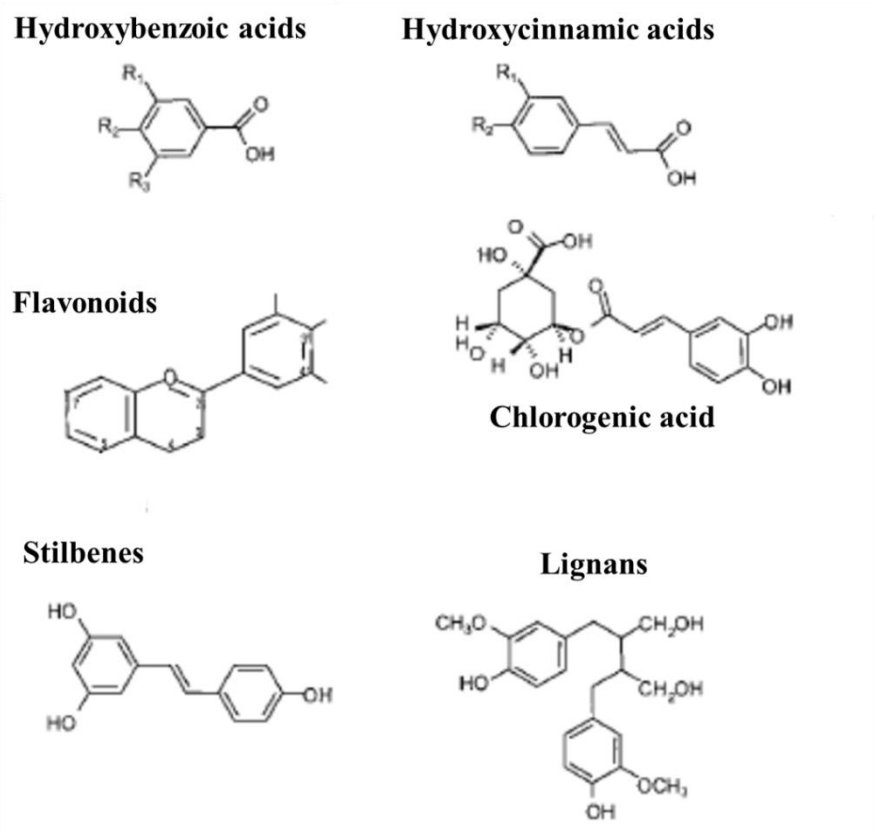


Figure 1.5. *Chemical structures of polyphenols [46].*

The biological activity of polyphenols has been largely attributed to their chemical structure. In fact, the aromatic ring and highly conjugated system with multiple hydroxyl groups are responsible for their intrinsic reactivity.

Due to the ability of the aromatic system to delocalize an unpaired electron, these molecules can easily donate hydrogen of the phenolic group to a radical, neutralizing free radicals. In fact, they are powerful reducing agents *in vitro*, able to neutralize free radicals by donating an electron or hydrogen atom to a wide range of reactive oxygen, nitrogen and chlorine species, including $O_2^{\bullet-}$, $OH^{\bullet}$, peroxyl radicals $RO_2^{\bullet}$, hypochlorous acid (HOCl). These redox properties of polyphenols are responsible for their reducing properties, acting as "quencher" of free radicals to end the chain of oxidative reactions, including the propagation stage of the lipid autoxidation chain reactions. They also act as "scavenger" of radicals, activating antioxidants enzymes and chelating free ions of transition metals, through the directly inhibition of Fe^{3+} reduction, resulting in the reduction of $OH^{\bullet}$ production by Fenton reaction [47]. **Figure 1.6** reports the tempted mesomeric structures of quercetin which contribute to stabilize the aroxyl radical [48].

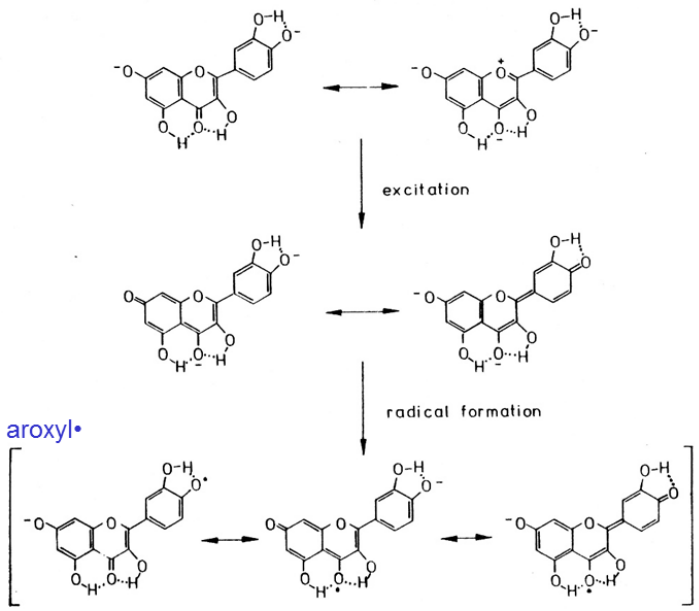


Figure 1.6. *Mesomeric structures of aroxyl radical generated from quercetin [48].*

Among polyphenols, particular relevance lies with anthocyanins, a flavonoid group, predominant in tea, honey, wines, fruits, vegetables, nuts, olive oil, cocoa and cereals. The anthocyanins are constituted by a molecule of benzene with a pyran (heterocyclic ring containing oxygen) connected with a phenyl group which can be linked to various substituents. This structure, called cation flavilio, is the base component of all anthocyanins (**Figure 1.7**). They are derived from the respective aglycones (anthocyanidins), from which they differentiate by the addition of a glycosidic group, such as glucose, rhamnose, xylose, galactose, arabinose and fructose.

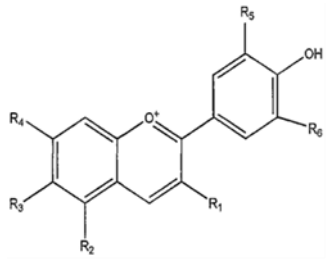


Figure 1.7. *Basic structure of the anthocyanin [49]*.

The antioxidant activity of anthocyanins closely depend on their basic structure, such as the ring orientation, hydroxyl groups and conjugated double bonds, presence of acyl groups, number of sugar residues [50].

As reported above, polyphenols are extensively metabolized either in tissues, after absorption through the gut barrier, or, the non-absorbed fraction and the fraction re-excreted in the bile, by the colonic microflora (**Figure 1.8**). Polyphenols are conjugated to form O-glucuronides, sulphate esters and O-methyl ether and all circulating polyphenols are glucuronidated and/or sulphated, and no free aglycones are found in plasma [51].

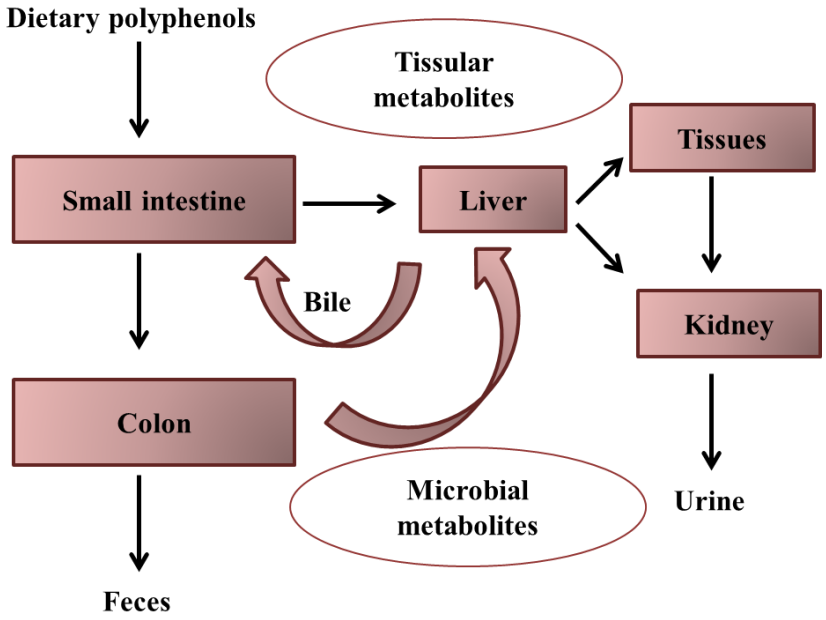


Figure 1.8. *Routes of dietary polyphenols in human body [51].*

1.2.2 Carotenoids

The second important class investigated in the present project is represented by carotenoids. Their name originates from carotene, a substance yellow-orange found for the first once in 1831 in the root of *Daucus carota*. They are pigments widespread in nature, with colour from yellow to orange, red to violet, responsible for the coloration of plants, fruits, flowers, roots, but also found in algae, bacteria, molds, yeasts, invertebrates, fish and birds [52]. They are lipid-soluble compounds, widely used in food, pharmaceutical and cosmetic industries. There are over 600 carotenoids occurring in nature and their basic structure is characterized by a long system of conjugated double bonds with p-electrons delocalized over the length of the polyene chain. Carotenoids are C40 tetraterpenoid pigments, belonging to isoprenoid

polyenes and the chemical structures of some carotenoids are reported in **Figure 1.9.**

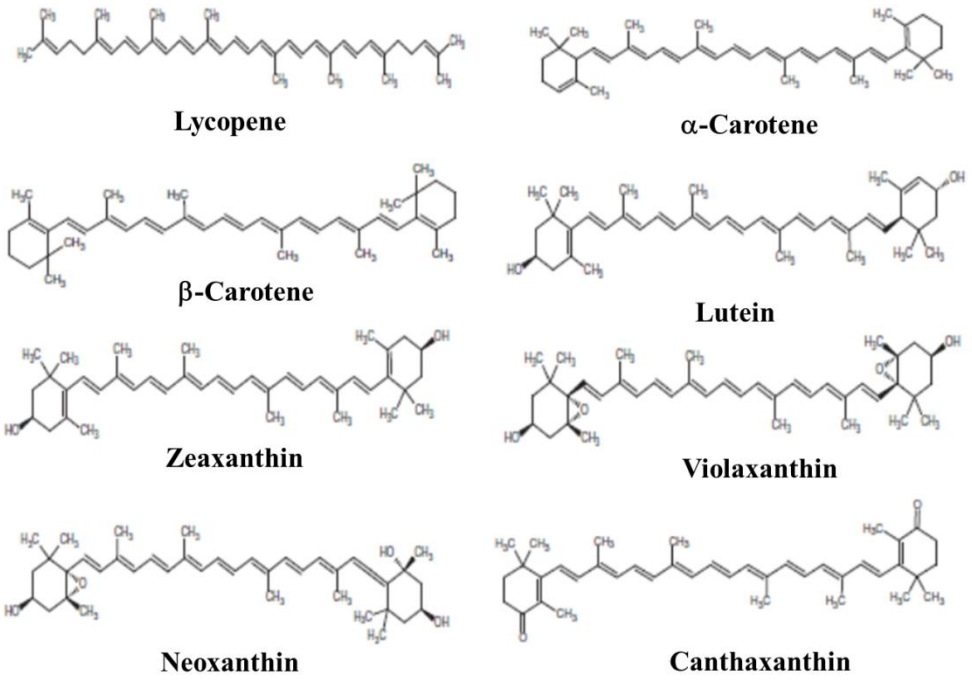


Figure 1.9. *Chemical structure of few among the most represented carotenoids in nature [52].*

The presence of numerous conjugated double bonds and cyclic end groups in their chemical structure allow the formation of some stereoisomers around the C=C bonds, such as geometric (E-Z), showing different chemical and physical properties, even if the isomeric form trans gives a more stable conformation than cis isomeric form in nature [53]. Addition of polar groups (epoxy, hydroxyl and keto) changes the polarity of carotenoids and influences their biological activity. In relation to the functional groups, they are divided in [54]:

- **Xanthophylls**, which contain oxygen as functional group and include lutein and zeaxanthin, neoxanthin and fucoxanthin;

- **Carotenes**, which contain only hydrocarbon chain without other functional groups, such as α-carotene, β-carotene and lycopene.

Due to the numerous conjugated double bonds and cyclic end groups, carotenoids present a variety of stereoisomers with different chemical and physical properties. The presence of the conjugated double bonds allow carotenoids to delocalize unpaired electrons, resulting excellent reducing agents, able to scavenge and neutralize free radicals, as largely demonstrated by *in vitro* and *in vivo* tests [55]. This property is related to the capacity to physically quench singlet oxygen and terminate reactive free radicals. For their reducing properties, carotenoids have been associated with potential protective effects against degenerative diseases, including atherosclerosis, cataracts, macular degeneration, MS and CVD [55].

From a functional point of view, they can divided into:

- **Vitamin A precursors,** such as β-carotene;
- **Pigments with partial vitamin A activity** such as cryptoxanthin, β-apo-8′-carotenoic acid ethyl ester;
- **Not vitamin A precursors** and not pigment, such as violaxanthin and neoxanthin;
- **Pigment without activity of vitamin A precursors**, such as lutein, zeaxanthin and canthaxanthin [53].

Regarding pro-vitamin A activity, part of carotenoids taken by enterocytes is converted into retinoid via two different mechanisms. In particular, β-carotene is the only carotenoid that can yield 2 molecules of retinaldehyde upon its symmetrical cleavage by the enzyme β-carotene-15,15′-oxygenase (CMOI or BCMO1 or BCO1) (**Figure 1.10**). β-Carotene is transformed into retinaldehyde, which is converted to retinol by a reductase dependent on NAD(P)H (**Figure 1.10**) [56]. However, β-carotene can also be cleaved asymmetrically by the enzyme β-carotene-9′,10′-oxygenase (CMOII or BCDO2 or BCO2), to generate apocarotenals, which can be converted to one

molecule of retinaldehyde. Retinaldehyde formed upon the cleavage of provitamin A can be oxidized by the action of enzymes belonging to the retinaldehyde dehydrogenase family (RALDH or ALDH 1 family) to generate all-trans retinoic acid (ATRA), the biologically active form of vitamin A [56].

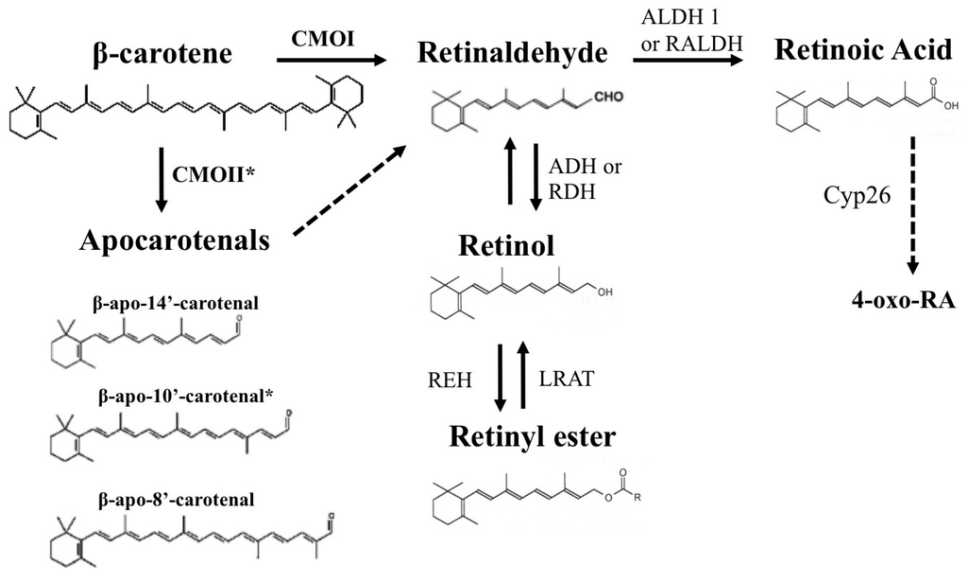


Figure 1.10. *β-Carotene as source of retinoids [56].*

The bioavailability of carotenoids after oral intake is influenced by several factors relating to the molecular linkage, the amount of carotenoids consumed in a meal, the matrix which incorporate them, the effectors of absorption and bioconversion, the genetic factors and the interactions with biological molecules. Their feature of lipid molecules allows their absorption by incorporation into mixed micelles. Recent studies have showed an increase of absorption of carotenoids when they are ingested with dietary lipids [57].

1.3 Functional foods, nutraceuticals and well-being

As schematically summarized in the **Figure 1.11**, the potential applications of food-derived bioactive compounds and/or extracts span from the pharmaceutical to the nutraceutical sectors. In both cases, it is possible to identify strengths and weaknesses due, for example, to the general low bioavailability and high pleiotropy of these compounds. The present thesis will explore the possibility that the biological properties of the polyphenolic and carotenoid extracts under investigation may find applicative outcomes in the areas of pharmacology and/or nutraceuticals/functional foods. The latter field will be shortly introduced in the following paragraphs.

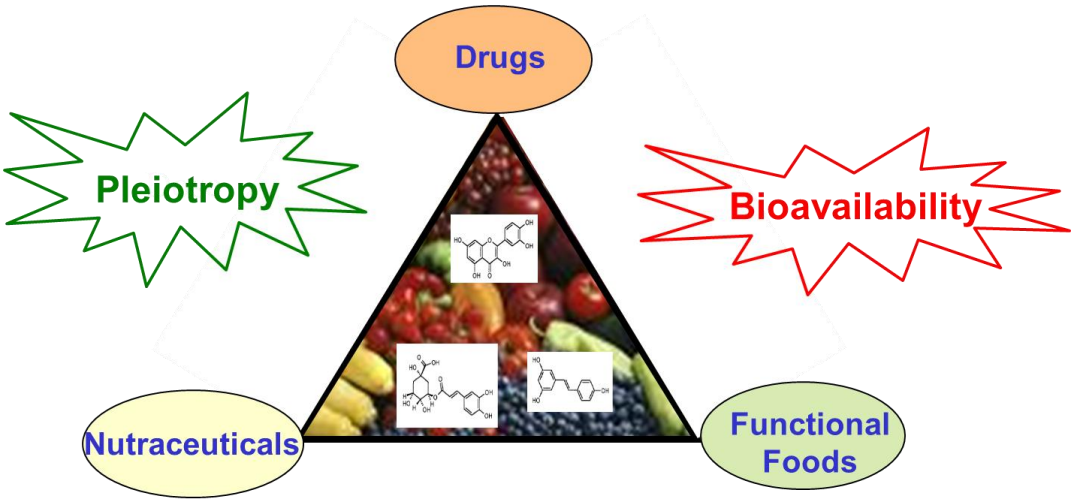


Figure 1.11. *Cartoon showing the possible applicative outcomes of food-derived natural compounds.*

The concept of "food as medicine" as a way to improve health and wellness was originally proposed by two medieval Persian practitioners, Avicenna and Rhazes, who developed the scientific guidelines about the use of natural products for the treatment of diseases and health problems [58]. Perhaps, this anecdotal episode represents the ancient origin of Nutritional Sciences with

the attempt to focus on dietary recommendations and foods to consume and others to avoid. Subsequently, as declared in 1947 by World Health Organization "Health is a state of physical, mental and social well-being and not merely the absence of disease", showing a pioneering perspective of nutrition respect to the last three decades, characterized by the many advances in medicine and drug discovery. The sanitary revolution of 19th century led to the development of a public health system, which provided the adequate treatment and drugs for most of diseases. In particular, the discovery of new synthetic drugs and a correct pharmacological approach to the main illnesses of the population, ensured better living conditions, resulting in a longer average lifespan [59].

The 20th century has been characterized by industrialized society, with changing dietary patterns and lifestyles due to a frenetic and stressful life, which resulted in an increase of diseases such as diabetes, CVD and obesity. For this reason, in recent years, the mission of Nutritional Science has been devoted to reduce malnutrition and diet-related diseases (obesity, diabetes, some forms of cancer) with cause an exponential growing of the health care costs. In partial contradiction to these considerations, at least in the Western world, the average life span is constantly extending, making the need for on improving of the quality of life especially during aging. This represents an additional pressure to the consumption of healthy foods. The lengthening of life expectancy and the emerging concept that therapeutic treatments can be supported by changes of lifestyle have contributed to focus the attention of the scientific community on the role of nutrition in maintaining of a health state on a specific category of foods, namely, functional foods [60].

Currently, the food and pharmaceutical industries are constantly looking for new products able to promote dietary patterns which include foods containing bioactive compounds, such as fruits and vegetables. In fact, many epidemiological studies have shown that a regular consumption of fruits and

vegetables is associated with a lower risk of coronary heart disease, obesity, diabetes and other age-related chronic and degenerative pathologies, like PD and AD [61] [62] [63, 64]. For these reasons, the consumers' demand changed dramatically over the years, resulting in a great attention to specific category of healthy promoting dietary products, namely functional foods and nutraceuticals [65].

1.3.1 Functional foods

The term "functional food" was first used in 1984 in Japan as a result of a study on the relationships between nutrition, sensory satisfaction, fortification and modulation of physiological systems in order to define those food products fortified with special constituents that possess advantageous physiological effects [66]. In 1991, the concept of Foods for Specific Health Use (FOSHU) was born in order to create a formal food category, in which a product must prove scientific evidences of its effectiveness. Functional foods should improve the general conditions of the body and decrease the risk of specific diseases [67]. For this reason, food products can be considered "functional" if, in addition to their nutritional provision, contain one or more components that positively influence one or more body functions, in order to promote a state of well-being and health, or to reduce the risk of degenerative disease. Based on these definitions, the terminology "physiologically functional foods" or simply "functional foods" is used to indicate an enhancer of physiological functions able to maintain and prolong health status, due to the presence of bioactive molecules [58]. The concept of "functional" indicates the *third* function of a food, related to the maintenance of a physical and mental well-being, in addition to the primary function of food, providing nutrition and caloric intake, and the secondary function of preference (taste) and sensory pleasure [68]. A new definition of "functional food" has been

provided recently by [69] as *"natural or processed food that contains known or unknown biologically active compounds, which, in defined, provides a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease."* Whereby, the idea of functional food aims to connect the function of ordinary food in maintaining a suitable nutrition status to pharmaceutical area reducing the risk of diseases. The category of functional foods generally includes conventional foods, consumed within the usual diet which can be classified as *natural*, if they *naturally* contain specific bioactive components or *processed*, if addition of beneficial effects have been reached by addition of beneficial components or removal of toxic ones, respectively. A functional food may also undergo chemical modifications aimed to improve the bioavailability of its bioactive compounds [69]. Basing on this definition, the category of "functional foods" includes fortified, enriched, altered and enhanced foods, as showed in **Table 1.3** [67]. Examples are fortified foods with vitamins and/or minerals, fortified beverages with calcium, enriched products, such as margarine with phytosterols, muffins with beta-glucan, yogurt with probiotics, margarines enriched with cholesterol-lowering phytosterols, cereals fortified with soluble fibres [65].

The functional ingredients are bioactive compounds, which taken in small quantities, may act synergistically to improve health well-being. They can be macronutrients, such as carbohydrates (soluble and insoluble fibers, prebiotics), monounsaturated (MUFA) and polyunsaturated (PUFA) lipids, peptides (caseinophosphopeptides (CPP), calmodulin-binding peptides), or micronutrients, such as vitamins (ascorbic acid and tocopherol) and natural phytochemical, such as polyphenols and carotenoids [69]. Examples of functional ingredients in specific foods, their sources and potential benefits are reported in **Table 1.4.**

Table 1.3. *Examples of functional foods [70].*

Type of functional food	Definition	Example
Fortified products	Foods fortified with additional nutrients	Fruit juices fortified with vitamin C
Enriched products	Foods added with new nutrients not naturally found in the same foods	Margarine with plant sterol ester, probiotics, prebiotics
Altered products	Foods containing a harmful element replaced by another with beneficial purpose	Fibers as fat releasers in meat or ice cream products
Enhanced commodities	Foods with a component naturally enhanced through special growing conditions, genetic manipulation, new feed composition	Eggs with increased omega-3 content achieved by altered chicken feed

Table 1.4. *Examples of functional ingredients of food, their sources and potential benefits [71].*

Bioactive components	Source	Potential benefits
	Carotenoids	
α-carotene/β-carotene	Carrots, pumpkin, paprika, chilli, spinach, apricots	Neutralize free radicals which may cause damage to cells
Lutein	Green vegetables	Reduce the risk of muscular degeneration
Lycopene	Tomato products	Reduce the risk of prostate cancer
	Non-starch polysaccharide	
Fucoidan (fucose)	Mushroom (maitake and reishi), brown seaweeds	Immune modulation; apoptosis of cancer cells; stimulates brain development; anti-clotting effect; lower blood cholesterol levels; decrease high blood pressure; stabilize blood sugar
Insoluble dietary fiber	Wheat bran	Reduces risk of breast or colon cancer
Soluble dietary fiber (β-Glucans)	Oat, barley	Reduces risk of cardiovascular desease; protects against heart desease and some cancers; lower LDL and total cholesterol
Soluble fiber	Psyllium	Reduces risk of cardiovascular desease; protects against heart desease and some cancers; lower LDL and total cholesterol
	Fatty acids	
Long chain omega-3 Fatty acids-DHA/EPA	Salmon and other fish oils	Reduce risk of cardiovascular desease; improve mental and visual functions
Conjugated Linoleic Acid (CLA)	Cheese, meat products	Improve body composition; decrease risk of certain cancers
	Phenolics	
Anthocyanidins	Fruits	Neutralize free radicals; reduce risk of cancer.
Catechins	Tea	Neutralize free radicals; reduce risk of cancer
Flavonones	Citrus	Neutralize free radicals; reduce risk of cancer
Flavones	Fruits/vegetables	Neutralize free radicals; reduce risk of cancer
Lignans	Flax, rye, vegetables	Prevention of cancer; renal failure
Tannins (proanthocyanidines)	Cranberries,cranberry products, cocoa, chocolate	Improve urinary tract health; reduce risk of cardiovascular desease
	Plant sterols	
Stanol ester	Corn, soil, wheat, wood oils	Lower blood cholesterol levels b inhibiting cholesterol absorption
	Prebiotics and probiotics	
Fructo-oligosaccharides (FOS)	shallots, onion powder	Improve quality of intestinal microflora; gastrointestinal health
	Lactobacillius	
Biofidobacterium	Yogurt, other dairy products	Improve quality of intestinal microflora; gastrointestinal health
	Soy phytoestrogens	
Isoflavones: Daidzein, Genistein	Soybean and soy-based foods	Menopause symptoms such as hot flashes; protection against heart disease and some cancers; lowering of LDL and total cholesterol

1.3.2 Nutraceuticals

Parallel to the role of functional foods, the global landscape has seen the advent of the new concept of "nutraceutical", term coined in 1989 by Stephen De Felice, founder and president of the Foundation for Innovation in Medicine, fusing the words "nutrition" and "pharmaceutical" [72]. It refers to health promoting bioactive compounds found in some foods, extracted, refined and finally sold as purified preparations, or added to other foods [9]. Nutraceuticals can be realized in form of pills, capsules, powders, as formulations that contain single or mixed compounds, but they can be also incorporated into foods or foods supplement as functional ingredients. Chemically nutraceuticals can be classified as isoprenoid derivatives (terpenoids, carotenoids, saponins, tocotrienols, tocopherols, terpenes), phenolic compounds (tannins, lignans, anthocyanins, isoflavones, flavonones, flavanoids), carbohydrate derivatives (oligosaccharides, not-starch polysaccharides), fatty acid and structural lipids, PUFA, conjugated linoleic acid (CLA), MUFA, sphingolipids, lecithins, amino acid derivatives (allyl-S compounds, capsaicinoids, isothiocyanates, indoles, folate, choline), microbes (probiotics, prebiotics) and minerals [73]. They play a crucial role in maintaining optimal immune response and they exert antioxidant, antibacterial, anti-inflammatory, anti-diabetic, antifungal, antiproliferative, hepato-protector, hypolipidemic and analgesic effects [74]. Examples of nutraceutical grouped by mechanism of action are reported in **Table 1.5.**

By definition, nutraceuticals may possess or not nutritional properties, but should provide clinical health benefits by means of their pharmaceutical activities. They are not synthetic drugs and represent the interface between food and medication (**Figure 1.12**) [75].

Table 1.5. *Examples of nutraceutical grouped by mechanism of action [73].*

Anticancer	Positive influence on blood lipid profile	Antioxidant activity	Anti inflammatory	Osteogenetic or Bone protective
Capsaicin	MUFA	CLA	Linoleic acid	CLA
Genestein	Quercetin	Ascorbic acid	EPA	Soy protein
Daidzein	Resveratrol	β-carotene	γ-linoleic acid	Fructooligosaccharides
α-Tocotrienol	Tannins	Polyphenols	Capsaicin	Inulin
γ-Tocotrienol	B-sitosterol	Gingerol	Curcumin	Calcium
Curcumin	Saponins	Hydroxytyrodol	Quercetin	Casein
Lutein	Pectin	Glutathione	Curcumin	Daidzein

Despite this definition, some important points relating to the differences between drugs and nutraceuticals, should be considered. In fact, a key feature of nutraceuticals compared to drugs regards the observation that the formers should not necessarily consist of a single active component, since the beneficial effects can be the result of synergistic actions of their specific components or classes of molecules.

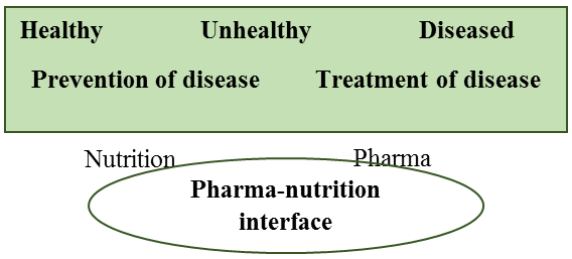


Figure 1.12. *The concept of nutraceutical agents [65].*

For drugs, we can tolerate the presence of side effects, while nutraceuticals must be safe, with minimal side effects compared to conventional drugs.

However, should be paid attention to the complexity of nutraceutical composition, whereby incorrect use could be toxic [66].
Pharmaceutical compounds and nutraceuticals may be able to treat and prevent pathological conditions, but only the formers foresee governmental sanctions, must prove to be safe and effective for human use, because of clinical trials. The lack of regulation leads to an incorrect approach to the nutraceutical products by the consumers who tend to use them without the required attention for the only reason that they are *natural* [66].

1.3.3 EU legislation, healthy claims and marketing implications

Nutraceutical products are widely available and minimally monitored since companies are not obligated to back claims about the function and effectiveness of their product. Despite this, Stephen DeFelice proposed in 2002 a first law on research and education Nutraceutical (NREA), focused to the creation of a Nutraceutical Commission (NUCOM) for the approval of nutraceuticals and also development of programs specifically addressed to encourage clinical research [76]. On the opposite, functional foods are subjected to a more detailed regulation than nutraceuticals [77]. In fact, in Japan, to be approved as FOSHU by the Minister of Health, Labor and Welfare these products must meet three specific criteria: assessment of the real efficacy in clinical trials; assessment of safety; determination of active functional ingredients [69]. Once approved, the FOSHU label contains: declared health claim, the food daily amount recommended, nutritional information, guidelines to healthy life and other information [69].
In EU government, the rules on nutrition and health claims have been established by Regulation (EC) No 1924/2006, and was applied starting from July, 1st 2007. This regulation is the legal framework used by food business operators when they want to highlight the particular beneficial effects of their

products, in relation to health and nutrition, on the product label or in their advertising. According to the Regulation, there are nutritional claims, such as "low fat", "high fiber", and health claims, such as "Vitamin D is needed for the normal growth and development of bone in children". These rules try to ensure that any claims made on a food labelling, presentation or advertising in the European Union is clear, accurate and based on scientific evidence. The main rules are the following:

- Regulation 1924/2006 on nutrition and health claims made on foods;
- Commission Regulation No 353/2008 establishing implementing rules for applications for authorization of health claims;
- Commission Regulation No 1169/2009 amending Regulation (EC) No 353/2008 establishing implementing rules for applications for authorization of health claims.

In particular, to define the health claims the European Union set up a European Commission Concerted Action coordinated by the International Life Sciences Institute (ILSI) Europe that establishes the Functional Food Science in Europe (FUFOSE). The aim was to develop and establish a science-based approach that supports the declaration of beneficial effect on an identified physiological function in the body, or to improve an individual state of health and well-being and/or reduce the risk of disease. The FUFOSE project looked at six areas of science and health: growth, development and differentiation, substrate metabolism, defense against ROS, functional foods and the cardiovascular system, gastrointestinal physiology and function, and the effects of foods on behavior and psychological performance. The final document was published in the British Journal of Nutrition [78]. The report indicates that functional foods should be in the form of normal foods and they must demonstrate their effects in amounts that can be normally consumed in the diet.

The new European Regulation on nutrition and health claims had come into force in January 2007. This followed a concerted action project supported by the European Commission titled “Process for the Assessment of Scientific Support for Claims on Foods” (PASSCLAIM) aiming to resolve some of the ongoing issues of validation, scientific substantiation of claims and communication to the consumer. The project started and will build upon the principle that "enhanced function" and "reduced risk of disease" claims should be based on designed clinical studies using appropriately identified, characterized and validated biomarkers. PASSCLAIM aims to establish common criteria to assess the scientific substantiation of health-claims, providing the framework to prepare scientific dossiers supporting claims [79]. The PASSCLAIM Consensus Document will assist those making claims and it will also improve the credibility of claims for consumers. This integrated strategy will generate more consumer confidence in the science base related to claims on foods and will better address the concerns of consumers [79].

The European Food and Safety Authority (EFSA) analyzes the evidence for the health claim. EFSA is responsible to verify the scientific substantiation of the submitted claims, some of which are currently in use, others are proposed by applicants who submit claims with the aim to authorize the commercialization of their products in EU. The EFSA opinions serve as basis for the European Commission and Member States, which will then decide whether to authorize the claims.

1.4 Specific aims of the Doctorate project

The main aim of the present Doctorate project is to evaluate the effects of bioactive extracts prepared from local Italian cultivars using pre-clinical

models resembling chronical and degenerative diseases, as summarized in **Figure 1.13**.

The initial goal is the identification of bioactive components with functional effects by a screening of Italian varieties of fruits and vegetables by the following steps.

-Preparation of bioactive extracts from selected food matrices;

-Identification of the most appropriate experimental models to assess the beneficial outcomes of food extracts, in details:

- Human Red Blood Cells (RBCs) and plasma, to evaluate the antioxidant response;
- Cell lines mimicking a neurodegenerative damage, to evaluate the protection from aging-related dysfunctions;
- Malignant cell lines to evaluate the chemopreventive effects of bioactive extracts.

The biological and biochemical characterization of bioactive extracts are relating to the following activities:

- Protection from oxidative stress;
- Protection from neurodegenerative damage;
- Protection from malignant transformation.

Among the typical Italian products/cultivars under investigation, two of them will be selected as rich sources of polyphenols and carotenoids, e.g. red wine obtained from local grapes and Neapolitan pumpkin.

Campania Region preserves old and treasured grape varieties, which are the basis of oenology with a strong typicality. The region is one of the oldest settlement areas of vines and is characterized by the presence of centenarians Gyves in many vineyards, including *Hellenica vitis*, *Vitis apiana*, the *Vinum album phalanginum* and *Aminea gemina*, the progenitors of Aglianico, Fiano, Falanghina and Greco. The real "dominus" of the Campania vineyard is the

Aglianico, an ancient vine, which has long been known in the international scene, giving rise to wines of excellent quality, such as Taurasi of Avellino, first DOCG among the southern Italian DOCG and Mastroberardino Aglianico *(http://www.agricoltura.regione.campania.it).*

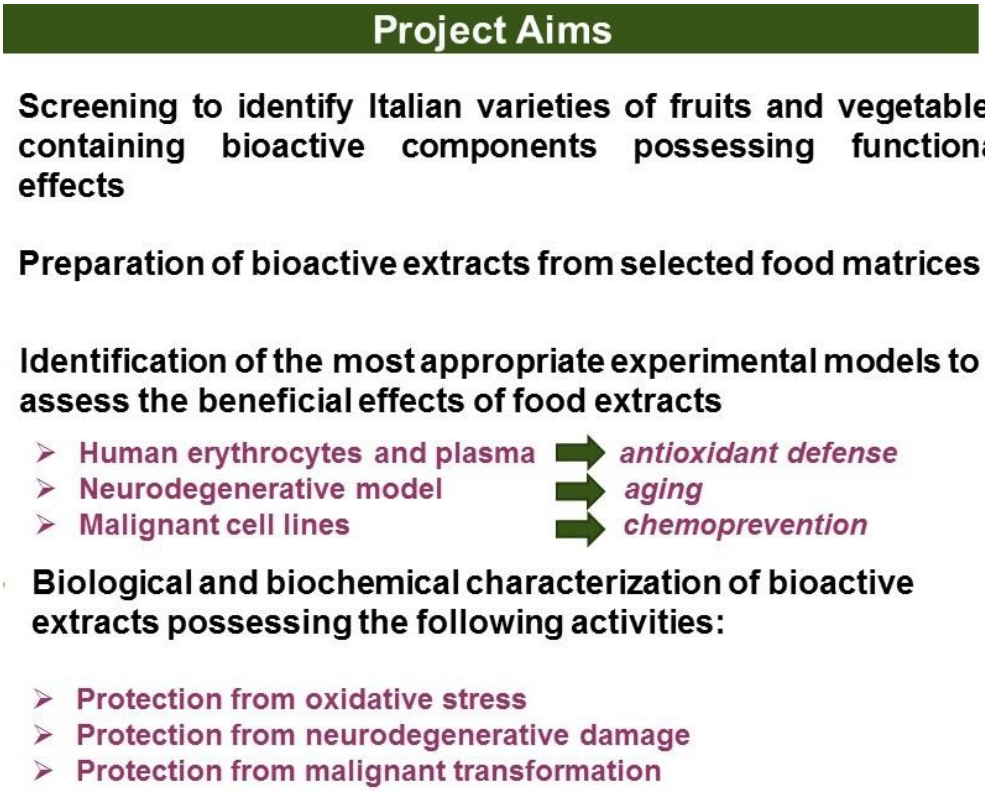


Figure 1.13. *Scheme summarizing the main aims of the project.*

Another typical product linked to the traditional cultivation of Campania Region is pumpkin, variety "Long Neapolitan Pumpkin" also known as pumpkin "full" of Naples. This cucurbitaceae, species *Cucurbita moschata*, is widely used in South Italy and enjoys a good reputation in Italy and abroad. Long Neapolitan Pumpkin in 2006 has been included in the list of traditional food products of the Campania Region. The Long Neapolitan Pumpkin is the

subject of a genetic stabilization program sponsored by Agriculture and Productive Activities of the Campania Region which was prompted by the recovery of semen found in the garden of a convent in the town of Visciano (Na) and it made landfall as early as the field cultivation on an experimental basis. The leaves are kidney-shaped, with 5-6 just mentioned lobes, dark green with lighter streaks, with strong presence of cirrus clouds. The flowers are carried axils of leaves and have a corolla bright yellow; male ones appear first and are characterized by a long stalk; female ones have a short stalk that holds the ovary from which will originate the result in fertilization took place. The fruit is a peponide, curved and swollen on both ends. It can be 60-70 cm long (exceptionally 100) with a diameter of 15-20 cm; its weight is, on average, of about 20-25 Kg. The skin is smooth, thin, light green or dark green. It is completely filled with orange-red flesh, firm, sweet and musky flavor. The internal cavity contains numerous seeds of white color, gray-brown, oval, flattened, slightly wrinkled and with the marginal rim clearly detected.

1.5 References

1. Russo, G.L., *Ins and outs of dietary phytochemicals in cancer chemoprevention.* Biochem Pharmacol, 2007. **74**(4): p. 533-44.
2. Sporn, M.B. and N. Suh, *Chemoprevention: an essential approach to controlling cancer.* Nat Rev Cancer, 2002. **2**(7): p. 537-43.
3. Russo, M., et al., *Phytochemicals in Cancer Prevention and Therapy: Truth or Dare?* Toxins 2010. **2**(4): p. 517-51.
4. Spagnuolo, C., et al., *Neuroprotective Role of Natural Polyphenols.* Curr Top Med Chem, 2016. **16**(17): p. 1943-50.
5. Russo, M., et al., *The flavonoid quercetin in disease prevention and therapy: facts and fancies.* Biochem Pharmacol, 2012. **83**(1): p. 6-15.
6. Ruiz, R.B. and P.S. Hernandez, *Cancer chemoprevention by dietary phytochemicals: Epidemiological evidence.* Maturitas, 2016. **94**: p. 13-19.
7. Howes, M.J. and M.S. Simmonds, *The role of phytochemicals as micronutrients in health and disease.* Curr Opin Clin Nutr Metab Care, 2014. **17**(6): p. 558-66.
8. Reis, J.F., et al., *Action mechanism and cardiovascular effect of anthocyanins: a systematic review of animal and human studies.* J Transl Med, 2016. **14**(1): p. 315.
9. Tome-Carneiro, J. and F. Visioli, *Polyphenol-based nutraceuticals for the prevention and treatment of cardiovascular disease: Review of human evidence.* Phytomedicine, 2016. **23**(11): p. 1145-74.
10. Goncharov, N.V., et al., *Reactive oxygen species in pathogenesis of atherosclerosis.* Curr Pharm Des, 2015. **21**(9): p. 1134-46.
11. Iriti, M. and F. Faoro, *Bioactivity of grape chemicals for human health.* Nat Prod Commun, 2009. **4**(5): p. 611-34.
12. Rasines-Perea, Z. and P.L. Teissedre, *Grape Polyphenols' Effects in Human Cardiovascular Diseases and Diabetes.* Molecules, 2017. **22**(1).
13. Renaud, S. and M. de Lorgeril, *Wine, alcohol, platelets, and the French paradox for coronary heart disease.* Lancet, 1992. **339**(8808): p. 1523-26.
14. Markoski, M.M., et al., *Molecular Properties of Red Wine Compounds and Cardiometabolic Benefits.* Nutr Metab Insights, 2016. **9**: p. 51-7.
15. Tousoulis, D., et al., *Acute effects of different alcoholic beverages on vascular endothelium, inflammatory markers and thrombosis fibrinolysis system.* Clin Nutr, 2008. **27**(4): p. 594-600.
16. Tousoulis, D., M. Charakida, and C. Stefanadis, *Endothelial function and inflammation in coronary artery disease.* Postgrad Med J, 2008. **84**(993): p. 368-71.

17. Russo, P., et al., *Effects of de-alcoholated red wine and its phenolic fractions on platelet aggregation.* Nutr Metab Cardiovasc Dis, 2001. **11**(1): p. 25-9.
18. Ferreira, S.T. and W.L. Klein, *The Abeta oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease.* Neurobiol Learn Mem, 2011. **96**(4): p. 529-43.
19. Libro, R., et al., *Natural Phytochemicals in the Treatment and Prevention of Dementia: An Overview.* Molecules, 2016. **21**(4): p. 518.
20. Perez-Hernandez, J., et al., *A Potential Alternative against Neurodegenerative Diseases: Phytodrugs.* Oxid Med Cell Longev, 2016. **2016**: p. 19.
21. Sorrentino, P., et al., *The dark sides of amyloid in Alzheimer's disease pathogenesis.* FEBS Lett, 2014. **588**(5): p. 641-52.
22. Pooler, A.M., W. Noble, and D.P. Hanger, *A role for tau at the synapse in Alzheimer's disease pathogenesis.* Neuropharmacology, 2014. **76 Pt A**: p. 1-8.
23. Gonzalez, H., et al., *Neuroimmune regulation of microglial activity involved in neuroinflammation and neurodegenerative diseases.* J Neuroimmunol, 2014. **274**(1-2): p. 1-13.
24. Greig, N.H., D.K. Lahiri, and K. Sambamurti, *Butyrylcholinesterase: an important new target in Alzheimer's disease therapy.* Int Psychogeriatr, 2002. **14 Suppl 1**: p. 77-91.
25. Lindsay, J., et al., *Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging.* Am J Epidemiol, 2002. **156**(5): p. 445-53.
26. Mandel, S.A., et al., *Understanding the broad-spectrum neuroprotective action profile of green tea polyphenols in aging and neurodegenerative diseases.* J Alzheimers Dis, 2011. **25**(2): p. 187-208.
27. Wang, J., et al., *Targeting multiple pathogenic mechanisms with polyphenols for the treatment of Alzheimer's disease-experimental approach and therapeutic implications.* Front Aging Neurosci, 2014. **6**: p. 42.
28. Kang, N.J., et al., *Polyphenols as small molecular inhibitors of signaling cascades in carcinogenesis.* Pharmacol Ther, 2011. **130**(3): p. 310-24.
29. Jayasena, T., et al., *The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease.* Ageing Res Rev, 2013. **12**(4): p. 867-83.
30. Moreira, P.I., et al., *Oxidative stress: the old enemy in Alzheimer's disease pathophysiology.* Curr Alzheimer Res, 2005. **2**(4): p. 403-8.
31. Smid, S.D., J.L. Maag, and I.F. Musgrave, *Dietary polyphenol-derived protection against neurotoxic beta-amyloid protein: from molecular to clinical.* Food Funct, 2012. **3**(12): p. 1242-50.

32. Mandel, S., et al., *Iron dysregulation in Alzheimer's disease: multimodal brain permeable iron chelating drugs, possessing neuroprotective-neurorescue and amyloid precursor protein-processing regulatory activities as therapeutic agents.* Prog Neurobiol, 2007. **82**(6): p. 348-60.
33. Lewandowska, U., J. Fichna, and S. Gorlach, *Enhancement of anticancer potential of polyphenols by covalent modifications.* Biochem Pharmacol, 2016. **109**: p. 1-13.
34. Abbott, N.J. and I.A. Romero, *Transporting therapeutics across the blood-brain barrier.* Mol Med Today, 1996. **2**(3): p. 106-13.
35. Faria, A., et al., *Insights into the putative catechin and epicatechin transport across blood-brain barrier.* Food Funct, 2011. **2**(1): p. 39-44.
36. Vauzour, D., et al., *The neuroprotective potential of flavonoids: a multiplicity of effects.* Genes Nutr, 2008. **3**(3-4): p. 115-26.
37. Siddiqui, I.A., et al., *Resveratrol nanoformulation for cancer prevention and therapy.* Ann N Y Acad Sci, 2015. **1348**(1): p. 20-31.
38. Surh, Y.J., *Cancer chemoprevention with dietary phytochemicals.* Nat Rev Cancer, 2003. **3**(10): p. 768-80.
39. Bilotto, S., et al., *Dietary Phytochemicals in Chemoprevention of Cancer: An Update.* Immun., Endoc. & Metab. Agents in Med. Chem., 2013. **13**: p. 2-24.
40. Umar, A., B.K. Dunn, and P. Greenwald, *Future directions in cancer prevention.* Nat Rev Cancer, 2012. **12**(12): p. 835-48.
41. Linnewiel-Hermoni, K., et al., *The anti-cancer effects of carotenoids and other phytonutrients resides in their combined activity.* Arch Biochem Biophys, 2015. **572**: p. 28-35.
42. Omenn, G.S., et al., *Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial.* J Natl Cancer Inst, 1996. **88**(21): p. 1550-9.
43. Pandey, K.B. and S.I. Rizvi, *Plant polyphenols as dietary antioxidants in human health and disease.* Oxid Med Cell Longev, 2009. **2**(5): p. 270-8.
44. Dai, J. and R.J. Mumper, *Plant phenolics: extraction, analysis and their antioxidant and anticancer properties.* Molecules, 2010. **15**(10): p. 7313-52.
45. Manach, C. and J.L. Donovan, *Pharmacokinetics and metabolism of dietary flavonoids in humans.* Free Radic Res, 2004. **38**(8): p. 771-85.
46. Manach, C., et al., *Polyphenols: food sources and bioavailability.* Am J Clin Nutr, 2004. **79**(5): p. 727-47.
47. Tsao, R., *Chemistry and biochemistry of dietary polyphenols.* Nutrients, 2010. **2**(12): p. 1231-46.
48. Bors, W., et al., *Flavonoids as antioxidants: determination of radical-scavenging efficiencies.* Methods Enzymol, 1990. **186**: p. 343-55.
49. Philpott, M., C.C. Lim, and L.R. Ferguson, *Dietary protection against free radicals: a case for multiple testing to establish structure-activity*

relationships for antioxidant potential of anthocyanic plant species. Int J Mol Sci, 2009. **10**(3): p. 1081-103.
50. Speciale, A., et al., *Bioavailability and molecular activities of anthocyanins as modulators of endothelial function.* Genes Nutr, 2014. **9**(4): p. 404.
51. Scalbert, A., et al., *Absorption and metabolism of polyphenols in the gut and impact on health.* Biomed Pharmacother, 2002. **56**(6): p. 276-82.
52. Niranjana, R., et al., *Carotenoids modulate the hallmarks of cancer cells.* NAO, 2015. **18 Pt B**: p. 968–85.
53. Tanaka, T., M. Shnimizu, and H. Moriwaki, *Cancer chemoprevention by carotenoids.* Molecules, 2012. **17**(3): p. 3202-42.
54. Saini, R.K., S.H. Nile, and S.W. Park, *Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities.* Food Res Int, 2015. **76**(3): p. 735–50.
55. Mezzomo, N. and S.R.S. Ferreira, *Carotenoids Functionality, Sources, and Processing by Supercritical Technology: A Review.* J Chem, 2016. **2016**: p. 1-16.
56. Shete, V. and L. Quadro, *Mammalian metabolism of beta-carotene: gaps in knowledge.* Nutrients, 2013. **5**(12): p. 4849-68.
57. Namitha, K.K. and P.S. Negi, *Chemistry and biotechnology of carotenoids.* Crit Rev Food Sci Nutr, 2010. **50**(8): p. 728-60.
58. Younesi, E. and M.T. Ayseli, *An integrated systems-based model for substantiation of health claims in functional food development.* Trends food sci tech, 2015. **41**(1): p. 95–100.
59. Pan, S.Y., et al., *New Perspectives on How to Discover Drugs from Herbal Medicines: CAM's Outstanding Contribution to Modern Therapeutics.* Evid Based Complement Alternat Med, 2013. **2013**(2013): p. 25.
60. Zamora-Ros, R., et al., *High concentrations of a urinary biomarker of polyphenol intake are associated with decreased mortality in older adults.* J Nutr, 2013. **143**(9): p. 1445-50.
61. Azqueta, A. and A. Collins, *Polyphenols and DNA Damage: A Mixed Blessing.* Nutrients, 2016. **8**(12).
62. Bellavia, A., et al., *Fruit and vegetable consumption and all-cause mortality: a dose-response analysis.* Am J Clin Nutr, 2013. **98**(2): p. 454-9.
63. Costamagna, M.S., et al., *Polyphenols rich fraction from Geoffroea decorticans fruits flour affects key enzymes involved in metabolic syndrome, oxidative stress and inflammatory process.* Food Chem, 2016. **190**: p. 392-402.
64. Giampieri, F., et al., *Polyphenol-rich strawberry extract protects human dermal fibroblasts against hydrogen peroxide oxidative damage and*

improves mitochondrial functionality. Molecules, 2014. **19**(6): p. 7798-816.
65. Eussen, S.R.B.M., et al., *Functional foods and dietary supplements: Products at the interface between pharma and nutrition.* Eur J Pharmacol, 2011. **668 Suppl 1**: p. S2-S9.
66. Hardy, G., *Nutraceuticals and functional foods: introduction and meaning.* Nutrition, 2000. **16**(7-8): p. 688-9.
67. Bigliardi, B. and F. Galati, *Innovation trends in the food industry: The case of functional foods.* Trends Food Sci Tech, 2013. **31**(2): p. 118–29.
68. Aronson, J.K., *Defining 'nutraceuticals': neither nutritious nor pharmaceutical.* Br J Clin Pharmacol, 2017. **83**(1): p. 8-19.
69. Martirosyan, D.M. and S. Jaishree, *A new definition of functional food by FFC: what makes a new definition unique?* Funct food health dis, 2015. **5**(6): p. 209-23.
70. Siro, I., et al., *Functional food. Product development, marketing and consumer acceptance--a review.* Appetite, 2008. **51**(3): p. 456-67.
71. Abuajah, C.I., A.C. Ogbonna, and C.M. Osuji, *Functional components and medicinal properties of food: a review.* J Food Sci Technol, 2015. **52**(5): p. 2522-9.
72. DeFelice, S., *The nutraceutical revolution: its impact on food industry R&D.* Trends Food Sci Tech, 1995. **6**(2): p. 59–61.
73. Keservani, R.K., et al., *Nutraceutical and Functional Food as Future Food: A Review.* Pharm Lett, 2010. **2**(1): p. 106-16.
74. Govender, M., et al., *A review of the advancements in probiotic delivery: Conventional vs. non-conventional formulations for intestinal flora supplementation.* AAPS PharmSciTech, 2014. **15**(1): p. 29-43.
75. Cencic, A. and W. Chingwaru, *The role of functional foods, nutraceuticals, and food supplements in intestinal health.* Nutrients, 2010. **2**(6): p. 611-25.
76. Santini, A., G.C. Tenore, and E. Novellino, *Nutraceuticals: A paradigm of proactive medicine.* Eur J Pharm Sci, 2017. **96**: p. 53-61.
77. Magnuson, B., et al., *Review of the regulation and safety assessment of food substances in various countries and jurisdictions.* Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 2013. **30**(7): p. 1147-220.
78. Bellisle, F., et al., *Functional Food Science in Europe.* Br J Nutr, 1998. **80 Suppl 1**: p. S1- S193.
79. *Scientific concepts of functional foods in Europe. Consensus document.* Br J Nutr, 1999. **81 Suppl 1**: p. S1-27.

Chapter II

Antioxidant activity of red wine polyphenols in human RBCs

2.1 Introduction

The protective role of red wine polyphenols against oxidative stress in RBCs has been reported in several *in vivo* and *in vitro* studies [1]. As mentioned in Chapter I, many epidemiological studies indicated that a moderate and regular consumption of red wine decreases cardiovascular and cerebrovascular ischemic events, through the ability to increase the antioxidant plasmatic potential, by improving endothelium dependent vasodilation [2] and inhibiting platelet aggregation [3] [4]. In addition, a regular intake of red wine inhibits lipoproteins oxidation, macrophage cholesterol accumulation and foam-cell formation [5]. These properties can be partially explained considering the presence of phenolic components in red wine, able to interact with physiological targets, through different mechanisms. For example, it has been reported that a daily intake of resveratrol protects Cu-oxLDL against peroxynitrite, copper, or 2-amidinopropane dihydrochloride-induced oxidation [6]. A recent review discusses the role of resveratrol in reducing organ damage following ischemia. Such effect is functionally related to a decreased formation of ROS and pro-inflammatory cytokines, as well as the mediation of a variety of intracellular signaling pathways, including NOS, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, MAPK and estrogen receptor-related pathways [7]. Furthermore, the red wine cardioprotective properties have been also referred to the presence of other phenolic compounds, such as anthocyanins. They are water soluble compounds, abundantly present in red wines and responsible for their red color. The main anthocyanins of a typical Italian red wine are malvidin-3-O-glucoside, cyanidin-3-O-glucoside, peonidin-3-O-glucoside and delphinidin-3-O-glucoside [8]. The presence of anthocyanins in red wines has been related to their ability to interact with endothelial functions, promoting vascular homeostasis through the release of NO and controlling platelet aggregation, as

reported above [9]. In addition, red wine anthocyanins exert antioxidant effects against H_2O_2-generated hemolysis of normal and catalase-inactive human RBCs [10]. Their antioxidant activity is closely connected to hydroxyl groups and to conjugated double bond system, even if it is assumed that their biological effects cannot be explained solely on the based on these features [9]. The inhibition of lipid peroxidation by red wine anthocyanin derivatives is also mediated by their interaction with lipid bilayer membrane [11]. One attractive hypothesis considers the possibility that phenolic groups of red wine components are oxidized by polyphenol oxidase to produce extremely reactive intermediates which, in turns, trigger the cellular antioxidant defenses [12].

For this reason, the role of antioxidant compounds, which modulate the cellular redox state has been considered [13]. In fact, in subjects receiving a diet low in antioxidants, a reduction in the activity of antioxidant enzymes, including catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), is measured in RBCs [14].

RBCs represent an ideal *in vitro* experimental model for the evaluation of the antioxidant defence mechanisms. In fact, they are constantly exposed to oxidative stress, due to their role of oxygen carriers and the high concentration of redox active haemoglobin with iron molecules [15]. These processes can catalyse free radicals reactions and produce ROS, responsible for oxidative stress when they are not removed by the endogenous defence systems [16]. ROS are a large family of highly reactive species, such as hydrogen peroxide, organic peroxide, superoxide anion and hydroxyl radical, that are formed through enzymatic and non-enzymatic reactions, through metabolic processes, or by exogenous sources such as food components, drugs, ultraviolet light and ionizing radiations. When present at not physiological concentrations, they can induce cell damage directly or through the formation of intermediates involved in different signalling pathways inducing the damage of RBCs [12]. In fact, elevated concentrations of ROS are involved in the damage of RBCs of sickle

cell anemia and β-thalassemia [17]. Furthermore, due to the absence of nuclei and mitochondria, oxidant molecules cause deformation of RBCs membrane and osmotic fragility, being the structural integrity particularly sensitive to changes of reactive oxygen content [16]. In particular, regarding their endogenous defences, RBCs are provided with several antioxidant enzymes, such as superoxide dismutase (SOD), CAT, GR, and GPx [18, 19] (**Figure 2.1**). An important role in maintaining cellular redox state is played by reduced glutathione (GSH), a peptide with cysteine residue containing a reactive thiol group. From the oxidation of GSH is produced the disulfide form (GSSG), which is converted back to GSH by the enzyme GR [20] (**Figure 2.1**). GSH is involved in cell detoxification processes, acting as antioxidant and scavenging free radicals. Indeed, a suitable ratio of GSH and GSSG is relevant for cell vitality and allows to maintain intracellular redox potential [21].

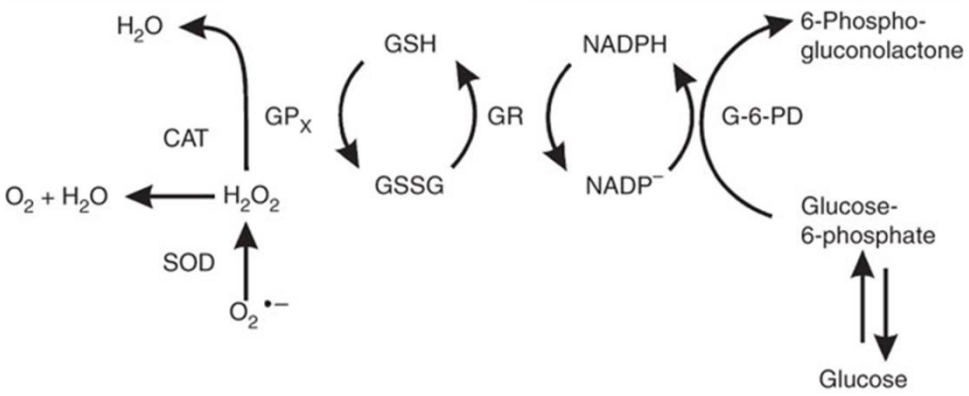


Figure 2.1. *Schematic activity of RBCs antioxidant enzymes [22].*

Furthermore, the surface of RBCs displays a cross-membrane electrons transport complex, named Plasma Membrane Redox System (PMRS) (**Figure 2.2**). PMRS has been suggested to play a vital role in reducing oxidative stress and this property has been hypothesized to control the rate of aging, lifespan,

and many pathological conditions associated with increased oxidative stress [23]. PMRS is not yet completely biochemically and functionally characterized and includes key enzymes differently expressed in various cell types, including membrane-associated oxidoreductase enzymes, such as NAD(P)H-dependent redox enzymes, cytochrome b5 reductase (b5R), a member of the cytochrome b561 family, and NAD(P)H-quinone oxidoreductase 1 (NQO1). The electron shuttles are coenzyme Q and cytochrome c and electrons donors, including NAD(P)H [24]. This enzymatic complex transfers electrons across the cell membrane from intracellular electron donors, such as NADH or ascorbate (ASC), GSH, to extracellular acceptors, like ascorbyl free radical (AFR), neutralizing free radical and maintaining the extracellular concentration of plasma antioxidant molecules, such as ASC and tocopherol [25] (**Figure 2.2**).

It is largely accepted that one of the main function of PMRS is the recovery of ASC, preventing its oxidation to AFR, which forms dehydroascorbate (DHA), a highly unstable compound [26]. More recently, ASC has been recognized as the most relevant intracellular electron donor in mammalian cells, with several mechanisms to maintain the intracellular ASC concentration in the millimolar range [26].

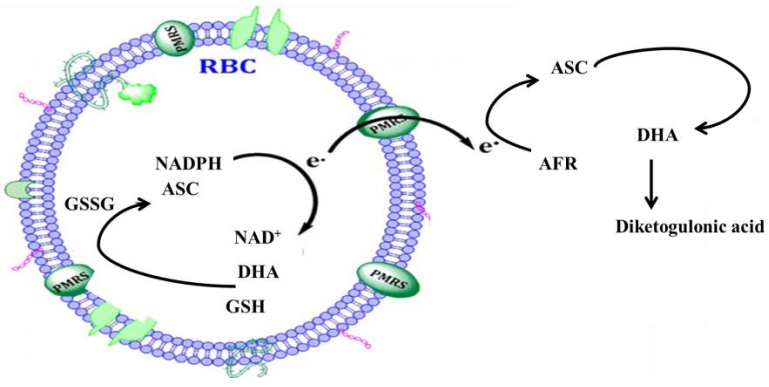


Figure 2.2. *Schematic diagram of PMRS in the maintenance of intracellular and extracellular redox state.*

Human RBCs are peculiar since they keep intracellular ASC concentrations equivalent to those present in plasma (40–80 μM) by a rapid and efficient uptake of extracellular DHA and reduce it to ASC [26]. The general mechanism implies the oxidation of extracellular ASC to AFR, which in the absence of the enzymatic reduction to ASC, can reacts with another AFR and forms one ASC and one DHA unit. DHA is highly unstable and it can undergo a double fate: rapid cellular uptake through the glucose transporter, GLUT-1 [27] and reduction to ASC, or hydrolysis to 2,3-diketogulonate, with irreversible loss of ASC (**Figure 2.3**). Once inside the RBCs, DHA is rapidly reduced, enzymatically or not enzymatically by GSH and reactions catalyzed by glutaredoxin and thioredoxin reductase to ASC, which becomes available for PMRS activity.

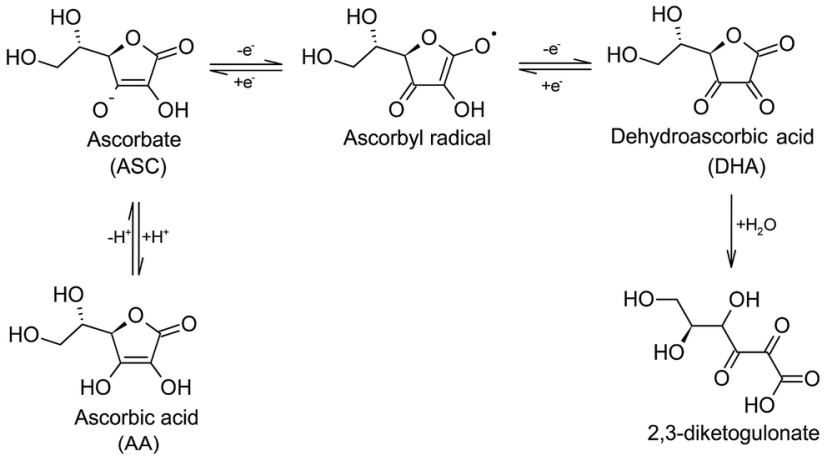


Figure 2.3. *Reactions of ascorbic acid [28]*.

The synergistic action of the PMRS and DHA reductase provides a recovery mechanism of ASC between the intra- and extracellular compartments. The ASC is able to regenerate the α-tocopherol and prevent the oxidative damage to cellular proteins from peroxyl radicals $ROO^{\bullet}$, which is firstly oxidized in plasma [29].

The importance of the PMRS is also highlighted by its key role in maintaining balanced NAD(P)/NAD(P)H ratio, essential for the normal energy metabolism and cell survival [30]. PMRS transports electrons from cytosolic NAD(P)H to extracellular electron acceptors, with the involvement at least two components, NADH-cytochrome b5 oxidoreductase and DT-diaphorase. It is also supported by NADPH oxidase, an enzyme complex on the plasma membrane, whose connection with the PMRS is still unclear [31].

It should be emphasized that the exact function of PMRS has not been fully understood, even if this complex is involved in many activities, such as: 1. maintenance of the redox state of sulfhydryl residues in membrane proteins; 2. neutralization of the oxidative molecules outside the cells; 3. recycle of the tocopherol; 4. reduction of lipid hydroperoxides; 5. maintenance of the extracellular concentration of ASC [29].

The induction of oxidative damage that occurs during aging is a condition characterized by a decrease of the antioxidant systems efficiency [32] [33]. However, it has been shown that PMRS activity reduces the oxidative damage during aging [34] [35]. Exogenous antioxidants, such as polyphenols, can modulate PMRS activity, improving the protection from the oxidative stress [29] [36]. The ability of polyphenols to provide electrons to this system is supported by the hypothesis that these molecules can cross the cell membranes [36]. In fact, the presence of caffeic acid, taxifolin, and ferulic acid has been evidenced into RBCs, even if only their metabolites, such as d-(3,4-dihydroxy-phenyl)-γ-valerolactone, show increased accumulation [37]. Probably, the uptake of these metabolites takes place via GLUT-1 transporter since their partitioning into RBCs is significantly inhibited by the presence of glucose [37]. Once inside the cells, polyphenols can donate electrons to extracellular electron acceptors through PMRS [29].

Other evidence also supports the hypothesis that red wine polyphenols, such as quercetin and resveratrol, can be incorporate into RBCs and activate PMRS

[29]. Therefore, considering the protective role of red wine polyphenols in CVD risk, it is reasonable to investigate the role of red wine polyphenols to protect human RBCs from oxidative stress through a potential activation of PMRS.

2.2 Aims of the Chapter

The aim of this Chapter has been the evaluation of the ability of red wine polyphenols to improve the antioxidant activity of RBCs, through a mechanism that involves the modulation of PMRS activity.

In particular, the specific aims of the present study have been:

-To evaluate the red wine ability to increase PMRS activity;

-To verify if red wine polyphenols are able to modulate RBCs antioxidant systems, such as GSH and GSH-dependent enzymes;

-To evaluate if red wine interfere with intracellular ROS in RBCs;

-To investigate the red wine components responsible for the antioxidant effects in RBCs.

Overall, in this Chapter, we investigated, at molecular level, if and how red wine components could potentiate antioxidant defenses in human RBCs.

2.3 Materials and Methods

2.3.1 Chemicals

Phosphate buffer saline (PBS) tablets (Life Technologies); Polyvinylpolypyrrolidone (PVPP); Gallic acid; Ferrous sulfate ($FeSO_4$); 1,1-diphenyl-2-picrilidrazilic (DPPH$^{\bullet}$) (Sigma-Aldrich); 5,5'-dithiobis(2-nitrobenzoic) acid (Nbs2); HCl; Ethanol; HOCl; Ascorbic acid; Ascorbate oxidase (AO); Xylenol orange (XO); Ammonium iron(II) sulfate hexahydrate; 2,6-di-tert-butyl-4-methylphenol; Potassium ferricyanide ($K_3Fe(CN)_6$); Sodium acetate (NaOAc); Citric acid; Ferric chloride ($FeCl_3$); Bathophenanthrolinedisulfonic acid disodium salt hydrate; Glucose; Iodoacetamide (IAC); N-acetyl-l-cysteine (NAC); Trichloroacetic acid (TCA); Phtaldialdehyde; Glutathione (GSH); Folin-Ciocalteau's reagent (FCR) (Sigma-Aldrich); 2,4,6-Tris (2-pyridyl)-triazine (TPTZ) (Sigma-Aldrich); Phtaldialdehyde; Sodium hypochlorite (NaOCl); Ethylenediaminetetraacetic acid (EDTA); Potassium metabisulfite (Carlo Erba); Dichlorofluorescein-diacetate (DCFDA); Nicotinamide adenine dinucleotide phosphate reduced (NADPH); Phenylmethylsulfonyl fluoride (PMSF); Flavin adenine dinucleotide (FAD); 3,5-Di-tert-butyl-4-hydroxytoluene (BHT); Oxidized glutathione (GSSG); Glutathione reductase (GR); Magnesium chloride ($MgCl_2$); Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl); Pyrogallol; Glucose-6-phosphate (G6P). All other chemicals used were of research highest purity grade.

2.3.2 Preparation of RBCs and plasma

Human blood samples were obtained from healthy donors, not smoking males and females of an average age of 41.2 (23–64). All subjects gave their

informed consents for this study, which are in custody of Blood Donation Centre at the Division of Onco-Hematology of "San Giuseppe Moscati" hospital in Avellino. Blood samples were collected in EDTA treated tubes and than prepared through consecutive centrifugations and washing in PBS to remove plasma, platelets, and buffy coat. The plasma were employed by the chloramine assay, while RBCs were washed for three times in PBS at 1800x*g* for 10 min and used for hemolysis, PMRS, GSH and ROS measurement, and determination of antioxidant enzymes activity.

2.3.3 Production of red wine and red wine fractionation

A selected red wine (**RW**), "Aglianico" (VCR 13 2011) vinified in 2010 from *Vitis vinifera* cultivars, grown in the vineyard of the Mastroberardino's cellar, located in the area surrounding the city of Avellino (Italy), were obtained by a microvinification process, which allows controlled and reproducible conditions. Grapes were destemmed and crushed; then they must be treated with potassium metabisulfite (100 mg/l of must) and 2g/hl Lafazym CL were added (Polsinelli, Frosinone, Italy). Subsequently, the must was transferred in stainless steel tanks (100 l) for fermentation that took place at 22-23 °C with 20g/hl of *Saccharomyces cerevisiae* strain D254 (Lallemand Inc., Castel d'Azzano, Italy) and the cap was immersed twice a day by pumping over. Maceration of the pomace lasted 10 days then they must be pressed to obtain the final wine. After 24 h, wines were racked from gross lees, added with 100 mg/l of potassium metabisulfite and bottled. No malolactic fermentation occurred. The wine was analyzed at bottling time. After opening, wine samples were divided in aliquots, stored in the dark at room temperature and used once for all experiments. In some cases, to avoid volatile components, aliquots of wines were lyophilized and resuspended in the same volume of 0.01 N HCl. In order to separate RW components, two preparations were

obtained by consecutive extractions with ethyl acetate: the aqueous phase, rich in total anthocyanins, monomers and polymers (**Fraction A**) and the organic phase, containing not-anthocyanin molecules and including flavanols, phenolic acids and catechins (**Fraction B**) [10]. After preparation, the two fractions were dried and dissolved in 0.01 N HCl. Polyphenols-stripped RW (**Fraction C**) was prepared using a column of PVPP, which was previously activated by several washings with 12% ethanol v/v and centrifugations [38]. To separate the high molecular weight components of RW, indicated as polymers (**RWP**), RW was dialyzed against 0.01 N HCl using a membrane of 3000 kDa cut-off and left under stirring for 72 h. When necessary, aliquots of RWP were dried and suspended in 0.01N HCl.

2.3.4 Determination of polyphenol content and antioxidant activity

Total polyphenols content was measured by Folin-Ciocalteu's assay [39] and the results were expressed as μg/ml gallic acid equivalent (GAE), a polyphenol abundant in RW. The anthocyanin content was estimated using a pH shift method [40] and the results were expressed as micromolar of malvidin-3-glucoside (M3GE), the main anthocyanin present in RW. The antioxidant activity *in vitro* was determined by FRAP (Ferric Reducing Antioxidant Power) [41] and DPPH radical scavenging (free radical 2,2-diphenyl-1-picrylhydrazyl) [42] assays and the results were expressed as millimolar of a solution of pure $FeSO_4$ and % quenching, respectively.

2.3.5 Determination of plasma chloramines formation

Chloramines concentration was measured using Nbs (5-thio-2-nitrobenzoic acid), prepared from Nbs2 with NaOH before dilution into PBS [43]. Samples

of plasma were incubated with the maximal volume of RW allowed by the assay (corresponding to 9.13 μg/ml GAE) for 10 min at 37 °C or with 50 μM quercetin, as positive control. After incubation, samples were treated with 1 mM HOCl for 15 min at 4 °C. Subsequently, Nbs was added to samples for 5 min at room temperature. The amount of Nbs remaining after plasma incubation was inversely related to chloramines formation and was measured spectrophotometrically at 412 nm, using the molar extinction coefficient of 8160 M^{-1} cm^{-1}.

2.3.6 Hemolysis assay

RBCs ($8x10^5$ RBCs/μl) were diluted to 0.6 ml with PBS and were incubated with RW (73 μg/ml GAE) for 45 min at 37 °C [44]. Samples were centrifuged at 1800xg for 2 min and were washed for three times with PBS, and incubated with 300 μM HOCl for 15 min at 37 °C. After centrifugation at 1800x*g* for 2 min, the absorbance of supernatants was measured at 540 nm and results were expressed as percentage of hemolysis, which was calculated by the ratio between the absorbance values of samples and the value of total hemolysis, obtained by osmotic shock using distilled water (100% hemolysis).

2.3.7 Evaluation of antioxidant activity in RBCs

RBCs ($8x10^5$ RBCs/μl) were incubated with RW (73 μg/ml GAE) for 10 min at 37 °C. Samples were centrifuged at 1800x*g* for 2 min and were washed for three times with PBS, and hemolyzed with 0.3 ml of cold water. After centrifugation at 11000x*g* for 5 min, the supernatants were treated for three times with the same volume of absolute ethyl acetate. The organic phases were collected, were dried and incubated with a methanolic solution of DPPH (100

mM) for 30 min at room temperature. The samples were centrifuged at 11000x*g* for 5 min and the absorbance of supernatants was measured at 517 nm. The results were expressed as percentage of quenching.

2.3.8 Determination of Plasma Membrane Redox System

PMRS activity was determined following different RBC treatments. RBCs ($8x10^5$ RBCs/μl) were diluted to 0.6 ml with PBS and incubated at 37 °C with different concentrations of: 1. RW (7.3, 18.25, 36.5, 73, 109.5, 182.5 μg/ml GAE) for 10 min; 2. same volume (20 μl) of Fraction A (48 μg/ml GAE), Fraction B (8.3 μg/ml GAE), and Fraction C (0.3 μg/ml GAE); 3. RW and RWP (20 μl corresponding to 73 and 27 μg/ml GAE, respectively) for 1-10 min; 4. 200 μM catechin, 20 μM quercetin, 7.3 μM resveratrol, and 290 μM gallic acid (final concentrations); 5. preincubated with 10 mM IAC, or 40 mM NAC at 37 °C for 1 h. In all cases, after treatment and washing with PBS, RBCs were incubated with a mixture containing PBS, 5 mM glucose and 1 mM $K_3Fe(CN)_6$ at 37 °C for 30 min. Samples were centrifuged at 1800x*g* for 2 min and the supernatants were collected for PMRS assay [29]. Briefly, the supernatants (25 μl) were incubated for 5 min with a mixture containing 750 mM NaOAc, pH 6.5; 500 mM citric acid; 0.41 mM $FeCl_3$; 0.775 mM bathophenanthroline disulfonic acid disodium salt hydrate. Absorbance was measured at 540 nm and results were expressed as picomoles ferrocyanide/10^6 RBCs/min.

2.3.9 Measurement of GSH levels

RBCs ($8x10^5$ RBCs/μl) were diluted to 0.6 ml with PBS and incubated with RW (73 μg/ml GAE) for 10 min at 37 °C. Samples were centrifuged at 1800x*g*

for 2 min and were washed for three times with PBS, and solubilized with TCA solution (5% v/v final concentration in 0.1 M HCl and 10 mM EDTA). For measurement of GSH [45] 0.5mg/ml phtaldialdehyde in PBS plus 10 mM EDTA were added to the samples, which was centrifuged at 11000x*g*, before measuring the fluorescence of supernatants at 340 nm (excitation wavelength) and 460 nm (emission wavelength). The micromolar concentration of GSH was calculated from a standard curve of pure GSH.

2.3.10 Reactive oxygen species (ROS) measurement

RBCs ($8x10^5$ RBCs/μl) were preincubated for 30 min with 20 μM DCFDA, a non-fluorescent compound able to cross the cell membrane. DCFDA is hydrolyzed to dichlorofluorescein, which reacts with the intracellular peroxide and gives rise to 2',7'-dichlorofluorescin (DCF), detected spectrofluorimetrically. After incubation, RBCs were centrifuged, washed with PBS and treated for 1 or 10 min with RW (73 μg/ml GAE). ROS production was measured fluorometrically with excitation and emission settings at 495 and 530 nm, respectively [44].

2.3.11 Activity of antioxidant enzymes

RBCs ($8x10^5$ RBCs/μl) were diluted to 0.6 ml with PBS and were treated at different times with RW (73 μg/ml GAE). After incubation, samples were centrifuged at 1800x*g* for 2 min, washed for three times with PBS and were lysed with 5 mM phosphate buffer, pH 8.0 containing PMSF. After centrifugation at 11000x*g* for 5 min, supernatant was used for enzymatic assays.

- *Activity of glutathione reductase*

GR activity was measured according to Lopez et al. [46]. The oxidation of NADPH to $NADP^+$ during the reaction of GSSG reduction, was measured at 340 nm. After 5 min of incubation at 37 °C with 5.1 μM FAD 0.16 mM NADPH 0.49 mM EDTA, the reaction was started with the addition of 1.95 mM GSSG. The absorbance was measured after 30 min and the specific enzymatic activity was expressed as nmol/min/ml RBCs.

- *Activity of glutathione peroxidase*

GPx activity was measured according to Lopez et al. [46]. After 5 min of incubation at 37 °C with 0.38 mM GSH, 0.38 mM NADPH, 1.25 mM EDTA, 1.37 U/ml GR, the reaction was started with the addition of 78 mM BHT. The absorbance was measured after 30 min and the specific enzymatic activity was expressed as nmol/min/ml RBCs.

- *Activity of glucose-6-phosphate dehydrogenase*

G6PD activity was measured according to Sirati-Sabet et al. [47], assaying the production of NADPH at 340 nm. After 5 min of incubation at 37 °C with 0.3 mM NADP, 10 mM $MgCl_2$ in 100 mM Tris-HCl, pH 8.0, the reaction was started with the addition of 1 mM G6P. The absorbance was measured after 30 min and the enzymatic activity expressed as nmol/min/ml RBCs.

- *Activity of superoxide dismutase*

SOD activity was measured as previously reported by Li [48]. Samples, treated with RW as reported above, were incubated for 5 min at 37 °C with 0.05 M Tris-HCl, pH 7.4; 1 mM EDTA; subsequently, the reaction was started with the addition of 50 mM pyrogallol at 37 °C. The absorbance was measured after 20min and the specific enzymatic activity expressed as U/ml RBCs.

2.3.12 Statistical analysis

Data are presented as mean values±standard error (SE) or values±standard deviation (SD) and the significance was measured by the use of Student's test of at least five determinations.

2.4 Results and discussion

2.4.1 Phenolic content and antioxidant activity of red wine

The total phenolic and anthocyanin contents of RW, determined as reported in "Materials and Methods" section, were of 2190±0.05 μg/ml GAE and 109.7±0.8 μM M3GE, respectively (**Table 2.1**). The antioxidant activity of RW, measured using FRAP assay, was of 6.62±0.38 mM Fe^{2+}equivalent (**Table 2.1**). Results reported in **Table 2.1** show that RW had phenolic content and antioxidant activity in the range of those previously measured in other wines [49]. In order to evaluate the activity of RW costituents, we separated RW components by different separation methods: a fraction containing total anthocyanins (monomers and polymers) (Fraction A), a fraction deprived of anthocyanins (Fraction B), a fraction deprived above 97% of polyphenols (Fraction C).

Table 2.1. *Phenolic, anthocyanin contents and antioxidant activity of total RW and fractions A, B, C and RWP.*

Samples	Total phenolic content[a]	Anthocyanin content [b]	FRAP[c]
RW	2190 ± 0.05	109.7 ± 0.81	6.62 ± 0.38
Fraction A	1430 ± 0.07	63.0 ± 1.56	4.38 ± 0.59
Fraction B	250 ± 0.01	8.4 ± 1.90	0.75 ± 0.05
Fraction C	10 ± 0.05	0.01 ± 0.02	0.02 ± 0.01
RWP	810 ± 0.03	--	--

Results are expressed as μg/ml GAE[a]; μM malvidin-3-glucoside equivalent (M3GE)[b]; mM Fe^{2+} equivalent[c]. All data are the means ± SD of at least five independent determinations.

As expected, Fraction A showed higher antioxidant power compared to

Fraction B and C, accordingly with the highest content of total phenols and anthocyanins (**Table 2.1**), while the absence of polyphenols of Fraction C showed the lower antioxidant activity. We also separated, through dialysis, the high molecular weight compounds of RW, RWP, and measured the phenolic content by Folin-Ciocalteu's assay (**Table 2.1**).

2.4.2 Protection from plasma chloramines formation and RBCs hemolysis

Based on the data reported in **Table 2.1**, we tested the ability of RW to improve the physiological antioxidant defenses. During the respiratory burst, activated macrophages and neutrophils trigger metabolic enzymatic events, including the activation of the enzyme myeloperoxidase, which catalyzes the reaction between H_2O_2 and Cl^-, resulting in the formation of the oxidizing HClO. This species is a potent antibacterial, antimicrobial and has highly reactive oxidant action, for which, an excessive production is responsible for the tissue damage. The thiol groups (-SH) of cysteine, methionine and the amino groups of the plasma proteins are the major targets of HClO. In particular, the free amino groups of the lysine residues react with HClO and form chloramines (RNHCl), very reactive oxidative agents [50] (**Figure 2.4**).

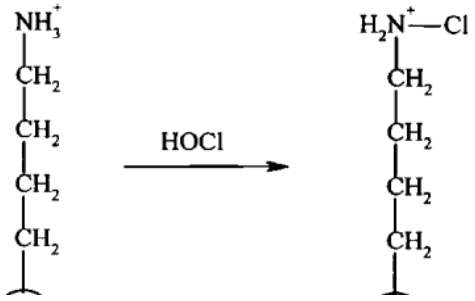


Figure 2.4. *Reaction of chloramine formation [50].*

We tested the ability of RW to protect against HOCl-induced plasma oxidation, measuring the reduction of N-chloramines formation in human plasma. The assay is an indirect measure of the concentration of chloramines, which is inversely proportional to the concentration of Nbs. After adding HClO to induce the formation of chloramines, it was added Nbs which interacts with the free amino groups of lysines plasma. The presence of many free groups which react with NBS corresponds to the lower chloramines formation. The plasma samples were preincubated with RW (9.13 μg/ml GAE) before the oxidative damage induced by HClO, as reported in "Materials and Methods" section. After incubation with RW, we observed a significant increase of Nbs concentration respect to HOCl-treated samples (**Figure 2.5**), which corresponded to a reduction of chloramines formation. As positive control of the assay, we used quercetin, a flavonoid present in RW and possing a strong antioxidant activity.

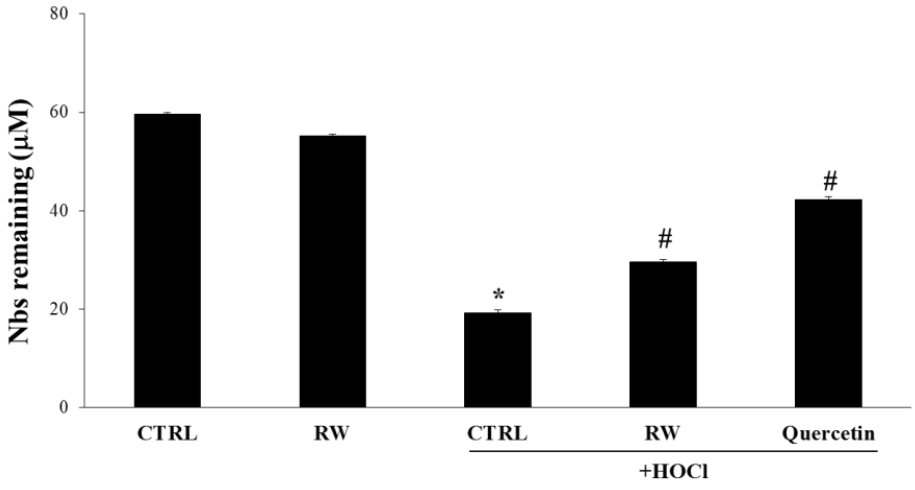


Figure 2.5. *Determination of chloramines in plasma after treatment with RW (9.13 μg/ml GAE). Chloramines were measured as reported in "Materials and Methods" and expressed as μM Nbs remaining. Quercetin (50 μM) was used*

*as positive control. Data are means of samples from 10 subjects (n=10) in duplicate ± SE. Symbols indicate significance: *p<0.05 respect to untreated (CTRL); #p<0.05 vs CTRL+HOCl-treatment.*

In order to evaluate the ability of RW to improve the physiological antioxidant defenses, we also verified the protection against hemolysis, treating RBCs with 300 μM HOCl, which induced a rapid hemolysis [51]. We detected an hemolysis of 8.6% that was significantly reduced to 5.4% by RW (73 μg/ml GAE) treatment (about 40% reduction), as showed in **Figure 2.6.**

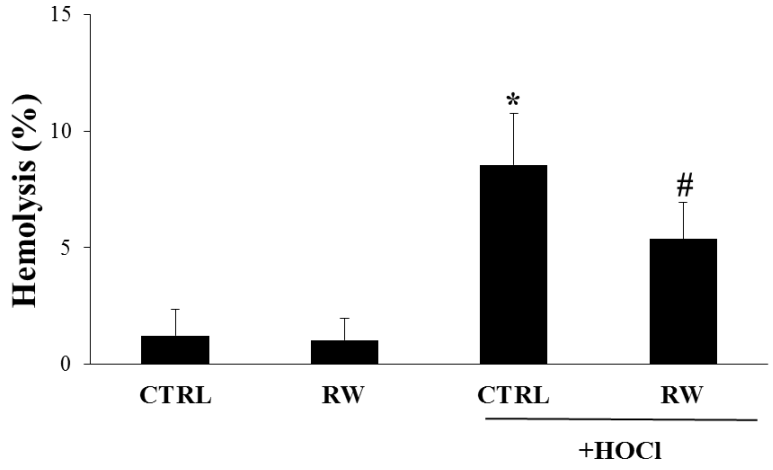


Figure 2.6. *Reduction of hemolysis induced by HOCl after treatment with RW (73 μg/ml GAE). The oxidative stress was induced by adding 300 μM HOCl for 15 min at 37 °C. Percent of hemolysis was calculated as reported in "Materials and Methods". Data are means of samples from 10 subjects in duplicate±SE. Symbols indicate significance: *p<0.05 respect to untreated (CTRL); #p<0.05 vs CTRL+HOCl-treatment.*

These experiments confirmed the protective effect of RW in RBCs against the formation of plasma chloramines and hemolysis induced experimentally by

HOCl. Therefore, we hypothesized that RW improved the antioxidant intracellular redox state, enhancing the physiological antioxidant defenses. To confirm this, we measured the total RBCs antioxidant activity by DPPH quenching assay and we observed that the treatment with RW (73 μg/ml GAE) increased (up to 3-fold) the intracellular antioxidant activity compared to untreated RBCs (**Table 2.2**).

Table 2.2. *Antioxidant capacity of RW into RBCs.*

Samples	DPPH
CTRL	12.8 ± 13.2
RW	39.6 ± 10.3

Results are expressed as quenching (%). All data are the means ± SD of four independent determinations.

2.4.3 Activation of PMRS activity by red wine

We evaluated the RW capability to interfere with PMRS activity, one of the major player in regulating RBCs antioxidant defences. The assay is based on the ability of ferricyanide (FIC), an oxidant molecule impermeable to plasma membrane, to accept electrons. The reduction of FIC to ferrocyanide (FOC), which occurs at the plasma membrane, was assessed spectrophotometrically and was related to the RW ability to donor electrons to the PMRS, increasing its activity. Therefore, we firstly performed a dose-response experiment (7.3-182.5 μg/ml GAE) to select the concentration of RW to be employed in the assays. We chose a concentration of 73 μg/ml GAE, which induced an increase of about 2-fold in PMRS activity (**Figure 2.7**).

To exclude the involvement of volatile compounds in increasing PMRS activity, samples were dried and suspended in the same volume of 0.01N HCl, before assaying the PMRS activity. No significant changes were observed,

confirming that the presence of all volatile components in wine did not interfere with PMRS activity (data not shown). We also studied the role of GSH, an important regulator of PMRS and RBCs antioxidant defenses, which plays a crucial role in reduction of DHA to ascorbate [26], as reported in the "Introduction" section. For this reason, we evaluated the role of RW polyphenols on GSH metabolism, using two GSH modulators, IAC, an alkylating agent that binds covalently the thiol group of cysteine inducing a depletion of GSH [52], and NAC, a synthetic precursor of intracellular cysteine and *de novo* synthesis of GSH [53] and we measured PMRS activity.

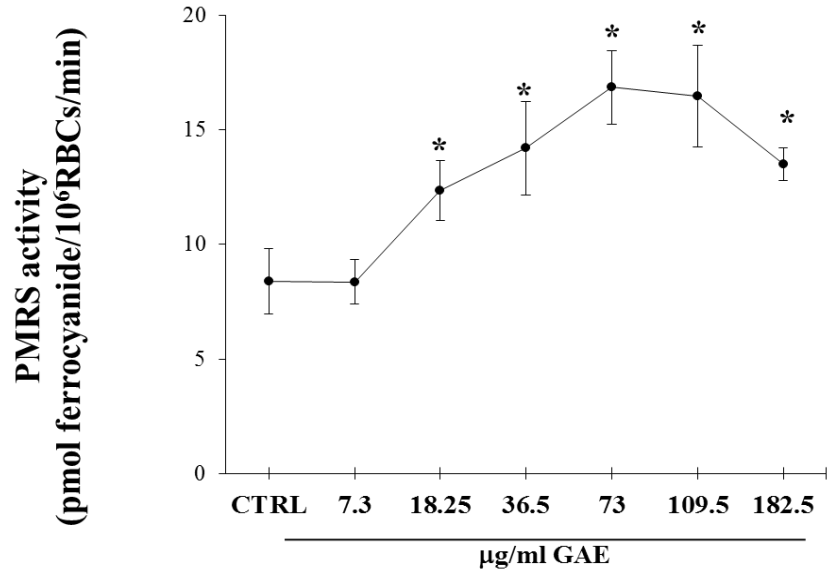


Figure 2.7. *Dose-response of RW on PMRS activity in RBCs. PMRS activity was evaluated as reported in "Materials and Methods" and expressed in terms of pmol ferrocyanide/10^6 RBCs/min. Data represent means of samples from 5 donors in duplicate ± SE (n=5). Symbols indicate significance: *p<0.05 respect to untreated (CTRL).*

The results in **Figure 2.8** confirmed the role of GSH in regulating PMRS,

since the incubation of RBCs with IAC (10 mM) determined a dramatic decrease of PMRS activity. This inhibitory effect was overcome by treatment with RW, suggesting a role GSH-like of RW polyphenols. The treatment with NAC increased PMRS activity, but at the tested concentration (40 mM), the molecule was less efficient (**Figure 2.8**). In fact, in the presence of NAC, PMRS activity increased by 1.58-fold. When we combined NAC plus RW, the increase of PMRS activity was comparable to that induced by RW single treatment.

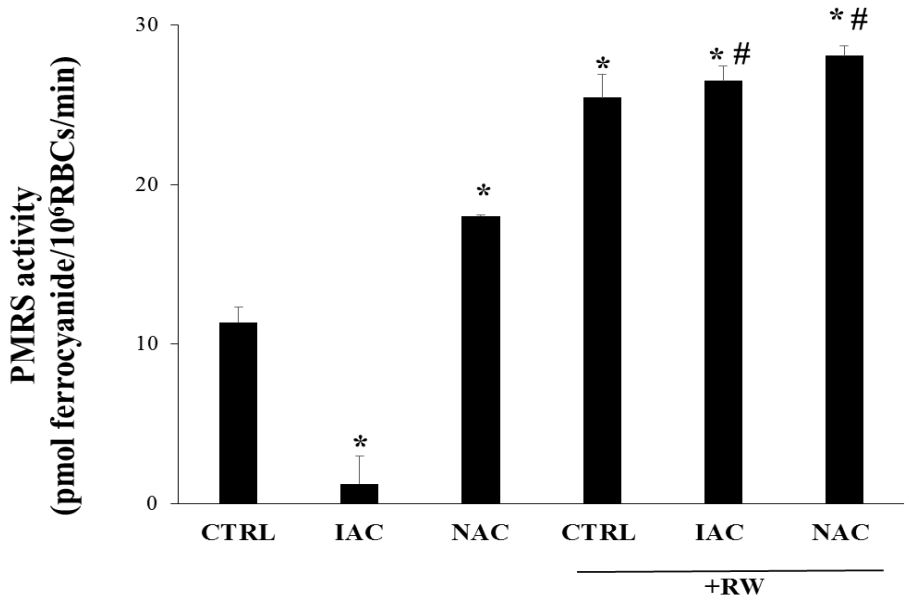


Figure 2.8. *Effects of IAC and NAC on PMRS activity. PMRS activity was described as reported in "Materials and Methods" and expressed in terms of pmol ferrocyanide/10^6 RBCs/min. Data are means of samples from 10 donors in duplicate ± SE (n=10). Symbols indicate significance: *p <0.05 respect to untreated (CTRL); #p<0.05 vs IAC and NAC single treatments.*

2.4.4 Red wine interaction with RBCs antioxidant systems

In the attempt to investigate the molecular mechanism triggered by RW and

mediated by PMRS, we reasoned that GSH levels could play a key role being among the most important non-enzymatic erythrocytic antioxidants. For this reason, we measured GSH concentration by a spectrofluorimetric method, after 10 min of incubation with RW (73 μg/ml GAE). As showed in **Figure 2.9**, GSH increased significantly compared to untreated control, as the result of RW treatment.

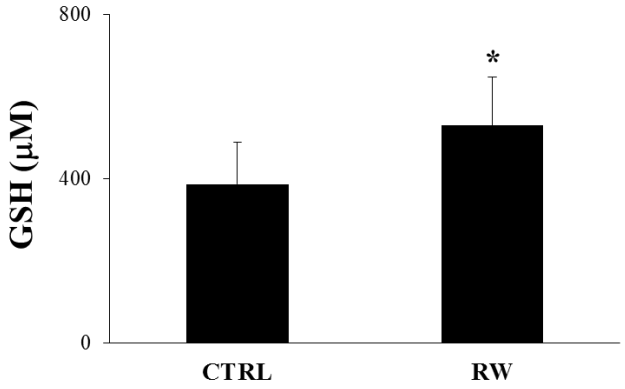


Figure 2.9. *Effect of RW (73 μg/ml GAE) on GSH concentration in RBCs. GSH micromolar concentration was measured spectrofluorimetrically at 340±20nm of excitation and 460±20nm of emission and calculated using a standard curve of GSH as reported in "Materials and Methods". Data are means of samples from seven donors in duplicate±SE. Symbols indicate significance: *p<0.05 respect to untreated (CTRL).*

Based on this evidence, we hypothesized that the activation of PMRS by RW polyphenols (**Figure 2.7**) was related to the GSH increase (**Figure 2.9**). Therefore, we investigated the possible mechanism triggered by RW to activate PMRS via an increase of GSH intracellular concentration. For this reason, we measured ROS production following RW treatment to assess if the response of GSH was due to an increase in radical molecules. As reported in **Figure 2.10**, the incubation of RBCs with RW for 1 and 10 min increased by

10% and 15% of intracellular ROS production, respectively.

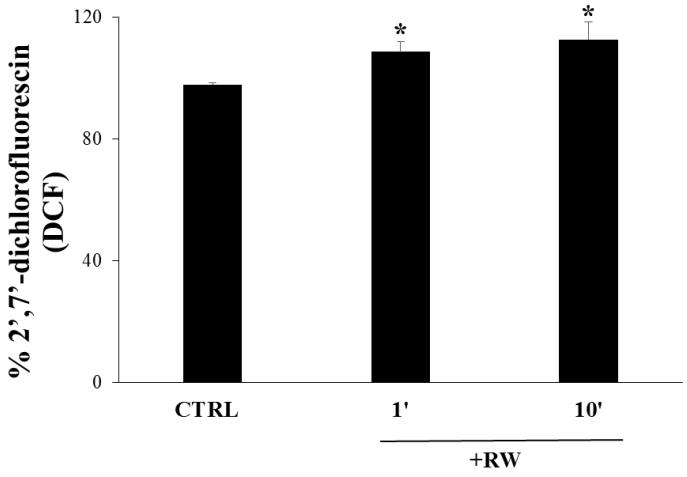


Figure 2.10. *ROS production in RBCs treated with RW (73 μg/ml GAE) at different times. ROS were measured using DCFDA/ DCF fluorescent method as reported in "Materials and Methods". Symbols indicate significance: *p<0.05 respect to untreated (CTRL). Data are means ± SD of at least five independent determinations. No significant difference was reported between treatment at 1 and 10 min (RW bars).*

Therefore, we suggested that this slight but significant pro-oxidant effect by RW could be responsible for the induction of an antioxidant response, as a RBC defense mechanism. Since the rapid activation of PMRS (**Figure 2.7**) and the observation that RW totally rescued the effect of IAC (**Figure 2.8**), we excluded the possibility of a *de novo* synthesis of GSH. In fact, L-buthionine sulfoximine (BSO), a specific inhibitor of glutamate-cysteine ligase, which is the key rate-limiting enzyme in GSH synthesis, did not significantly inhibit the activation of PMRS by RW (data not shown). Alternatively, we considered that the conversion from GSSG to GSH could be involved in the activation of PMRS via increased levels of GSH. To explore this possibility, we measured the activity of GR, the enzyme responsible for the conversion of GSSG to

GSH (**Figure 2.1**). As reported in **Figure 2.11,** RW increased GR activity significantly in the range 1-10 min compared to the untreated control with a peak at 2 min corresponding to 29% increase compared to 1 min. We also showed that the enzymatic activity of GPx, the complementary enzyme in the cycle of GSH (**Figure 2.1**) did not increase at the time of incubation tested (data not shown).

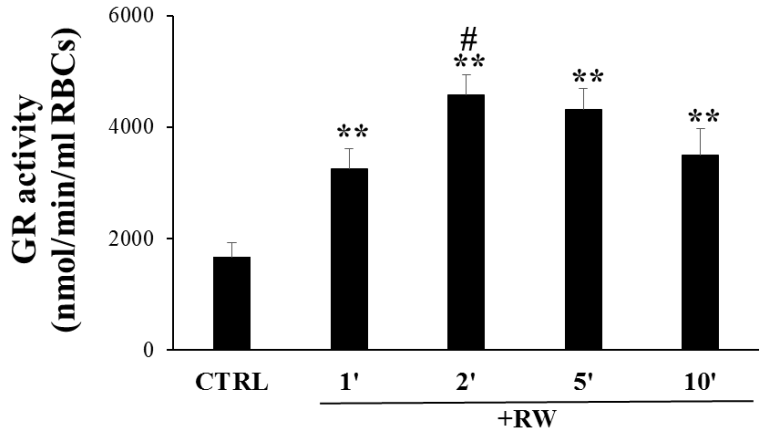


Figure 2.11. *GR activity of RBCs after incubation with RW (73 μg/ml GAE). GR activity was expressed as nmol/min/ml RBCs. Symbols indicate significance with **p<0.005 respect to untreated (CTRL); #p<0.05 significance between 1 and 2 min of incubation with RW. Data are means ± SD of at least seven independent determinations.*

We also hypothesized the involvement of G6PD, an enzyme involved in maintaining the normal levels of NADPH, cofactor of the enzyme GR (**Figure 2.1**). G6PD enzyme is required to produce NADPH in the pentose phosphate pathway and protects RBCs from oxidative stress. **Figure 2.12** shows a significant increase of G6PD activity at 2, 5 and 10 min of treatment with RW. The increase of G6PD activity peaked at 2 min with 47% increased respect to 1 min.

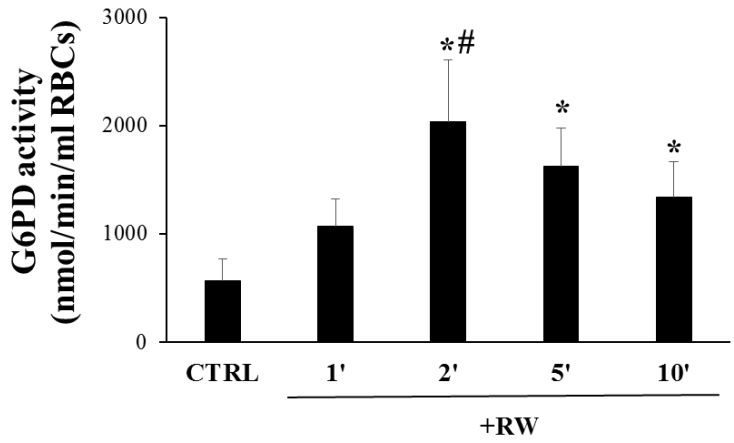


Figure 2.12. *G6PD activity of RBCs after incubation with RW (73 μg/ml GAE). G6PD activity was expressed as nmol/min/ml RBCs. Symbols indicate significance with *p<0.05 respect to untreated (CTRL); #p<0.05 significance between 1 and 2 min of incubation with RW. Data are means ± SD of at least seven independent determinations.*

Finally, we detected the increase of SOD activity, an important antioxidant enzyme, which catalyzes the dismutation of the superoxide radical into molecular oxygen or hydrogen peroxide (**Figure 2.1**). In **Figure 2.13** a kinetic of SOD activity after treatment with RW is reported. The only significant difference compared to untreated control was detected after 10 min of incubation (38% of increase).

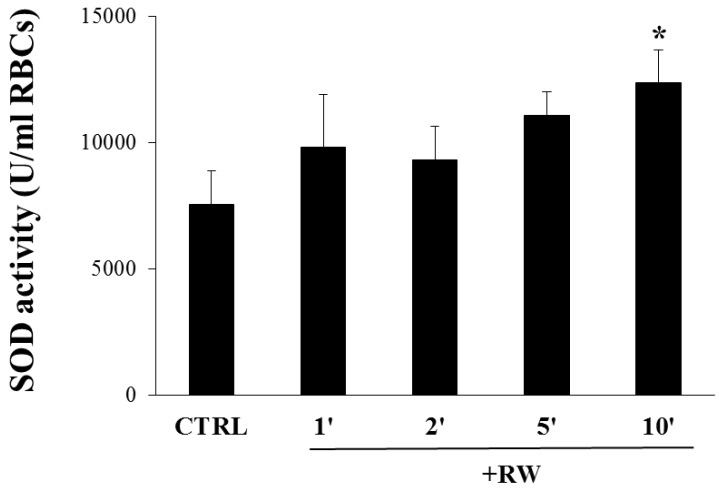


Figure 2.13. *SOD activity of RBCs after incubation with RW (73 μg/ml GAE). SOD activity was expressed as U/ml RBCs. Symbols indicate significance with *p<0.05 respect to untreated (CTRL). Data are means ± SD of at least seven independent determinations.*

2.4.5 PMRS activity by red wine anthocyanins and polymers

To investigate the RW components potentially responsible for the activation of PMRS, we tested the capacity of individual antioxidant molecules, such as quercetin, resveratrol, gallic acid and catechin to activate PMRS. These molecules were selected since they are among the most representative in RW and were applied in the range of concentrations usually found in an Aglianico wine [54-56]. **Figure 2.14** shows that single molecules had not a significant effect on PMRS activity respect to RW mixture. In one case (resveratrol), even at a concentration 3-fold higher than that normally present in RW, no effect on PMRS activity was detected (data not shown).

We also calculated by FRASC method the concentration of ASC, one of the major non-phenolic antioxidant, whose value was about 280 μM, in good agreement with other values reported in literature [57]. Since human RBCs do

not express sodium-dependent vitamin C transporters (SVCTs), they cannot take up ASC; therefore, it was useless to test its ability to directly activate PMRS. Instead, we assayed DHA as a positive control and, as expected, we found that DHA was able to activate PMRS (data not shown). Previous evidence suggested that phenols also present in RW, such as quercetin and resveratrol, are effectively incorporated into RBCs and activate PMRS [23]. However, the concentrations applied were far from those present in RW.

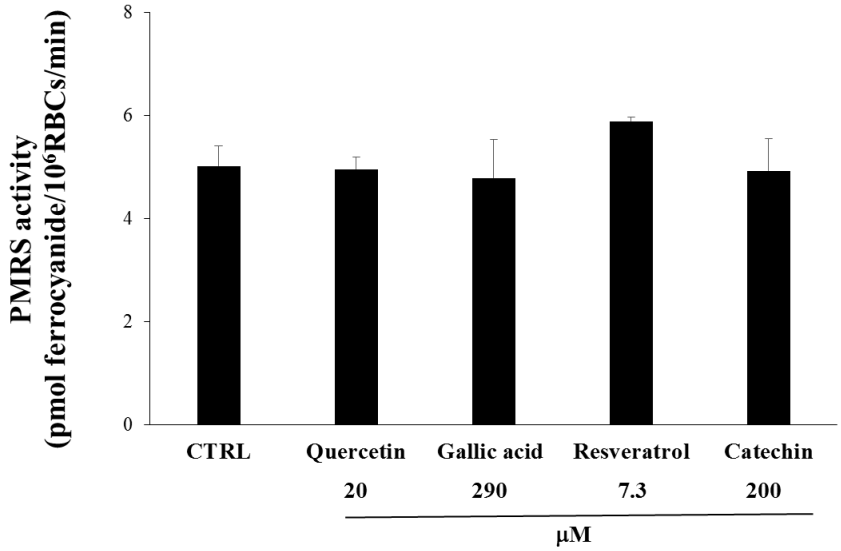


Figure 2.14. *Effects of antioxidant compounds present in RW on PMRS activity. RBCs were incubated for 10 min with 20 µl of RW (73 µg/ml GAE) or single compounds (200 µM catechin, 20 µM quercetin, 7.3 µM resveratrol, 290 µM gallic acid). PMRS activity was described as reported in "Materials and Methods" and expressed in terms of pmol ferrocyanide/10^6 RBCs/min. Data are means of samples from 5 donors duplicate±SE.*

Based on these results, we investigated the possibility that other classes of polyphenols could be involved in the activation of PMRS. Therefore, we applied a separation method, based on ethyl acetate extraction, which allowed

us to obtain a fraction enriched in total anthocyanins (Fraction A) and a fraction without anthocyanins and rich in flavonols, phenolic acids, and catechins (Fraction B) [8, 10] (**Table 2.1**). A third fraction deprived of polyphenols (Fraction C) was also prepared by mixing RW with a column of PVPP [38]. The total polyphenol content of the RW was determined before and after the phenolic compounds were stripped and >97% of phenols were removed by this procedure (**Table 2.1**).

We tested the PMRS activity of these fractions normalizing to the original volume of RW used at the beginning of the separation procedure. As reported in **Figure 2.15**, Fraction A (48 μg/ml GAE) increased PMRS activity of 3-fold respect to untreated samples, while Fraction B (8.3 μg/ml GAE) did not induce a significant increase of PMRS. We confirmed that RW polyphenols were able to modulate PMRS activity and the anthocyanins components were responsible for the increase of PMRS, since the Fraction C (0.33 μg/ml GAE), deprived of polyphenols, was completely inactive (**Figure 2.15**).

Figure 2.15 shows that more than 95% of the PMRS modulating effect of RW was associated with Fraction A, containing monomeric and polymeric anthocyanins. A residual activity, although not significant, was associated to the fraction without anthocyanins (Fraction B) indirectly confirming the data reported in **Figure 2.14**, which refer to the inability of well-known bioactive and antioxidant compounds, such as phenolic acids, flavonoids, catechins, and stilbenes, to activate PMRS at the concentrations normally present in RW. These molecules are been *bona fide* present in Fraction B. A corollary of data shown in **Figure 2.15** regards the observation that the activity of RW is specifically associated to polyphenols, not to other compounds present in the tested sample, since stripping of polyphenols from RW (Fraction C) abolished its ability to activate PMRS.

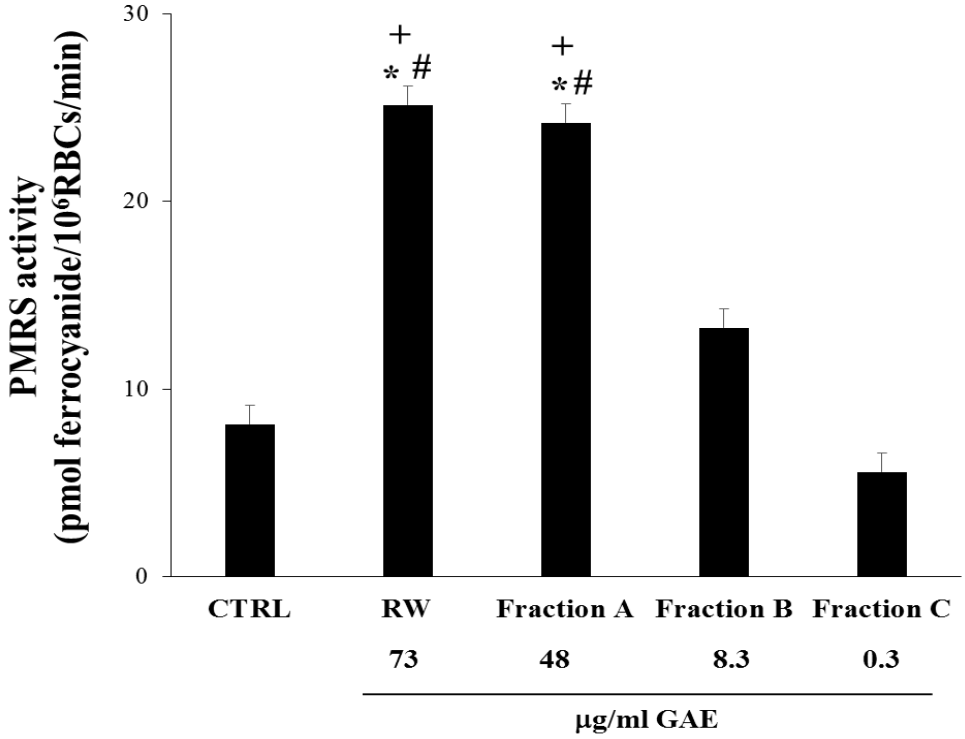


Figure 2.15. *Effects on PMRS activity of RW components. RBCs were incubated for 10 min with 20 µl of RW (73 µg/ml GAE), Fraction A (47.6 µg/ml GAE), Fraction B (8.33 µg/ml GAE) and Fraction C (0.3 µg/ml GAE). PMRS activity was described as reported in the section ''Materials and Methods'' and expressed in terms of pmol ferrocyanide/10^6 RBCs/min. Data are means of samples from 5 donors duplicate±SE. Symbols indicate significance: *p<0.05 respect to untreated (CTRL); #p<0.05 and +p<0.05 vs fractions B and C, respectively.*

We further investigated the contribution of bioactive RW components, after separating in two groups: high molecular weight (RWP) and monomers by dialysis, as reported in "Materials and Methods" section. After dialysis, RWP were dried and suspended in 0.01N HCl and used for the assay normalizing to the original volume of RW used at the beginning of the separation procedure. **Figure 2.16** shows a kinetic of PMRS activity after treatment with RW and

RWP (73 and 27 μg/ml GAE, respectively) for the indicated times. In particular, RW and RWP increased PMRS activity significantly starting from 1 min of incubation and the activation persisted up to 10 min. The differences in PMRS activity among the two groups, e.g. RW versus RWP were not significant.

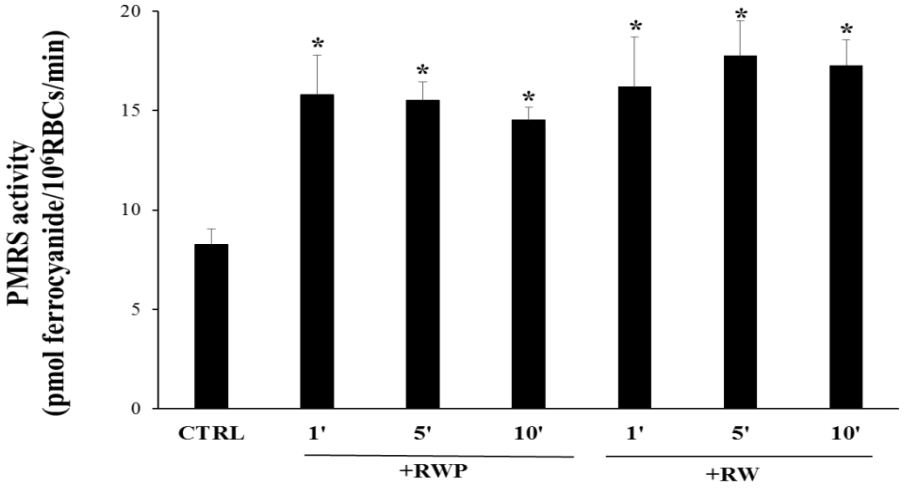


Figure 2.16. *Kinetic of PMRS activity in RBCs after incubation with RW and RWP (73 μg/ml GAE for RW, 27 μg/ml GAE for RWP). PMRS activity was expressed in terms of pmol ferrocyanide/10^6RBCs/min. Symbols indicate significance with *p<0.05 respect to untreated (CTRL). Data are means ± SD of at least four independent determinations.*

We also tested the ability of RWP to increase GSH intracellular concentration. As showed in **Figure 2.10,** GSH increased significantly of 27% and 25% compared to untreated control, as the result of RW and RWP treatment, respectively. No significant difference was measured between RW and RWP, confirming the contribution of these molecules to biological activity of RW.

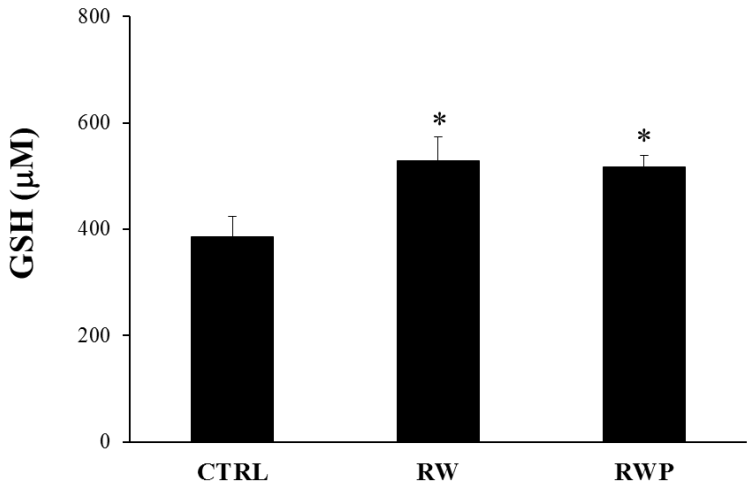


Figure 2.17. *GSH levels after incubation with RW and RWP (73 μg/ml GAE for RW, 27 μg/ml GAE for RWP).GSH levels were expressed in terms of μM GSH as reported in "Materials and Methods". Symbols indicate significance with *p<0.05 respect to untreated (CTRL). Data are means ± SD of at least four independent determinations.*

2.5 Conclusions

In the present Chapter, we demonstrated that RW possesses pleiotropic functions able to protect human RBCs against oxidative stress and/or capable to potentiate their antioxidant defenses acting extra- and intracellularly on multiple mechanisms (**Figure 2.18**). The data reported demonstrate, for the first time to our knowledge, that the protective antioxidant effect exerted by RW on human RBCs is mediated by PMRS. In particular, we suggested that the increase of ROS by RW induces an adaptive response in RBCs, which results in an increase of their antioxidant defenses protecting them from oxidative stress (**Figure 2.18**). In fact, we showed that RW activates GR (**Figure 2.11**), the enzyme responsible for the conversion of GSSG to GSH, suggesting that compounds present in RW contribute to increase the level of GSH, directly or indirectly. In support of this view, the rapid activation of PMRS and the observation that RW totally rescued the effect of IAC (**Figure 2.8**) make unlikely that the *de novo* synthesis of GSH can play an important role. In addition, RW is capable of increasing the intracellular concentration of GSH even more efficiently than NAC, since the combined treatment, NAC plus RW, does not significantly enhance the effect of RW mono-treatment (**Figure 2.8**).

Among the several red wine components, the anthocyanin fraction appears the most likely responsible for stimulating the PMRS enzymatic activity. Future work will be devoted to the purification and characterization of specific compounds present in RW and/or in the anthocyanin-enriched fraction, with high molecular weight, and able to interfere with specific PMRS components.

We cannot exclude a synergistic effect of bioactive compounds present in RW, which may explain its capacity to increase the PMRS activity and the protection from oxidative stress. However, further studies are going in order to measure the concentration of RW polyphenols into RBCs, not excluding

possible interactions at the membrane bilayer by RWP.

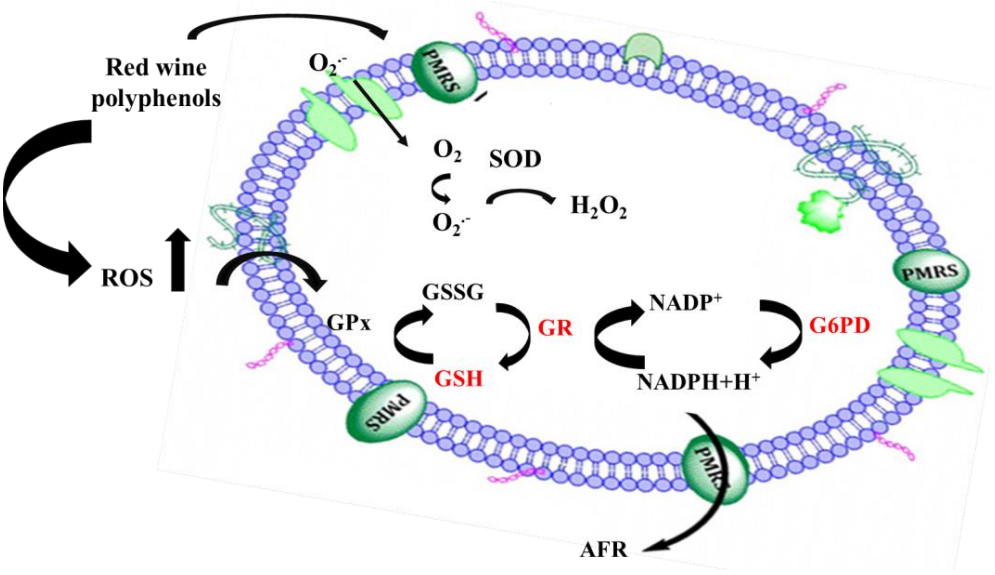


Figure 2.18. *Proposed mechanism triggered by RW components to enhance antioxidant defences in RBCs mediated by PMRS.*

2.6 References

1. Artero, A., et al., *The impact of moderate wine consumption on health.* Maturitas, 2015. **80**(1): p. 3-13.
2. Botden, I.P., et al., *Daily red wine consumption improves vascular function by a soluble guanylyl cyclase-dependent pathway.* Am J Hypertens, 2011. **24**(2): p. 162-8.
3. Russo, P., et al., *Effects of de-alcoholated red wine and its phenolic fractions on platelet aggregation.* Nutr Metab Cardiovasc Dis, 2001. **11**(1): p. 25-9.
4. Kutil, Z., et al., *Impact of wines and wine constituents on cyclooxygenase-1, cyclooxygenase-2, and 5-lipoxygenase catalytic activity.* Mediators Inflamm, 2014. **2014**: p. 8.
5. Lippi, G., et al., *Moderate red wine consumption and cardiovascular disease risk: beyond the "French paradox".* Semin Thromb Hemost, 2010. **36**(1): p. 59-70.
6. Zou, J., et al., *Effects of resveratrol on oxidative modification of human low density lipoprotein.* Chin Med J (Engl), 2000. **113**(2): p. 99-102.
7. Liu, F.C., H.I. Tsai, and H.P. Yu, *Organ-Protective Effects of Red Wine Extract, Resveratrol, in Oxidative Stress-Mediated Reperfusion Injury.* Oxid Med Cell Longev, 2015. **2015**: p. 568634.
8. Ghiselli, A., et al., *Antioxidant Activity of Different Phenolic Fractions Separated from an Italian Red Wine.* J Agric Food Chem, 1998. **46**(2): p. 361-367.
9. Speciale, A., et al., *Bioavailability and molecular activities of anthocyanins as modulators of endothelial function.* Genes Nutr, 2014. **9**(4): p. 404.
10. Tedesco, I., et al., *Antioxidant effect of red wine anthocyanins in normal and catalase-inactive human erythrocytes.* J Nutr Biochem, 2001. **12**(9): p. 505-511.
11. Ossman, T., G. Fabre, and P. Trouillas, *Interaction of wine anthocyanin derivatives with lipid bilayer membranes.* Comput Theor Chem, 2016. **1077**: p. 80–86.
12. Rodrigo, R., A. Miranda, and L. Vergara, *Modulation of endogenous antioxidant system by wine polyphenols in human disease.* Clin Chim Acta, 2011. **412**(5-6): p. 410-24.
13. Lobo, V., et al., *Free radicals, antioxidants and functional foods: Impact on human health.* Pharmacogn Rev, 2010. **4**(8): p. 118-26.
14. Fernandez-Pachon, M.S., et al., *Changes in antioxidant endogenous enzymes (activity and gene expression levels) after repeated red wine intake.* J Agric Food Chem, 2009. **57**(15): p. 6578-83.
15. Sivilotti, M.L., *Oxidant stress and haemolysis of the human erythrocyte.* Toxicol Rev, 2004. **23**(3): p. 169-88.

16. An, F., et al., *Attenuation of Oxidative Stress of Erythrocytes by Plant-Derived Flavonoids, Orientin and Luteolin.* Evid Based Complement Alternat Med, 2016. **2016**: p. 8.
17. Fibach, E. and E.A. Rachmilewitz, *The role of antioxidants and iron chelators in the treatment of oxidative stress in thalassemia.* Ann N Y Acad Sci, 2010. **1202**: p. 10-6.
18. Zheng, L., et al., *Radical scavenging activities of Tyr-, Trp-, Cys- and Met-Gly and their protective effects against AAPH-induced oxidative damage in human erythrocytes.* Food Chem, 2016. **197 Pt A**: p. 807-13.
19. Devasagayam, T.P., et al., *Free radicals and antioxidants in human health: current status and future prospects.* J Assoc Physicians India, 2004. **52**: p. 794-804.
20. Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.
21. Kalinina, E.V., N.N. Chernov, and M.D. Novichkova, *Role of glutathione, glutathione transferase, and glutaredoxin in regulation of redox-dependent processes.* Biochemistry (Mosc), 2014. **79**(13): p. 1562-83.
22. Weydert, C.J. and J.J. Cullen, *Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue.* Nat Protoc, 2010. **5**(1): p. 51-66.
23. Rizvi, S.I., et al., *Erythrocyte plasma membrane redox system may determine maximum life span.* Med Hypotheses, 2011. **76**(4): p. 547-9.
24. Fatima, M., et al., *Protective effect of theaflavin on erythrocytes subjected to in vitro oxidative stress.* Biochem Res Int, 2013. **2013**: p. 7.
25. Asha Devi, S., C.S. Shiva Shankar Reddy, and M.V. Subramanyam, *Peroxyl-induced oxidative stress in aging erythrocytes of rat.* Biogerontology, 2011. **12**(4): p. 283-92.
26. Lane, D.J. and A. Lawen, *Ascorbate and plasma membrane electron transport--enzymes vs efflux.* Free Radic Biol Med, 2009. **47**(5): p. 485-95.
27. Wann, J.G., et al., *Enhanced expression of glucose transporter 1 on erythrocyte membrane in hemodialysis patients: the possible role in erythrocyte ascorbate recycling.* Am J Kidney Dis, 2006. **47**(6): p. 1055-63.
28. Levine, M., et al., *Cellular functions of ascorbic acid: a means to determine vitamin C requirements.* Asia Pac J Clin Nutr, 1993. **2 Suppl 1**: p. 5-13.
29. Rizvi, S.I. and K.B. Pandey, *Activation of the erythrocyte plasma membrane redox system by resveratrol: a possible mechanism for antioxidant properties.* Pharmacol Rep, 2010. **62**(4): p. 726-32.

30. Del Principe, D., et al., *Trans-plasma membrane electron transport in mammals: functional significance in health and disease.* Antioxid Redox Signal, 2011. **14**(11): p. 2289-318.
31. de Grey, A.D., *The plasma membrane redox system: a candidate source of aging-related oxidative stress.* Age (Dordr), 2005. **27**(2): p. 129-38.
32. Inal, M.E., G. Kanbak, and E. Sunal, *Antioxidant enzyme activities and malondialdehyde levels related to aging.* Clin Chim Acta, 2001. **305**(1-2): p. 75-80.
33. Gil, L., et al., *Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes.* Free Radic Res, 2006. **40**(5): p. 495-505.
34. De Cabo, R., et al., *Calorie restriction attenuates age-related alterations in the plasma membrane antioxidant system in rat liver.* Exp Gerontol, 2004. **39**(3): p. 297-304.
35. Lopez-Lluch, G., et al., *Mouse liver plasma membrane redox system activity is altered by aging and modulated by calorie restriction.* Age (Dordr), 2005. **27**(2): p. 153-60.
36. Fiorani, M. and A. Accorsi, *Dietary flavonoids as intracellular substrates for an erythrocyte trans-plasma membrane oxidoreductase activity.* Br J Nutr, 2005. **94**(3): p. 338-45.
37. Kurlbaum, M., M. Mulek, and P. Hogger, *Facilitated uptake of a bioactive metabolite of maritime pine bark extract (pycnogenol) into human erythrocytes.* PLoS One, 2013. **8**(4): p. e63197.
38. Caccetta, R.A., et al., *Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect ex vivo lipoprotein oxidizability.* Am J Clin Nutr, 2000. **71**(1): p. 67-74.
39. Singleton, V.L. and J.A. Rossi, *Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents.* Am J Enol Vitic, 1965. **16**: p. 144-158.
40. Lee, J., R.W. Durst, and R.E. Wrolstad, *Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study.* J AOAC Int, 2005. **88**(5): p. 1269-78.
41. Benzie, I.F. and J.J. Strain, *The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay.* Anal Biochem, 1996. **239**(1): p. 70-6.
42. Wan-Ibrahim, W.I., K. Sidikb, and U.R. Kuppusamy, *A high antioxidant level in edible plants is associated with genotoxic properties.* Food Chem, 2010. **122**(2): p. 1139–44.
43. Hawkins, C.L. and M.J. Davies, *Hypochlorite-induced oxidation of proteins in plasma: formation of chloramines and nitrogen-centred radicals and their role in protein fragmentation.* Biochem J, 1999. **340 Pt 2**: p. 539-48.

44. Tedesco, I., et al., *Antioxidant effect of red wine polyphenols on red blood cells.* J Nutr Biochem, 2000. **11**(2): p. 114-9.
45. Tedesco, I., et al., *Protective Effect of gamma-Irradiation Against Hypochlorous Acid-Induced Haemolysis in Human Erythrocytes.* Dose Response, 2012. **11**(3): p. 401-12.
46. Lopez, O., et al., *Changes in antioxidant enzymes in humans with long-term exposure to pesticides.* Toxicol Lett, 2007. **171**(3): p. 146-53.
47. Sirati-Sabet, M., et al., *The Effect of Lead on Erythrocyte Glucose-6-Phosphate Dehydrogenase Activity in Rats.* Biotech Health Sci, 2014. **1**: p. 191-92.
48. Li, X., *Improved pyrogallol autoxidation method: a reliable and cheap superoxide-scavenging assay suitable for all antioxidants.* J Agric Food Chem, 2012. **60**(25): p. 6418-24.
49. Tedesco, I., et al., *Dealcoholated red wine induces autophagic and apoptotic cell death in an osteosarcoma cell line.* Food Chem Toxicol, 2013. **60**: p. 377-84.
50. Thomas, E.L., M.B. Grisham, and M.M. Jefferson, *Preparation and characterization of chloramines.* Methods Enzymol, 1986. **132**: p. 569-85.
51. Vissers, M.C., et al., *Membrane changes associated with lysis of red blood cells by hypochlorous acid.* Free Radic Biol Med, 1994. **16**(6): p. 703-12.
52. Palmen, N.G. and C.T. Evelo, *Glutathione depletion in human erythrocytes and rat liver: a study on the interplay between bioactivation and inactivation functions of liver and blood.* Toxicol In Vitro, 1996. **10**(3): p. 273-81.
53. Atkuri, K.R., et al., *N-Acetylcysteine--a safe antidote for cysteine/glutathione deficiency.* Curr Opin Pharmacol, 2007. **7**(4): p. 355-9.
54. De Nisco, M., et al., *Nutraceutical properties and polyphenolic profile of berry skin and wine of Vitis vinifera L. (cv. Aglianico).* Food Chem, 2013. **140**(4): p. 623-9.
55. Gambelli, L. and G.P. Santaroni, *Polyphenols content in some Italian red wines of different geographical origins.* J Food Compost Anal, 2004. **17**: p. 613–18.
56. Gambuti, A., et al., *trans-Resveratrol, quercetin, (+)-catechin, and (-)-epicatechin content in south Italian monovarietal wines: relationship with maceration time and marc pressing during winemaking.* J Agric Food Chem, 2004. **52**(18): p. 5747-51.
57. Liguori, L., et al., *Evolution of quality parameters during red wine dealcoholization by osmotic distillation.* Food Chem, 2013. **140**(1-2): p. 68-75.

Chapter III

Neuroprotective effects of a polyphenolic extract from *Sambucus nigra*

3.1 Introduction

Several studies report the potential role of polyphenols in the prevention of the occurrence of degenerative diseases [1]. In fact, a growing body of evidence suggests that the intake of polyphenols may contribute to counteract the neuronal injuries, involved in neurodegeneration [2]. This term refers to a pathological condition characterized by dysfunction and death of neurons in the brain and spinal cord [3]. The exact aetiology of this pathogenic process is not defined; however, both environmental and genetic risk factors seem to play a fundamental role. The neuronal deterioration is due to a fatal and inevitable damage of brain function, with cognitive deficits, dementia, motor abnormalities and behavioural and psychological disorders. Neurodegeneration underlies various diseases such as PD, AD, Huntington's, Amyotrophic Lateral Sclerosis [4]. In particular, AD is the most common type of dementia, characterized by the progressive neuronal dysfunction occurring in adulthood or old age. The memory deficits and the progressive loss of brain function, typical of AD, are due to the degeneration of a population of neurons, called cholinergic, which release acetylcholine, the neurotransmitter involved in complex functions such as memory and reasoning [5]. This disease was studied for the first time by Alois Alzheimer, a german neurologist, who in 1907 described the symptoms and neuropathological aspects of the disease: the presence of neurofibrillary tangles and senile plaques (**Figure 3.1**), that progress from the brain stem to the inner parts of the temporal lobes [6]. These two neuropathological markers are derived from the accumulation of aberrant cellular proteins, resulting in the deposition of insoluble material, which interferes with neuronal function and synaptic plasticity [7].

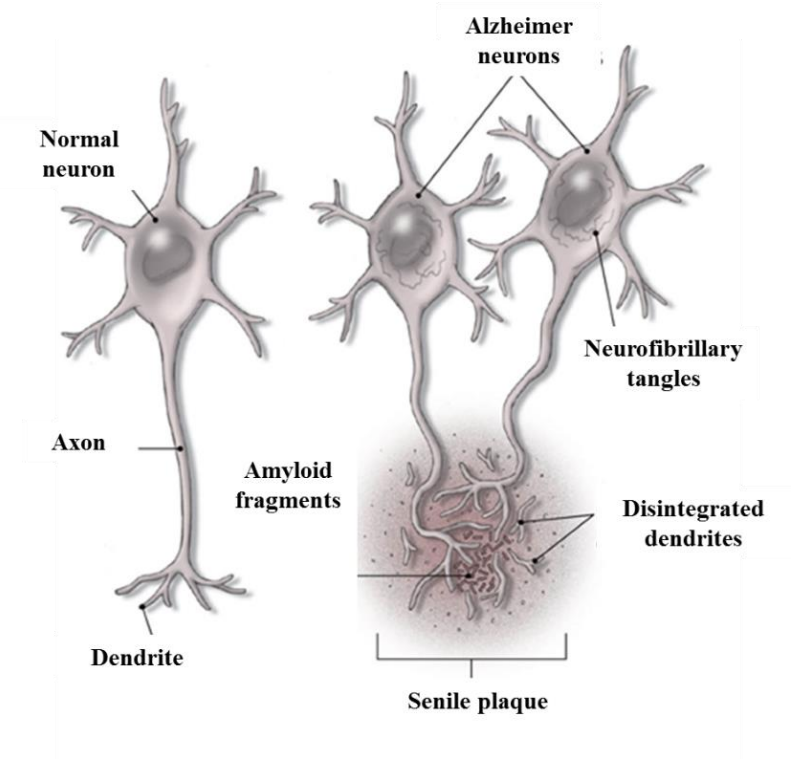


Figure 3.1. *Neurofibrillary tangles and senile plaque (provided by Alzheimer's association: https://www.alz.org/braintour/early_stage.asp).*

The neurofibrillary tangles derive from several isoforms of hyperphosphorylated tau protein, which is generally associated with microtubules and exerts two basic functions, supporting the neuron structure and regulating the transport of nutrients. The tau protein hyperphosphorylation decreases its ability to bind to microtubules, resulting in aggregates, formed by paired helical filaments, which induce the collapse of microtubules and the reduction of axonal transport [8]. Since the efficiency of this transport is necessary for maintaining neuronal connections, the degeneration of neuronal network, involved in progression of AD, is promoted by tau hyperphosphorylation and senile plaques. In detail, the central part of a senile plaque is a fibrous structure, called amyloid body, whose main protein is a 4 kDa peptide, called Aβ protein purified and sequenced in 1984 by George

Glenner, and derived from the proteolytic cleavage of APP [9]. The APP protein is the substrate of three different proteolytic activities, α, β and γ-secretase, in relation to the release of several secreted forms of APP [10]. The activity of α-secretase induces the proteolytic cut between the Lys16 and Leu17, situated inside the portion of the Aβ peptide, thus preventing its formation. For this reason, the APP degradation promoted by α-secretase activity is related to a not-amyloidogenic process (**Figure 3.2**). Instead, the β-secretase enzyme induces the amyloidogenic pathway, cutting APP to the terminal amino portion of Aβ peptide and generating a soluble form of shorter APP, APPsβ and a fragment of C99 residues, which becomes the substrate of γ-secretase, releasing the Aβ peptide (**Figure 3.2**) [11].

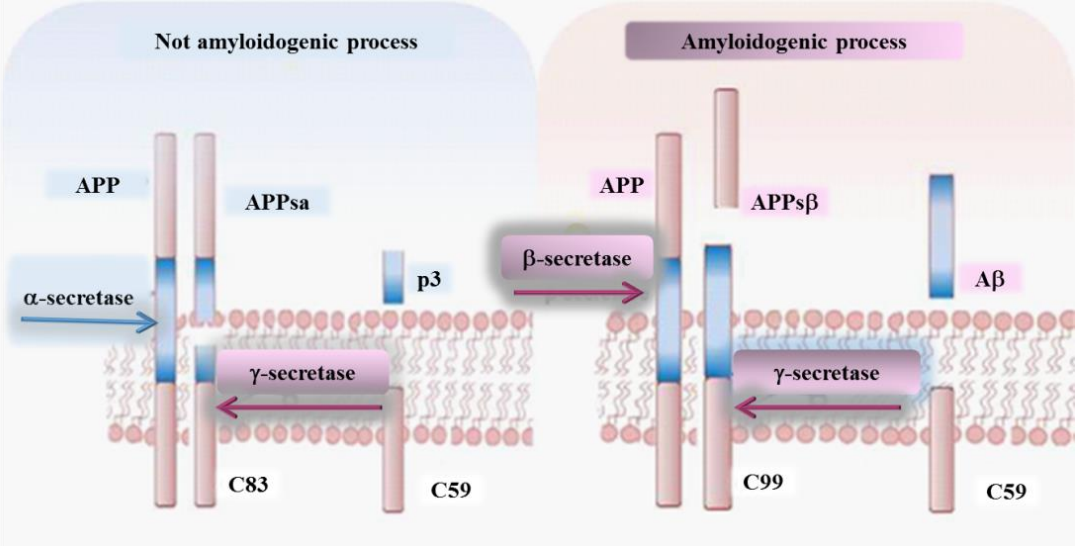


Figure 3.2. *Not amyloidogenic and amyloidogenic process [12].*

During the last two decades, most researches support the "amyloid hypothesis", that considers the deposition of Aβ peptide the initial event responsible for the neurodegeneration process (**Figure 3.3**) [13].

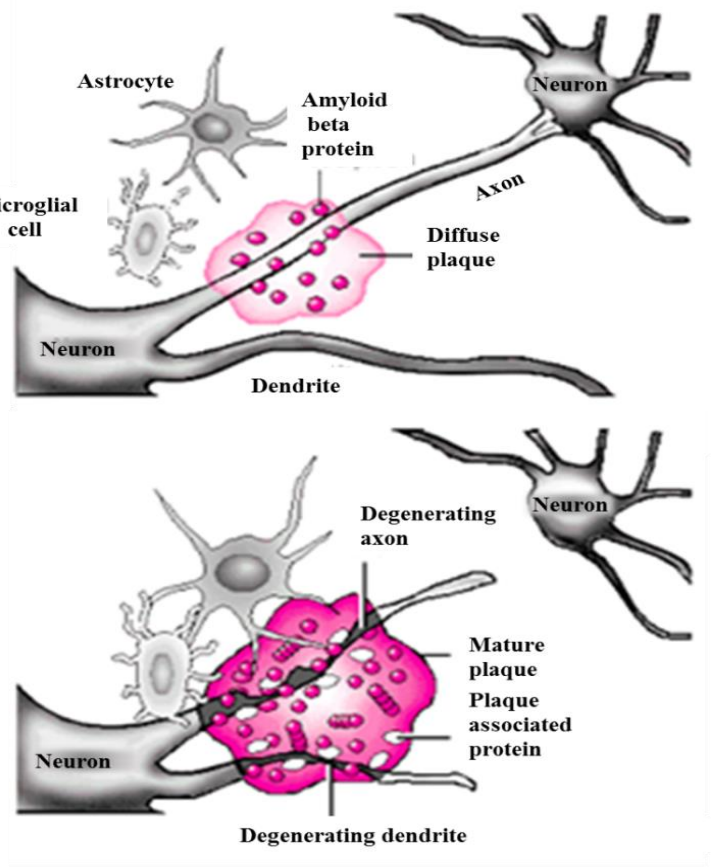


Figure 3.3. *Deposition of amyloid plaque, the initial event of neurodegeneration [14].*

Normal levels of Aβ peptide are involved in the regulation of excitatory neurotransmission in the brain, preventing the hyperactivity of synapses. In patients with AD, however, the abnormal amount of oligomers are toxic to neurons, causing synaptic dysfunction, inflammation and release of ROS [10]. In fact, the presence of Aβ peptide is associated with neuronal inflammation, excitotoxicity and oxidative stress [4]. As reported above, the oxidative stress, strongly related to neuroinflammation, plays a key role in the AD pathogenesis. For example, ROS at elevated concentrations induce the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a hydroxyl radical obtained by the nucleoside guanine. Several studies have shown that the level

of 8-OHdG is elevated in the brain of the elderly, four times higher than in young [15]. Furthermore, the damage caused by a massive build-up of free radicals implements the apoptotic cell death, the main cause of neuronal loss observed in AD [16]. The apoptotic process is based on the activity of specific enzymes, caspases, and it has been observed that flavonoids protect against neuronal oxidative damage by preventing caspase-3 activation, providing evidences in support of their powerful anti-apoptotic action [17].

Considering the prominent role of tau proteins and Aβ, they represent preferred targets for drug development. However, in many cases, innovative drugs against these two proteins fail in clinical trials. The limited effectiveness of current therapies against AD highlights the need to intensify research aimed to develop new agents able to prevent or retard the disease progression. Thus, considering the high complexity and the multiple etiological nature of AD and other aging-related ND, novel therapeutic approaches suggest the use of a cocktail of naturally antioxidant compounds, such as polyphenols.

These compounds are potential neuroprotective agents, probably for their ability to influence several cellular pathways, such as proliferation, apoptosis, redox balance and differentiation [18]. As reported above, polyphenols protect from the neuronal damage induced by neurotoxins, reducing the inflammation and the oxidative stress, through the regulation of pro-inflammatory genes and the modulation of the antioxidant enzymes activity, respectively [19] [20]. These effects seem to take place throughout two main mechanisms. In the first place, they interact with proteins that lead to the inhibition of apoptosis triggered by neurotoxic agents and promote neuronal survival. Secondly, they induce beneficial effects on the vascular system leading to changes in blood flow cerebrovascular, capable of causing angiogenesis, neurogenesis, and changes in neuronal morphology [21].

Among polyphenols, those from red berries play an important role in the protection from degenerating processes related to oxidative stress. In fact, as

shown by *in vivo* studies, the supplementation of highly antioxidant polyphenolic extracts, such as blueberries and strawberries, may protect from oxidative stress in brain aging [22]. In another study, the effects of daily consumption of wild blueberry juice on older adults with early memory changes have been investigated, demonstrating an improvement of memory and learning, after 12 weeks, with additional positive trends regarding reduced depressive symptoms and lowered glucose levels [23].

The antioxidant potential of berries is related to the presence of different families of compounds: flavonoids, proanthocyanidins, ellagitannins, gallotannins, phenolic acids, vitamins A, C and E, folic acid, calcium and selenium, α and β-carotene, lutein, phytosterols (β-sitosterol and stigmasterol), triterpenic esters [24]. As an example, the anti-oxidative and anti-inflammatory effect of elderberry extracts, through the inhibition of the microglial activation is probably related to the high content of anthocyanins, flavonoids and other polyphenols [25]. The major flavonoids in elderberry are quercetin and rutin, while the primary anthocyanins are cyanidin-3-O-sambubioside and cyanidin-3-O-glucoside [26]. Therefore, it is reasonable to assume that polyphenols-rich foods and products as nutraceuticals and supplementary treatments can potentially be used in the prevention the occurrence and in the management of aging-related neurodegeneration. For this reason, in this Chapter, we evaluated the potential role of elderberries polyphenol extract in the neuroprotection induced by oxidizing agents.

3.2 Aims of the Chapter

The main aim of this section of the thesis has been the evaluation of the potential neuroprotective effect of a polar extract enriched in polyphenols obtained from *Sambucus nigra* berries (**Figure 3.4**).

Figure 3.4. *Sambucus nigra berries.*

In detail, the specific objectives have been the following:

-To prepare the polyphenolic extract from *S. nigra* berries and test its antioxidant activity *in vitro*;

-To reproduce an *in vitro* model which resembles the pathological process of neurodegeneration using the human cell line IMR-32;

-To evaluate the potential protective effect of *S. nigra* polyphenol extract against the neurotoxicity induced by specific oxidizing agents, such as Aβ peptide and hydrogen peroxide;

-To decipher the protective molecular mechanisms triggered by *S. nigra* polyphenol extract against neurodegenerative damage induce in IMR-32 cells.

3.3 Materials and Methods

3.3.1 Chemicals

Phosphate buffer saline (PBS) tablets (Life Technologies); Minimum Essential Medium (MEM) (SAFC Bioscence); Fetal bovine serum (FBS) (HyClone); Glutamine (Life Technologies); Penicillin/streptomycin solution (Life Technologies); N6,2' Dibutyryladenosine-O-3 ', 5'-cyclic monophosphate (dbcAMP) (Sigma); 5-bromodeoxyuridine (BrdU) (Sigma-Aldrich); Dimethyl sulfoxide (DMSO); Bromide of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT); ABTS (2,2'-azinobis (3-etilbenzotiazolin-6-sulfonate); Methanol; Hydrochloric acid (HCl); Trypsin-EDTA; Trizol reagent (Life Technologies); Chloroform; Isopropanol; SuperScriptTM III kit (Life Technologies); SYBR Green PCR Master Mix (Applied Biosystems); Folin-Ciocalteu's Reagent (FCR) (Sigma-Aldrich); Potassium chloride (KCl); Sodium acetate (CH_3COONa); Sodium bicarbonate (Na_2CO_3); Fe(III)-TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) (Sigma- Aldrich); Ferric chloride ($FeCl_3$); 2,2'-azinobis(3-etilbenzotiazolin-6-sulfonate) (Sigma-Aldrich); 1,1-diphenyl-2-picrilidrazilic ($DPPH^{\bullet}$) (Sigma-Aldrich); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium); Isopropanol; 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); Ethylenediaminetetraacetic acid (EDTA); 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); Dithiothreitol (DTT); Methylphenylsulfonylfluoride (PMSF); Pepstatin A; Aprotinin; Leupeptin; Bio-Rad Protein reagent (Bio-Rad); Bovine serum albumin (BSA); Glycerol; AFC (amino-4-trifluoromethylcoumarin); Z-DEVD-AFC peptide (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluormethylcoumarin); Ac-IETD AFC-peptide (Ac-Ile-Glu-Thr-Asp-AFC); LEHD-AFC peptide (Leu-Hys-Glu-Asp-7-amino-a- trifluormethylcoumarin); Hoechst dye (Sigma-Aldrich); Dichlorofluorescein-diacetate (DCFDA). All

other chemicals used were of research highest purity grade.

3.3.2 Cell culture and differentiation

The IMR-32 cell line, derived from human neuroblastoma [27], was employed in the present study. For proliferation assays, cells were maintained at low confluence in polystyrene Petri plates (Corning, Milan, Italy) treated to ensure an optimal adhesion. The culture medium used for their growth was MEM, enriched to 10% FBS containing growth factors necessary for the growth of cells, 1% glutamine, 1% solution penicillin/streptomycin. The cells were maintained in proliferation at a density of $1x10^6$/ml, at a temperature of 37 °C, with a percentage of 100% humidity and 5% CO_2. In order to reproduce *in vitro* the pathological condition of neurodegenerative process aging-related, the IMR-32 cells were induced to differentiate to neuron-like cells. In this cell line, the amyloid precursor protein is expressed by 58% in the form APP695, APP751 and 38% <4% APP770 [28]. Cells were plated at 40% of confluency and differentiation was induced by treating the cells for a week with a mixture of N6,2' Dibutyryladenosine-O-3', 5'-cyclic monophosphate (dbcAMP) and 5-bromodeoxyuridine (BrdU):

-1 mM dbcAMP an analogue of cyclic-AMP intracellular mediator. The stock solution, dissolved in H_2O, was stored at -20 °C. Considering the data reported in the literature, dbcAMP was used extensively in the differentiation of a variety of stem cells or neural progenitor cells into neurons [29].

-2.5 μM of BrdU, a synthetic analogue of thymidine. The mother concentration was dissolved in DMSO. The BrdU is commonly used in the detection of proliferating cells in living tissues [30]. The molecule can be incorporated in the newly synthesized DNA during the S phase of the cell cycle by replacing thymidine.

In order to asses the IMR-32 differentiation process, the expected morphological changes were observed every 24 h using a microscope by phase contrast (inverted microscope AXIOVERT, 200X phase contrast).

3.3.3 Real time PCR

The real-time PCR (RT-PCR) is used to quantify the expression of the messenger of a given gene by amplification of specific sequences [31]. This technique allows to monitor the PCR reaction while it is still in progress and the data obtained at the end of the cycles can be used for a relative quantification of the amplified fragment. The levels of expression of the γ-enolase, also known as neuron-specific enolase (NSE) or enolase 2 (ENO2), an highly expressed gene in neurons, were evaluated on days 0 -3 -7 [32]. The differentiation was induced with 1 mM dbcAMP and 2.5 μM BrdU, as reported above. At the end of the stimulation, the cells were collected by trypsinization (trypsin-EDTA) and the obtained pellets were lysed using 1 ml of Trizol reagent, a mixture of phenol and guanidine isothiocyanate, specific for RNA extraction. The addition of chloroform, followed by centrifugation, separated the organic phase from the aqueous phase (RNA), which was treated with isopropanol to precipitate the RNA. The pellet was washed with 70% ethanol and the amount of RNA is determined spectrophotometrically at a wavelength of 260 e 280 nm. The RNA was reverse transcribed, using the SuperScriptTM III kit, in order to obtain the complementary DNA (cDNA). The amplification of the fragment of interest by RT-PCR was carried out using the SYBR Green PCR Master Mix, a fluorescent aromatic organic compound which during the amplification reaction intercalated into the double-stranded DNA that was formed at each cycle. The primers used were: for the NSE gene, forward (F) ACTTTGTCAGGGACTATCCTGTG; reverse (R) TCCCTACATTGGCTGTGAACT; for GAPDH (glyceraldehyde-3-phosphate

dehydrogenase) housekeeping gene, used as reference for normalization: (F) CACCAACTGCTTAGCACCCC; (R) TCTTCTGGGTGGCAGTGATG (designed using Primer3web). In the step of denaturing the Sybr Green is free in the reaction mixture, in the phase of "annealing" is positioned in a non-specific minor groove of DNA, and finally in the phase of "elongation" and the fluorescence due to the presence of SYBR Green within the double-stranded amplicon was observed and was directly proportional to the amount of DNA present. The gene expression levels were calculated by the method of 2^-ΔΔCt [33].

3.3.4 Preparation of S. nigra polyphenol extract

The elderberry (*Sambucus nigra*), belonging to *Caprifoliaceae* family, is a perennial shrub, very vigorous, common in uncultivated areas of Italy, Europe, Asia, North Africa and North America. The elderberry plant grows ubiquitously, from sea level up to an altitude of 1200 m, mainly near the beaches, chestnut and oak. The experimental samples of *S. nigra* berries, provided from University of Basilicata, was carried out in the period of August to September. In order to preparate the *S. nigra* polyphenolic extract (**SnPE**), the berries were weighed (10 g) and homogenized with mortar and pestle [34]. Three subsequent extractions were carried out using acidified methanol, (90% absolute methanol, 10% 0.01 N HCl) at room temperature. After vigorous mixing, the samples were centrifuged at 11000xg (Biofuge Stratos Heraeus Instruments) for 10 min before removing the supernatant and frozen at -20 °C. For the assays, the methanolic extract was centrifuged at 11000xg for 10 min, transferred to microcentrifuge tubes (Eppendorf) and freeze-dried to remove the solvent of extraction and the volatile components, and than solubilized using 0.01 N HCl to the final concentration of 30 mg/ml (w/v) or H_2O to a

final concentration of 1 mg/ml (w/v).

3.3.5 Polyphenol content and antioxidant activity

The total polyphenol content was determined using the Folin-Ciocalteu's method [35]. The samples were added to an aqueous solution containing 5% (v/v) of Folin-Ciocalteau's reagent (FCR) with 2% (w/v) of Na_2CO_3. The FCR consists of a mixture of fosfotunstic acid and phosphomolybdic acid, which, in an alkaline environment, oxidize various substances containing the phenolic ring, by interacting with the hydroxyl groups and are reduced to tungsten oxides and molybdenum, emitting the blue color. The mixture containing the samples was incubated in the dark at room temperature for 2 h, before spectroscopically measurement at wavelength of 760 nm of the polyphenolic content, expressed as the micromolar concentration of equivalents of quercetin (μM EqQ).

The anthocyanin content was determined by pH shift method [36]. The samples were parallel incubated with two different solutions: 0.4 M KCl (pH 1.0) and 0.4 M CH_3COONa (pH 4.5). The absorbance of both solutions was measured spectrophotometrically to the respective wavelengths of 510 and 700 nm. The total content of anthocyanins is expressed as micromolar of Cyanidin-3-O-glucoside equivalent.

For the evaluation of antioxidant activity, three different *in vitro* methods were employed.

- *FRAP assay*

The FRAP assay is a direct measurement of the reducing power of a solution and is based on the reduction of the iron of the complex Fe(III)-TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in an acid environment (pH 3.6), by reducing

compounds that induce the formation of the Fe(II)-TPTZ. This complex exhibits a maximum absorption peak at 593 nm, measurable by spectrophotometry. The samples were added to 1 ml of FRAP reagent (20 mM $FeCl_3$; TPTZ in 10 mM acetate buffer pH 4.6) and the absorbance was determined after 6 min of incubation at 37 °C. To quantify this value, a quercetin standard curve, one of the flavonoids largely present in elderberry, was determined and reported to the concentration of 1 mg/ml of dry weight.

- *ABTS assay*

The ABTS (2,2'-azinobis (3-etilbenzotiazolin-6-sulfonate) assay is an analytical method based on a spectrophotometric measurement to determine the antioxidant capacity of the sample. Under normal conditions, the oxidation of a colourless solution containing ABTS (2,2'-azinobis (3-etilbenzotiazolin-6-sulfonate) generates the formation of the radical $ABTS^{\bullet+}$, with a characteristic purple colour. The addition of the samples to the $ABTS^{\bullet+}$ solution (5.5 g/l in an aqueous solution pH 3.6 containing 2.4 mM H_2O_2) may induce the reduction of the radical, with decoloration of the reaction mixture, measured spectrophotometrically as decrease in absorbance at a certain time at a specific wavelength (660 nm). The concentration of $ABTS^{\bullet+}$ in the reaction medium is expressed as a percentage of $ABTS^{\bullet+}$ remaining ($ABTS^{\bullet+}$/Rem), calculated using the following expression:

$\%ABTS^{\bullet+}_{Rem} = ([ABTS^{\bullet+}]_0-[ABTS^{\bullet+}]_{t=6min})/[ABTS^{\bullet+}]_0 \times Fd$

T = time required, determined in preliminary experiments, so that the reaction reaches the stationary state (6 min); Fd = dilution factor.

- *DPPH assay*

The antioxidant activity of the extract was also determined using the DPPH test performed after 30 min by measuring the decrease in absorbance (517 nm) of a methanolic solution of the stable radical $DPPH^{\bullet}$ (1,1-diphenyl-2-

picrilidrazilic). The decrease of absorbance was directly proportional to the antioxidants properties of the sample. SnPE, previously diluted 1:10 by HCl, was added to a methanolic DPPH solution (100 μM). The concentration of $DPPH^{\bullet}$ in the reaction medium is expressed as the percentage of $DPPH^{\bullet}$. Remaining (% $DPPH^{\bullet}$/Rem), was calculated by the following expression:

$\%DPPH^{\bullet}_{Rem} = ([DPPH^{\bullet}]_0-[DPPH^{\bullet}]_{t=30min})/[DPPH^{\bullet}]_0 \times Fd$

T = time required, determined in preliminary experiments, so that the reaction reaches the stationary state (30 min); Fd = dilution factor.

3.3.6 Viability assay (MTT)

Cell viability was measured by the MTT assay (bromide of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) [37]. The oxidizing agent chromogen (MTT bromide), corresponding to a polycyclic system, contains a tetrazole ring which may be easily reduced by the mitochondrial enzyme succinate dehydrogenase, which is active only in living cells. The opening of tetrazole ring results in the formation of a compound chromogen nitrogen, said formazan, which forms insoluble crystals, impermeable to the cells. The cells were plated at a concentration of 30,000 cells in a volume of 500 μl/well and were incubated for 24 h at 37 °C to allow the adhesion to the plate and the cell differentiation was induced by dbcAMP and BrdU, as described previously. At the end of differentiation, the cells were stimulated with increasing concentrations of SnPE (100, 250, 500, 1000 μg/ml, w/v) for 24 h, before induction of neurodegeneration obtained adding 20 μM Aβ peptide for 48 h or 100 μM H_2O_2 for 18 h. In fact, in order to reproduce *in vitro* the pathologic condition, the damage was induced with two different agents: 1. the oxidizing agent H_2O_2, which is involved in the processes of the plaque formation; 2. the Aβ peptide fragment 25-35. To induce the oligomerization, the peptide was prepared in PBS (0.5 mM) and incubated at 37 °C for 4 days (see Results

section). After incubation with SnPE (µg/ml, w/v) and oxiding agents, MTT solution was added to samples and incubated at 37 °C for 3 h. Subsequently, the cells were treated with 0.1% isopropanol and 4 mM HCl. The absorbance of the solution was determined by spectrophotometric reading at 595 nm.

3.3.7 Caspase assays

For the measurement of the enzymatic activity of caspases-3, -8 and -9, the IMR-32 cells were plated at a density of 50,000 cells/ml medium. The cells were pre-incubated for 24 h with SnPE (500 µg/ml, w/v) and subsequently with oxiding agent H_2O_2 (100 µM) for 5 h. After incubation, the cells were trypsinized, centrifuged at 11000x*g* for 5 min and washed in PBS. The pellet was resuspended in the following lysis buffer: 10 mM HEPES, pH 7.4; 2 mM EDTA; 0.1% CHAPS; 5 mM DTT; 1 mM PMSF; 10 µg/ ml pepstatin A; 10 μml^{-1} aprotinin; 20 µg ml^{-1} leupeptin. Following the cell lysis, the samples were centrifuged at 11000x*g* for 5 min and kept on ice. The protein concentration was determined spectrophotometry according to [38], using the reagent "Bio-Rad Protein Assay", before assaying the enzymatic activity. The protein concentration of samples, expressed as µg protein/µl, was obtained by interpolation of the absorbance values in a calibration line of BSA, previously realized. For testing the enzymatic activity of caspases, cell extracts (10 µg) were added with a reaction buffer (100 mM HEPES; 20% v/v glycerol; 0.5 mM EDTA; 5 mM DTT; pH 7.4) and the substrate were conjugated with the fluorochrome AFC (amino-4-trifluoromethyl coumarin). The substrates used were the following: for caspase-3, Z-DEVD-AFC peptide (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluormethylcoumarin); for caspase-8, Ac-IETD AFC-peptide (Ac-Ile-Glu-Thr-Asp-AFC); for the caspase-9, LEHD-AFC peptide (Leu-Hys-Glu-Asp-7-amino-a- trifluormethylcoumarin). Upon

proteolytic cleavage of the substrates by the caspases, the free fluorochrome AFC was detected spectrophotometrically with excitation 395±20nm and emission and 530±20nm. To quantify enzymatic activities, an AFC standard curve was determined. Caspase-specific activities were calculated as nmol of AFC produced per min per μg proteins at 37 °C at saturating substrate concentrations (50 μM; with a Specific activity measured as mean fluorescence/time*1000/10744/μg protein). Fold increase in caspase activity was determined by direct comparison with the level of the respective controls.

3.3.8 Haechst assay

To highlight the presence of the apoptotic bodies, the Hoechst dye, that binds to nucleic acids, highly akin to the bases adenine and thymine, was used. The IMR-32 cells were plated at a density of 30,000 cells/500 μl medium. Following the treatment with SnPE (500 μg/ml, w/v), the cells were incubated with 100 μM H_2O_2 for 5 h. Hoechst dye was added to a final concentration of 1 μg/ml and incubated in the dark for 10 min. After incubation, cells were observed at the fluorescence microscope (Zeiss Axiovert 200), counted in three different fields and expressed as percentage of apoptotic bodies compared to not apoptotic cells.

3.3.9 Reactive oxygen species (ROS) measurement

To detect intracellular ROS production, we used a spectrofluorimetric method [39]. ROS production was assayed using DCFDA a not fluorescent compound that freely permeates the cells. DCFDA is hydrolyzed to dichlorofluorescein, which reacts with the intracellular peroxide and gives rise to DCF, detected spectrofluorimetrically. Cells were plated at a density of 60,000 cells/ml

medium and pre-incubated for 24 h with SnPE (500-1000 μg/ml, w/v). Subsequently, 20 μM Aβ peptide (25-35 fragment) was added for 7 h, before the incubation of cells with of 20 μM DCFDA for 30 min. To determine the intracellular ROS, samples were spectofluorimetrically read with an excitation and emission setting of 495 and 530 nm, respectively.

3.3.10 Statistical analysis

Data are presented as mean values ±s tandard error (SE) and the significance was measured by the use of Student's test of at least five determinations.

3.4 Results and discussion

3.4.1 Neuronal differentiation of IMR-32

To study the neurodegenerative disorders, we used an appropriate *in vitro* model that resembles a neuronal phenotype. The differentiation process involves several changes that occur during cell development and induce specialization; in particular, the differentiation of a neuroblastoma cell line involves many morphological and biochemical changes, including the axonal extensions. Therefore, when we induced the differentiation of the IMR-32 cells with dbcAMP and BrdU, they showed a neuronal phenotype, through the expression of neuronal specific proteins. For this reason, the differentiated IMR-32 cells represented an adequate model for the study of the potential protective effect of SnPE from cytotoxicity induced by Aβ peptide and hydrgen peroxide in aging-related neurodegenerative conditions, as confirmed by other studies [40] [41].

As reported previously, the differentiation was induced treating the cells for one week with 1 mM dbcAMP and 2.5 μM BrdU. Examining the different stages of the process, several morphological changes were observed by the phase contrast microscope. As shown in **Figure 3.5**, after 7 days of treatment, the small cell processes, resembling neuritis, were highlighted and indicated by the yellow arrows.

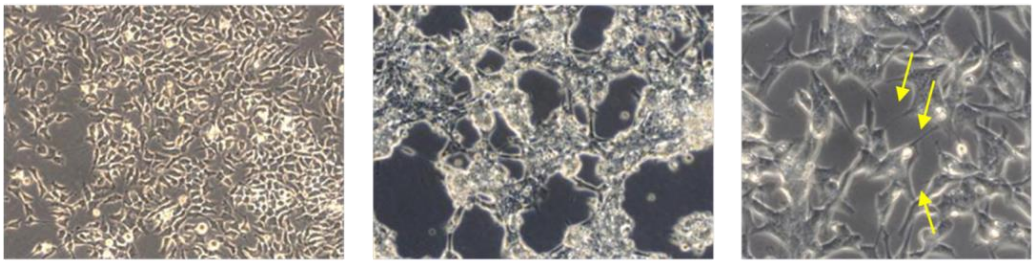


Figure 3.5. *Differentiation of the IMR-32 cells. The morphological changes*

and the presence of cellular extensions in IMR-32 cells treated with 1 mM dbcAMP and 2.5 μM BrdU after 3 and 7 days (inverted microscope AXIOVERT, 200X phase contrast) are indicated by the yellow arrows.

During the differentiation process, we measured by RT-PCR the mRNA level of the NSE gene, highly expressed in neurons, after 3 and 7 days of treatment with differentiating agents [32]. As shown in **Figure 3.6**, the increased levels of NSE indicated that, after a week of stimulation, the IMR-32 cells were differentiated and therefore ready to be used for the stimulation with oxidizing agents.

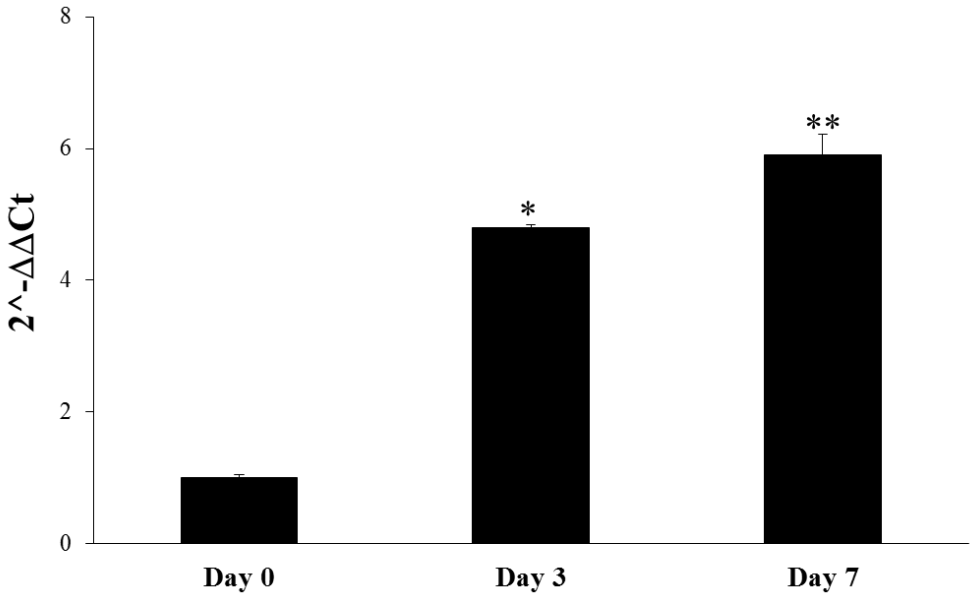


Figure 3.6. *Level of mRNA expression of NSE of the IMR-32 cells at day 0 and after 3 and 7 days of treatment with 1 mM dbcAMP and 2.5 μM BrdU. The bars in the graphs indicate the SE; symbols indicate significance: *p<0.05 and **p<0.01 vs Day 0.*

3.4.2 Phenolic content and antioxidant activity of S. nigra extract

Considering the promising role of red berries in the protection from oxidative damage during the brain aging reported by several *in vivo* studies (Introduction section), we evaluated the polyphenol content and antioxidant activity of *S. nigra*, whose neuroprotective role has not yet been studied, comparing to those of other more common berries including blackberries, *Rubus procerus*, whose neuroprotective effect was already studied in literature [42].

As reported in **Table 3.1**, the polyphenol content of SnPE, measured by Folin-Ciocalteu's assay, was of 72.14±2.93 μM EqQ, higher compared to *R. procerus* (data not shown). We also measured the total anthocyanin content by pH shift method, which was of 52.19±5.72 μM Cyanidin-3-O-glucoside equivalent, as showed in **Table 3.1.** In order to evaluate the antioxidant properties of SnPE, three different *in vitro* assays were performed, FRAP, ABTS and DPPH assys (**Table 3.1**). The antioxidant activity was higher in SnPE respect to *R. procerus* (data not shown), probably related to its higher polyphenols content. These data have provided a first indication on the potential protective role of SnPE in neurodegeneration respect to other berries.

Table 3.1. *Phenolic, anthocyanin contents and in vitro antioxidant activity of SnPE.*

Sample	Total phenolic content[(a)]	Anthocyanin content[(b)]	FRAP assay[(c)]	ABTS assay[(d)]	DPPH assay[(e)]
SnPE	72.14±2.93	52.19±5.72	293.66±8.99	29.66±0.48	8.87±0.27

Results are expressed as μM EqQ[(a)]; μM Cyanidin 3-O-glucoside equivalent[(b)]; μM EqQ [(c)]; quenching (%)[(d)]; quenching (%)[(e)]. All data are the means ± statistic error (SE) of at least five independent determinations.

3.4.3 Evaluation of the protective effect of S. nigra extract in neuronal differentiation of IMR-32

The potential neuroprotective effect of SnPE to reduce the toxicity induced by neurotoxic molecules was evaluated. The differentiated IMR-32 cells were pre-treated with increasing concentrations of extract (µg/ml w/v) for 24 h as reported in "Materials and Methods". To reproduce *in vitro* the pathological condition, the neurotoxic damage was induced by treating the cells with two agents, Aβ peptide, involved in the formation of amyloid plaque, or H_2O_2, which plays a role in the neurodegenerative process. In particular, the oxidative stress is the primary event involved in the cellular injury which induces neuropathology conditions [43]. However, it is not only the primary cause, but it is also implicated in phathology mechanism that occurs after the deposition of amyloid plaque. In particular, a current hypothesis considers the possibility that proteins involved in protofibril formation are responsible for the oxidative stress, confirming the production of H_2O_2 induced by Aβ peptide [43]. Therefore, after the incubation with SnPE, the cells were treated with 20 µM Aβ peptide for 48 h or 100 µM H_2O_2 for 18 h. As report in **Figure 3.7**, the mono-treatment with SnPE did not affect cell viability, determined by MTT assay, compared to untreated control, showing the absence of toxicity by SnPE. Furthermore, the pre-incubation with SnPE significantly protected against toxicity induced by Aβ in a dose dependent manner (**Figure 3.7, A**). Similarly, while the treatment with H_2O_2 induced a significant reduction in cell viability (67%), the pre-treatment with SnPE rescued the cytotoxicity of H_2O_2 of about 30% (**Figure 3.7, B**). These data demonstrated that SnPE was able to significantly reduce the citotoxicity induced by both Aβ peptide and H_2O_2, probably interfering with the deposition of the plaque and the oxidative stress.

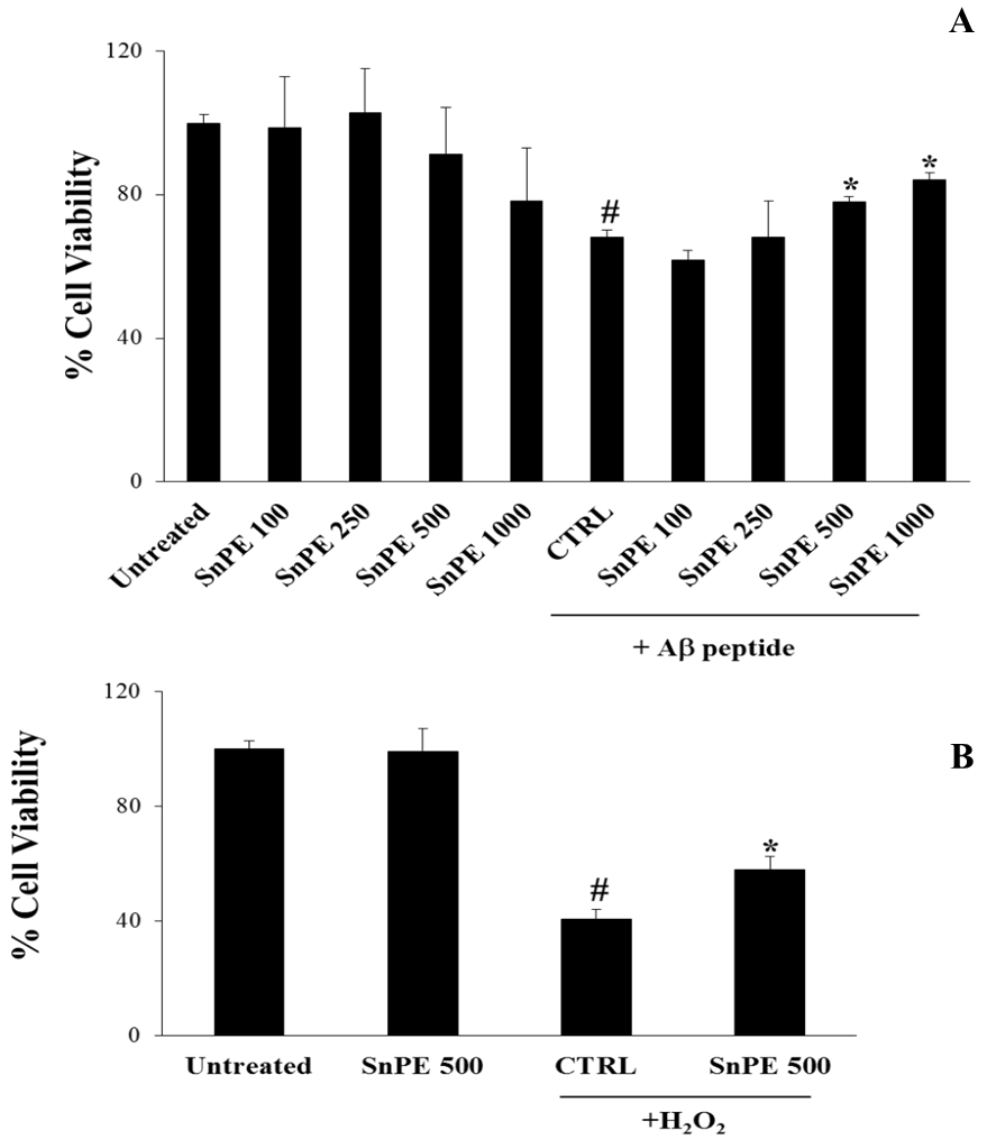


Figure 3.7. *Neuroprotective effect of SnPE against toxicity induced by Aβ peptide* ***(A)*** *and* H_2O_2 ***(B)***. *The differentiated IMR-32 cells were pre-incubated for 24 h with SnPE at the indicated concentrations (μg/ml, w/v). Subsequently, 20 μM Aβ peptide (25-35 fragment) was added for 48 h in* ***A*** *and 100 μM* H_2O_2 *for 18 h in* ***B***. *After incubation, the cell viability was determined by MTT assay, as described in "Materials and Methods" and the results are expressed*

*as % of cell viability. The bars in the graphs indicate SE; symbols indicate significance: #p<0.001 vs untreated, *p<0.05 vs CTRL+Aβ or CTRL+H_2O_2.*

It should also be considered the increased susceptibility to mitochondrial dysfunction and inflammatory insults induced by oxidative stress as major contributing factors involved in brain aging [44]. Indeed, age-related deficits in brain might be due to the injuries by oxidative damage, with a decrease of normal antioxidant defense mechanisms. For this reason, the cabability of SnPE to reduce the toxicity by H_2O_2 highlights its potential protective role from neurodegenerative mechanisms aging-related.

3.4.4 Protection of S. nigra extract from neuronal cell death

We reasoned that the reduction of cell viability induced by the neurotoxic molecules could be attributable to the activation of an apoptosic process. In fact, as reported in the Introduction section, the neuronal damage by oxidizing agents induces the apoptotic process, the main mechanism of death activated in AD [16], characterized by chromatin condensation and nuclear fragmentation into the apoptotic bodies. In order to verify the ability of SnPE to reduce apoptosis, we firstly verified the presence of the apoptotic bodies after treatment with 100 μM H_2O_2 using the nuclear stain Hoechst. The **Figure 3.8** shows that H_2O_2 induced an increase of about 53% of apoptotic cells compared to untreated cells. The pre-treatment with SnPE (500 μg/ml, w/v), significantly reduced the apoptotic bodies induced by H_2O_2 by 32%.

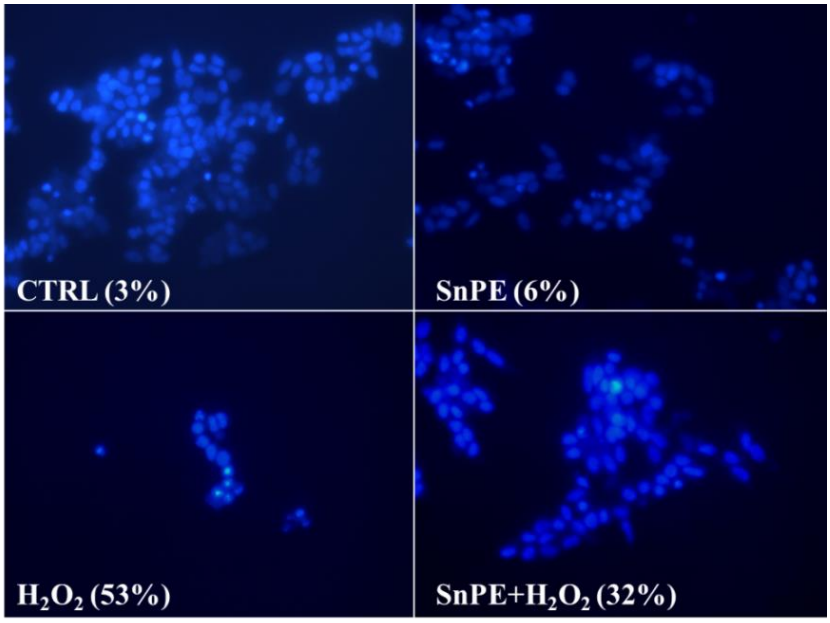


Figure 3.8. *Apoptotic nuclei evidenced by Hoechst staining. Representative image from three independent experiments (Inverted microscope AXIOVERT, Fluorescence 200X). The numbers indicate the % of apoptotic bodies determined as reported in "Materials and Methods".*

To confirm the protection from H_2O_2-induced apoptosis by SnPE, we evaluated additional apoptotic markers, measuring level of activation of the caspase-3, -8 and -9. In fact, these enzymes are essential to implement the apoptosis, through the proteolitic cleavage after an aspartic acid residue of proteins that induce the fragmentation of nuclear material and cell death. In particular, the capsases -8 and -9 belong to the "initiators" group of caspases which activate, by proteolitic cut, other caspases, called "effectors", such as caspase-3. During the execution phase of apoptosis a series of enzymatic caspase reactions occurs, leading to cell death cascade [45]. Several authors have documented the activities of caspases in the brain of AD patients [46].

The **Figure 3.9** shows how the treatment with H_2O_2 induced a significantly increase in the caspase-3 activity compared to untreated cells, while the pretreatment with the SnPE significantly reduced of about 4-fold the activation

of caspase-3. These data confirm that SnPE was able to reduce the activaction of apoptosis induced by H_2O_2.

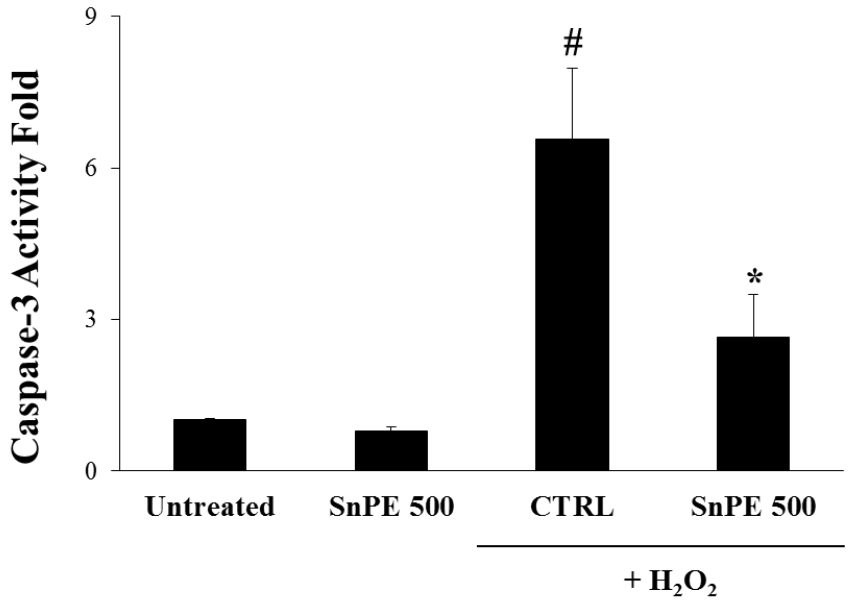


Figure 3.9. *Protection of SnPE from apoptosis induced by H_2O_2. The differentiated IMR-32 cells were pre-incubated for 24 h with SnPE at 500 μg/ml (w/v); subsequently, 100 μM H_2O_2 was added for 5 h. Caspase-3 activity was determined by enzymatic assay as described in "Materials and Methods" and was expressed as nmol AFC/min/μg total proteins. The bars in the graphs indicate SE; symbols indicate significance: #p<0.001 vs untreated; *p<0.05 vs CTRL+H_2O_2.*

The effector caspase-3 can be activated by two different pathways: the death-receptor pathway (extrinsic pathway) mediated by death receptors and caspase-8 and the mitochondrial pathway triggered by pro-apoptotic protein Bcl-2, with a formation of a complex called the "apoptosome", consisting of the cytochrome c, caspase 9 and APAF-1 (apoptotic protease activating factor 1), involving oxidative stress and activation of caspase-9 [47]. Several

evidences report that the oxidative damage in the AD brain is possibly related to direct oxidizing effects of β-amyloid [47], leading to the hypothesis that the mitochondrial pathway of apoptosis may be activated in AD. The role of mitochondrial dysfunction and oligomerization of Aβ peptide result in high susceptibility to oxidative stress and translate into cell death. In AD brain has been observed the mitochondrial dysfunction and the stimulation of the mitochondrial pathway of apoptosis, by activation of caspase-9 [47]. Furthermore, several studies have also reported the involvement of the death receptor-mediated pathway in AD brain, suggesting that β-amyloid may activate the initiator procaspase-8 by cross-linking death receptors such as Fas/TNF family, with a sequential activation of both caspase-8 and caspase-3 [48]. An alternative explanation may suggest a cross-link between the two pathways. In fact, according to **Figure 3.10**, it has been proposed by [49] that the mitochondrial pathway is not the only way to trigger apoptosis in AD, suggesting that, following stimulation of the mitochondrial pathway, activation of caspase-9 may lead to processing of caspase-8 downstream of caspase-3. Therefore, it is plausible that β-amyloid may initiate the activation of both apoptotic pathways, through either cross-linking of death receptors or through the generation of oxidative damage.

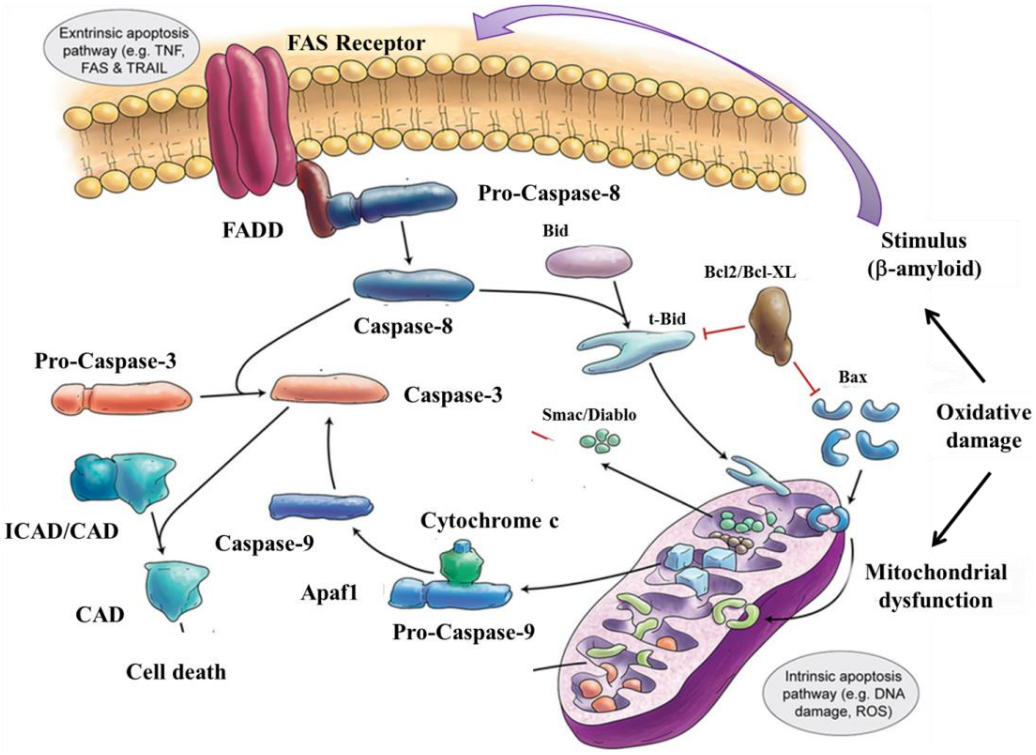


Figure 3.10. *Proposed pathway of apoptotic activation by β-amyloid in the AD brain [49].*

Based on these hypotheses, we investigated if the treatment with SnPE could interfere with the two different pathways, measuring the activation levels of caspase-8 and -9. As reported in **Figure 3.11**, the pre-treatment with SnPE (500 μg/ml, w/v) was able to significantly reduce the activation of both apoptotic pathways, inducing the decrease of caspase-8 (**Figure 3.11, A**) and caspase-9 (**Figure 3.11, B**) activities respect to treatment with H_2O_2. The decrease of caspase activities was of 30% and 22% for caspase-8 and -9, respectively.

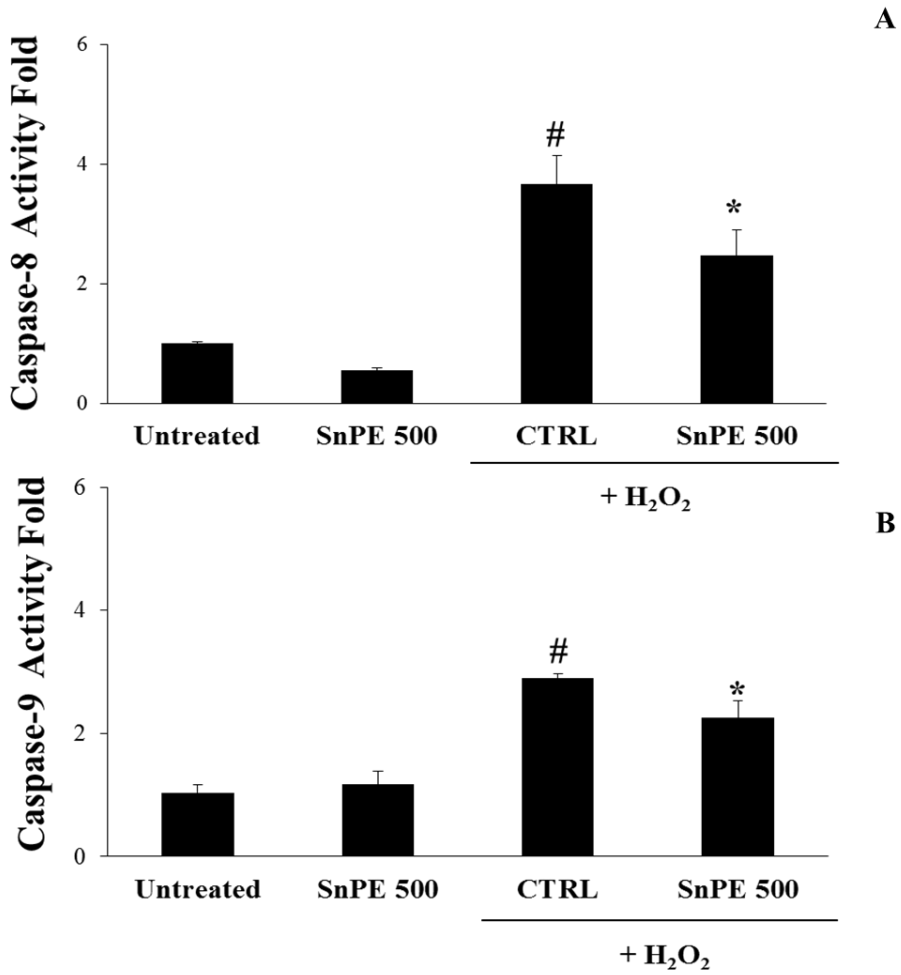


Figure 3.11. *Protection of SnPE from apoptosis induced by H_2O_2. The differentiated IMR-32 cells were pre-incubated for 24 h with SnPE at 500 μg/ml (w/v); subsequently, 100 μM H_2O_2 was added for 5 h. Caspase-8* ***(A)*** *and -9* ***(B)*** *activity were determined by enzymatic assay as described in "Materials and Methods" and were expressed as nmol AFC/min/μg total proteins. The bars in the graphs indicate SE; symbols indicate significance: #p<0.001 vs untreated, *p<0.05 vs CTRL+H_2O_2.*

3.4.5 Protection of S. nigra extract from oxidative stress

The brain is overly susceptible to the ROS damages and in particular, the oxidative stress has been linked to neuronal cell death, activating intracellular signaling pathways. To demonstrate whether the protection of SnPE against the toxicity induced by oxidizing agents was related to its antioxidant capacity, we measured the levels of intracellular ROS by a fluorimetric assay, as reported in "Materials and Methods". The **Figure 3.12** shows that the treatment of the IMR-32 differentiated cells with Aβ peptide increases ROS by 40% compared to untreated control. We observed a significant reduction of ROS levels, in a dose-dependent manner, when cells were pre-incubated with SnPE at concentrations of 500 and 1000 μg/ml (w/v) compared to Aβ treatment (**Figure 3.12**). Furthermore, **Figure 3.12** also shows that the mono-treatment with SnPE increased slightly but significantly the intracellular ROS (about 10-20%), suggesting that this limited icrement is enough to induce an adaptive cellular response, resulting in an improvement of cellular antioxidant defenses and in protection from the oxidative stress induced by oxidizing agents.

We concluded that the potential protective role of SnPE was mediated by the reduction of oxidative stress, implicated in aging-related degenerative diseases.

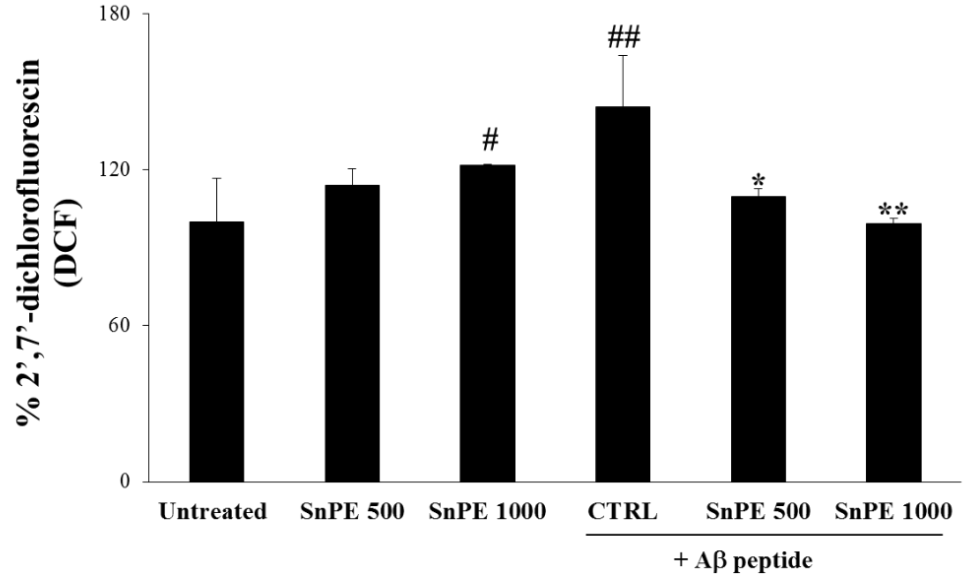


Figure 3.12. *Reduction of intracellular ROS formation induced by Aβ peptide after treatment with SnPE. The differentiated IMR-32 cells were pre-incubated for 24 h with SnPE at the indicated concentrations (μg/ml, w/v); subsequently, 20 μM Aβ peptide (25-35 fragment) was added for 7 h. ROS intracellular levels were measured as reported in "Materials and Methods" and expressed as fluorescence of DCF (% control). The bars in the graphs indicate SE; symbols indicate significance: #p<0.001 and ##p<0.05 vs untreated ,*p<0.05 and **p<0.005 vs CTRL+Aβ.*

3.5 Conclusions

The novelty of the present work resides in the demonstration, for the first time to our knoeledge, that SnPE exerts potential neuroprotective effects in an *in vitro* experimental model of neurodegeneration. We demostrated that the efficacy of SnPE to effectively protect neuronal cells from apoptosis induced by Aβ peptide and H_2O_2, is probably related to its ability to modulate the cellular antioxidant response. In fact, the oxidative stress plays an important role in neurodegenerative disordes, even if the absolute assumption whether this event is the cause or the consequence of the neurodegenerative process is still under discussion [43]. Our results suggest that the protective effect of SnPE against apoptosis induced by neuritoxic agents, is probably mediated by a reduction of oxidative stress, resulting in the decrease of ROS induced by Aβ peptide. Considering the *in vitro* and *in vivo* data referring to the capability of polyphenols to modulate the activity of specific antioxidant enzymes, we hypothesized the interaction with cellular antioxidant systems, such as GSH, and further studies are currently underway in this direction. Certainly, it will be necessary to study the molecular mechanisms triggered by SnPE to elucidate the molecular basis of its neuroprotective effects. Therefore, these results represent a first indication that SnPE can inspirate future clinical trials in order to confirm the preventive and/or therapeutic potential of this natural extract against neurodegenerative aging-related diseases and its therapeutic application alone or in combination with other neuroprotective compounds.

3.6 References

1. Lindsay, J., et al., *Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging.* Am J Epidemiol, 2002. **156**(5): p. 445-53.
2. Gu, Y., et al., *Food combination and Alzheimer disease risk: a protective diet.* Arch Neurol, 2010. **67**(6): p. 699-706.
3. Breydo, L. and V.N. Uversky, *Role of metal ions in aggregation of intrinsically disordered proteins in neurodegenerative diseases.* Metallomics, 2011. **3**(11): p. 1163-80.
4. Cifra, A., G.L. Mazzone, and A. Nistri, *Riluzole: what it does to spinal and brainstem neurons and how it does it.* Neuroscientist, 2013. **19**(2): p. 137-44.
5. Reitz, C. and R. Mayeux, *Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers.* Biochem Pharmacol, 2014. **88**(4): p. 640-51.
6. Bramblett, G.T., et al., *Abnormal tau phosphorylation at Ser396 in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding.* Neuron, 1993. **10**(6): p. 1089-99.
7. Ferreira, S.T. and W.L. Klein, *The Abeta oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease.* Neurobiol Learn Mem, 2011. **96**(4): p. 529-43.
8. Meraz-Rios, M.A., et al., *Tau oligomers and aggregation in Alzheimer's disease.* J Neurochem, 2010. **112**(6): p. 1353-67.
9. Glenner, G.G., et al., *The amyloid deposits in Alzheimer's disease: their nature and pathogenesis.* Appl Pathol, 1984. **2**(6): p. 357-69.
10. De Strooper, B., R. Vassar, and T. Golde, *The secretases: enzymes with therapeutic potential in Alzheimer disease.* Nat Rev Neurol, 2010. **6**(2): p. 99-107.
11. Bergmans, B.A. and B. De Strooper, *gamma-secretases: from cell biology to therapeutic strategies.* Lancet Neurol, 2010. **9**(2): p. 215-26.
12. Pescosolido, N., et al., *Critical Review on the Relationship between Glaucoma and Alzheimer's Disease.* Adv Ophthalmol Vis Syst, 2014. **1**(4).
13. Golde, T.E., D. Dickson, and M. Hutton, *Filling the gaps in the abeta cascade hypothesis of Alzheimer's disease.* Curr Alzheimer Res, 2006. **3**(5): p. 421-30.
14. Boyd, T.D., et al., *GM-CSF upregulated in rheumatoid arthritis reverses cognitive impairment and amyloidosis in Alzheimer mice.* J Alzheimers Dis, 2010. **21**(2): p. 507-18.
15. Khadem-Ansari, M.H., et al., *Elevated levels of urinary 8-hydroxy-2'-deoxyguanosine and 8-isoprostane in esophageal squamous cell carcinoma.* J Carcinog, 2011. **10**: p. 14.

16. Axelsen, P.H., H. Komatsu, and I.V. Murray, *Oxidative stress and cell membranes in the pathogenesis of Alzheimer's disease.* Physiology (Bethesda), 2011. **26**(1): p. 54-69.
17. Schroeter, H., et al., *Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3.* Biochem J, 2001. **358 Pt 3**: p. 547-57.
18. Kang, N.J., et al., *Polyphenols as small molecular inhibitors of signaling cascades in carcinogenesis.* Pharmacol Ther, 2011. **130**(3): p. 310-24.
19. Scapagnini, G., et al., *Modulation of Nrf2/ARE pathway by food polyphenols: a nutritional neuroprotective strategy for cognitive and neurodegenerative disorders.* Mol Neurobiol, 2011. **44**(2): p. 192-201.
20. Jayasena, T., et al., *The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease.* Ageing Res Rev, 2013. **12**(4): p. 867-83.
21. Spencer, J.P., *Food for thought: the role of dietary flavonoids in enhancing human memory, learning and neuro-cognitive performance.* Proc Nutr Soc, 2008. **67**(2): p. 238-52.
22. Joseph, J.A., B. Shukitt-Hale, and L.M. Willis, *Grape juice, berries, and walnuts affect brain aging and behavior.* J Nutr, 2009. **139**(9): p. 1813S-7S.
23. Krikorian, R., et al., *Concord grape juice supplementation improves memory function in older adults with mild cognitive impairment.* Br J Nutr, 2010. **103**(5): p. 730-4.
24. Seeram, N.P., *Berry fruits: compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease.* J Agric Food Chem, 2008. **56**(3): p. 627-9.
25. Simonyi, A., et al., *Inhibition of microglial activation by elderberry extracts and its phenolic components.* Life Sci, 2015. **128**: p. 30-8.
26. Wu, X., G. Cao, and R.L. Prior, *Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry.* J Nutr, 2002. **132**(7): p. 1865-71.
27. Tumilowicz, J.J., et al., *Definition of a continuous human cell line derived from neuroblastoma.* Cancer Res, 1970. **30**(8): p. 2110-8.
28. Asami-Odaka, A., et al., *Long amyloid beta-protein secreted from wild-type human neuroblastoma IMR-32 cells.* Biochemistry, 1995. **34**(32): p. 10272-8.
29. Kume, T., et al., *Dibutyryl cyclic AMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype.* Neurosci Lett, 2008. **443**(3): p. 199-203.
30. Lehner, B., et al., *The dark side of BrdU in neural stem cell biology: detrimental effects on cell cycle, differentiation and survival.* Cell Tissue Res, 2011. **345**(3): p. 313-28.

31. Zipper, H., et al., *Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications.* Nucleic Acids Res, 2004. **32**(12): p. e103.
32. Maresca, G., et al., *LMNA knock-down affects differentiation and progression of human neuroblastoma cells.* PLoS One, 2012. **7**(9): p. e45513.
33. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
34. Wu, Q.K., et al., *Berry phenolic extracts modulate the expression of p21(WAF1) and Bax but not Bcl-2 in HT-29 colon cancer cells.* J Agric Food Chem, 2007. **55**(4): p. 1156-63.
35. Singleton, V.L. and J.A. Rossi, *Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents.* Am J Enol Vitic, 1965. **16**: p. 144-158.
36. Lee, J., R.W. Durst, and R.E. Wrolstad, *Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study.* J AOAC Int, 2005. **88**(5): p. 1269-78.
37. Morgan, D.M., *Tetrazolium (MTT) assay for cellular viability and activity.* Methods Mol Biol, 1998. **79**: p. 179-83.
38. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
39. Russo, M., et al., *Quercetin and anti-CD95(Fas/Apo1) enhance apoptosis in HPB-ALL cell line.* FEBS Lett, 1999. **462**(3): p. 322-8.
40. Misiti, F., et al., *Protective effect of rhubarb derivatives on amyloid beta (1-42) peptide-induced apoptosis in IMR-32 cells: a case of nutrigenomic.* Brain Res Bull, 2006. **71**(1-3): p. 29-36.
41. Kataria, H., et al., *Water extract from the leaves of Withania somnifera protect RA differentiated C6 and IMR-32 cells against glutamate-induced excitotoxicity.* PLoS One, 2012. **7**(5): p. e37080.
42. Tavares, L., et al., *Neuroprotective effects of digested polyphenols from wild blackberry species.* Eur J Nutr, 2013. **52**(1): p. 225-36.
43. Andersen, J.K., *Oxidative stress in neurodegeneration: cause or consequence?* Nat Med, 2004. **10 Suppl**: p. S18-25.
44. Mariani, E., et al., *Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview.* J Chromatogr B Analyt Technol Biomed Life Sci, 2005. **827**(1): p. 65-75.
45. Calissano, P., C. Matrone, and G. Amadoro, *Apoptosis and in vitro Alzheimer disease neuronal models.* Commun Integr Biol, 2009. **2**(2): p. 163-9.

46. Su, J.H., et al., *Activated caspase-3 expression in Alzheimer's and aged control brain: correlation with Alzheimer pathology.* Brain Res, 2001. **898**(2): p. 350-7.
47. Rohn, T.T., et al., *Caspase-9 activation and caspase cleavage of tau in the Alzheimer's disease brain.* Neurobiol Dis, 2002. **11**(2): p. 341-54.
48. Rohn, T.T., et al., *Caspase Activation in the Alzheimer's Disease Brain: Tortuous and Torturous.* Drug News Perspect, 2002. **15**(9): p. 549-57.
49. Rohn, T.T., et al., *Activation of caspase-8 in the Alzheimer's disease brain.* Neurobiol Dis, 2001. **8**(6): p. 1006-16.

Chapter IV

Antiproliferative effect of carotenoids from *Cucurbita moschata*

4.1 Introduction

Studies on the anticancer role of natural compounds rapidly expanded in the last decade due to their possible applications in chemoprevention. This term refers to the ability of chemically synthesized or naturally occurring agents to block, invert or retard one or more mechanisms regulating cancer onset, development and progression [1]. Among phytochemicals, carotenoids, such as lycopene, β-carotene, ATRA and their derivatives, show cancer-preventive activity reported in several *in vitro* and *in vivo* studies [2, 3]. Lycopene has been one of the most studied carotenoids. Epidemiological studies have linked the increased assumption of lycopene with a reduction of prostate and breast cancer risk. Also *in vivo* studies have shown that lycopene significantly reduces the prostate cancer incidence, through the ability to decrease androgen signaling, IGF-I expression, and basal inflammatory signals in a mouse model of prostate cancer [4]. However, there is still no clearly proven clinical evidences to support the use of lycopene in the prevention or treatment of prostate cancer, due to the limited number and quality of published randomized clinical trials. In particular, four randomized controlled clinical trials analyze the effects of lycopene on the secondary prevention of prostate cancer, showing contrasting results. In fact, only one of these studies reports that the administration of 4 mg of lycopene for one year reduced the incidence of prostate cancer, which was of 10% in the intervention and 30% in the control groups (**Table 4.1**). Other studies shows a significant reduction of serum prostate antigen specific (PSA) in patients with prostate hyperplasia after lycopene administration (**Table 4.1**) [4].

Table 4.1. *Randomized controlled clinical trials analyzing the effects of lycopene on the prevention of prostate cancer [4].*

Patients	Follow-up	Lycopene formulation and source	Outcome
40 patients with high-grade intraepithelial neoplasia	2 years	4 mg lycopene (Lyc-O-Mato, LycoRed Natural Products Industries,Beer-Sheva, Israel)	The incidence of prostate cancer was 10%in the intervention and 30% in the control group. Decreased PSA levels in the intervention group
80 patients with high-grade intraepithelial neoplasia	4 months	15 mg lycopene (Lyc-O-Mato, LycoRed Natural Products Industries,Beer-Sheva, Israel) twice a day	No difference in PSA levels between groups. Incidence of prostate cancer was not reported
40 patients with proven benign prostate hyperplasia	6 months	15 mg microencapsulated synthetic lycopene (LycoVit, Germany) once a day	Significant reduction in PSA levels of intervention group. Incidence of prostate cancer was not reported
131 patients with elevated PSA levels	21 days	15 mg lycopene (Lyc-O-Mato, LycoRed Natural Products Industries,Beer-Sheva, Israel) twice a day	Significant increase of lycopene serum levels and prostate tissue in the intervention group. No significant difference in prostate cancer incidence

Furthermore, some randomized controlled clinical trials emphasize the possible therapeutic effects of lycopene in prostate cancer, reporting the reduction of PSA serum levels and a significant decline in the rate of PSA increase from pre-therapy to post-therapy in patient with prostate cancer (**Table 4.2**) [4]. In a recent systematic review about dose-response meta-analysis of observational studies, the authors conclude that only α-carotene and lycopene, not β-carotene, show an inverse association with the risk of prostate cancer, although these compounds are not able to reduce the risk in advanced forms of this cancer [5]. The other side of the coin is represented by an old but still emblematic interventional study, the ATBC (Alpha-Tocopherol Beta-Carotene) Cancer Prevention Study, where 29,133 male smokers, ages

50-69, were treated daily with β-carotene (20 mg/day as all-trans-β-carotene), α-tocopherol (50 mg/day as DL-α-tocopheryl acetate), both β-carotene and α-tocopherol, or placebo.

Table 4.2. *Randomized controlled clinical trials analyzing the therapeutic effects of lycopene in prostate cancer [4].*

Patients	Follow-up	Lycopene formulation and source	Outcome
26 patients with clinically diagnosed prostate cancer prior to scheduled prostatectomy	3 weeks	15 mg lycopene (Lyc-O-Mato, LycoRed Natural Products Industries,Beer-Sheva, Israel) once a day	Decreased PSA levels in the intervention group. 73% of the intervention group had free resection margins
54 patients with prostate cancer metastases and orchidectomy	24 months	2 mg lycopene (no information about formulation) twice a day	Decreased PSA levels in the intervention group. Survival rate was higher in the intervention group
71 patients with histologically proven prostate cancer	6 months	15 mg lycopene (Lyc-O-Mato, LycoRed Natural Products Industries,Beer-Sheva, Israel) twice a day	No decline in serum PSA levels. Survival rate was higher in the intervention group
41 patients with recurrent prostate cancer	8 weeks	Tomato containing 25 mg lycopene per day	Significant increase of lycopene serum levels. No decline in serum PSA levels.

The alarming results obtained after 5-8 years of follow-up, caused the immediate end of the trial since β-carotene supplementation was associated with an increased risk of lung cancer development (**Table 4.3**) [6, 7]. Unexpected results were also obtained from the β-Carotene and Retinol Efficacy Trial (CARET), where the combination of 30 mg β-carotene and 25,000 IU retinyl palmitate (vitamin A) daily in 18314 men and women at high risk of developing lung cancer was tested (**Table 4.3**). This study was terminated 21 months early because of clear evidence of no benefit and of possible harm. In fact, a 28% increase of lung cancers was measures with 17%

more deaths in the active intervention group [8]. Other long-term large randomized intervention trials were designed to test the efficacy of high doses of β-carotene (20–30 mg/day) in the prevention of cancer. The Linxian (Chinese) Cancer Prevention Study have shown that supplementation with β-carotene, vitamin E and selenium led to a significant reduction in total mortality (9%), especially from total cancer (13%) and stomach cancer in particular (21%) (**Table 4.3**). The positive results of the Chinese study probably reflected the correction of a vitamin A deficiency in the study population. The Women's Health Study indicated no statistically significant differences in incidence of cancer, although the treatment duration was short (a median treatment duration of 2.1 years and a median total follow-up of 4.1 years) (**Table 4.3**).

Table 4.3. *β-carotene supplementation trials* [9].

Studies	Population	Intervention	Duration	Outcome
ATBC	29,133 Finish male smokers (50-69 years of age)	20 mg/day β-carotene; 50 mg/day vitamin E	5-8 years	18% increase in lung cancer; 8% increase in mortality
CARET	18,314 men and women and asbestoss workers (45-74 years of age)	30 mg/day β-carotene; 25,000 IU vitamin E	4 years	28% increase in lung cancer; 17% increase in mortality
PHS	22,071 male physicians (40-84 years of age)	50 mg b-carotene on alternate days	12 years	No effect of supplementation in incidence of cancer
Linxian	29,584 men and women, vitamin and mineral deficient (40-69 years of age)	15 mg/day β-carotene; 50 mg/day selenium; 30 mg/day vitamin E	5 years	13% decrease in total cancers; 9% decrease in overall deaths
Women's Health Study	39,876 female health professionals (over 45 years of age)	50 mg β-carotene on alternate days	4 years(2 years of treatment and 2 years of follow-up)	No effect of supplementation in incidence of cancer

On the opposite, ATRA, derived from carotenoids as described in Chapter I, when administered as co-adjuvant in chemotherapy against acute promyelocytic leukemia (APL) resulted in absence of cytotoxicity and an increase of complete remission 93% to 100% in 5 years [10]. Considering these results, future research could focus on the potential application of ATRA as adjuvant in therapy of solid tumors. Moving from clinical to pre-clinical studies, numerous *in vitro* and *in vivo* investigations show the antiproliferative activity of carotenoids, e.g., as α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein, zeaxanthin, violaxanthin, neoxanthain, canthaxanthin, astaxanthin, fucoxanthin, in different cancer models, such as colon, liver, breast, prostate, cervix and leukemia [9]. The *in vitro* studies on cancer cell lines reveal that carotenoids affect cell growth or cell death. In particular, carotenoids interfere with different aspects of cancer biology, such as hormone and growth factor signalling, regulatory mechanisms of cell cycle progression, cell differentiation, apoptosis and autophagy (**Figure 4.1**).

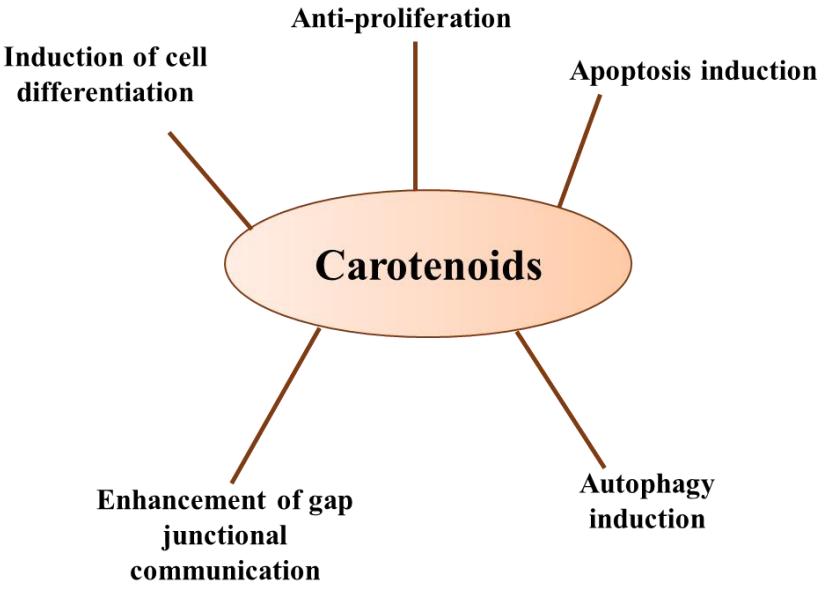


Figure 4.1. *Proposed mechanisms by which carotenoids suppress*

carcinogenesis [9].

- *Antiproliferative effect*

Dysregulation of cell cycle was one of the major hallmarks of cancer and carotenoids inhibit the growth of tumor cells by interfering at different levels with this process [11]. In human colon adenocarcinoma cells, β-carotene induced cell cycle arrest in the G2/M phase by reducing the expression of cyclin A, one of the key regulator of the G2/M transition [11]. The mechanisms by which lycopene may reduce the risk of prostate cancer also involves the arrest of cell cycle progression in G0/G1 phase mediated by the decrease of cyclins D1 and E [11]. Moreover, lycopene exerts the anti-proliferative effect in human colon cancer cells reducing the expression of cyclin D1, inhibiting the phosphorylation of pRB protein and increasing the expression of $p27^{KIP}$ [11]. The cell cycle arrest induced by fucoxanthin in hepatocarcinoma cells is accompanied with an enhanced gap junctional intracellular communication linked to the increased expression of gap junction proteins, Cx43 and Cx32, and also by decreased activation of c-Jun N-terminal kinase (JNK) and extracelluar signal-regulated kinase (ERK) [11].

Other examples exist in the literature indicating that the antiproliferative effect of carotenoids can be the result of differentiating processes. This is the case of ATRA which, as reported for the first time in 1980 [12], at micromolar concentrations induced granulocytic differentiation in HL-60 cell line, derived from a patient with APL. Although preliminary, our research group also showed that the radio-sensitizing effects of ATRA on human chronic lymphocytic leukemia and osteosarcoma cell lines induces cell differentiation [13].

- *Activation of apoptosis*

A well-studied process which can help to explain the anticancer effects of carotenoids in cancer cell lines is apoptosis. *In vitro* studies have shown that carotenoids, such as β-carotene, neoxanthin, fucoxanthin, canthaxanthin and lutein, induce apoptosis in different malignant cell lines, such as human cervical cancer line, colon adenocarcinoma, mammary carcinoma cells [14]. These compounds trigger different mechanisms of caspase cascade activation by the interaction with both apoptotic pathways, the extrinsic and mitochondrial ones. β-Carotene activates caspase-3, the executioner of the apoptotic process, necessary for the chromatin condensation and nuclei fragmentation [15]. Caspase-3 cascade is activated by carotenoids mainly through the interaction with the receptor complex on cell membrane, which induces caspase-8 activation, and also through a non-receptor signalling pathway, which induces intrinsic pathway through caspase-9 activation [15]. The involvement of mitochondrial pathway has been clearly demonstrated by β-carotene with the alteration of the mitochondrial membrane potential and release of cytochrome c from mitochondria in different tumor cells [14]. Other carotenoids also induce apoptosis. As an example, in sarcoma 180 xenograft-bearing mice, fucoxanthin interacts with the ROS-mediated Bcl-xL pathway and JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling pathway, through the down-regulation of EGFR, as reported by an *in vivo* study [16]. A different mechanism explaining the apoptosis induction by carotenoids refers to conformational changes of some membrane-associated "death" receptors, resulting in the activation of caspase 8. It has also been suggested that carotenoids could modulate the expression of the pro-apoptotic proteins, Bid, Bad, and Bax, in different experimental cell models such as leukemia (HL-60) and colon adenocarcinoma cell lines (HT-29) [14].

- *Carotenoids and autophagy in cancer cell lines*

Recent studies have reported the involvement of some carotenoids in the autophagic processes [17]. The ability to modulate the autophagy has been shown by not-retinoid precursors of carotenoids, such as astaxanthin [18] and fucoxanthin [19]. In particular, the treatment with flucoxanthin induces autophagy-dependent cytotoxic effects in human epithelial cervical cancer, HeLa cells, through the inhibition of Akt/mTOR signaling pathway [19]. Furthermore, ATRA induces autophagy through the interaction with the receptor mannose-6-phosphate cation-independent, a glycoprotein constitutively produced by the cells, which plays a key role in endocytosis and mediates the transport of proteins bound to the mannose-6-phosphate from the trans-Golgi to endosomes. Finally the induction of a cytotoxic autophagy, associated to a reduction of the cell viability by ATRA and γ-radiations treatment has been recently reported by [13], showing its radio-sensitizing effects on human chronic lymphocytic leukemia (HG3) and osteosarcoma cell lines (SAOs). A part from the above cited papers, the role of carotenoids in inducing autophagy represents a field only barely studied. Since this topic will be experimentally investigated in the present Chapter, it is useful to summarize the key events regulating the different forms of autophagy in cancer cells. The term autophagy means "eating oneself" and was coined by the Nobel Prize Christian de Duve in the '60s. It describes a catabolic process, activated by stress conditions aimed to maintain cellular integrity through the regeneration of metabolic precursors (amino acids, nucleic acids, fatty acids) and the elimination of sub-cellular detritus or damaged organelles (mitochondria, endoplasmic reticulum, peroxisomes), or pathogenic organisms (viruses, bacteria) by the lysosomal enzymes. This process is essential for tissue homeostasis and for regulation of differentiation and development of multicellular organisms and can be divided into several steps, governed by

about 30 genes (autophagy related gene, ATG), first discovered in yeast and then in higher vertebrates [20]. The importance of this process in cell biology is confirmed by the assignment of Nobel prize for medicine and physiology in 2016 to professor Oshumi who studied autophagy in yeast following nutrient starvation [21]. The lack of nutrients is a powerful physiological regulator of autophagy and this signal converges on the mTOR protein (mammalian target of rapamycin), located in a macromolecular complex called mTORC1. Therefore, the depletion of energy (a decrease of ATP and an increase of AMP) activate AMPK (adenosine 5'-monophosphate-activated protein kinase) [22]. This kinase binds and phosphorylates UNC-51 like kinase 1 (ULK1), which leads the direct activation of ULK1 by AMPK bypassing mTOR-inhibition. The ULK1 complex is a crucial factor in the early stages of autophagy (**Figure 4.2**). In fact, ULK1 and Beclin-1 molecular complex (Beclin-1 interacting complex), induce the initial stage of autophagy, involved in the nucleation of autophagosomes, which are vesicles of a phospholipid bilayer surrounding the cytoplasmic material. The autophagosome merge with lysosomes to create the microenvironment necessary for the degradation and recycling of their content (**Figure 4.2**).

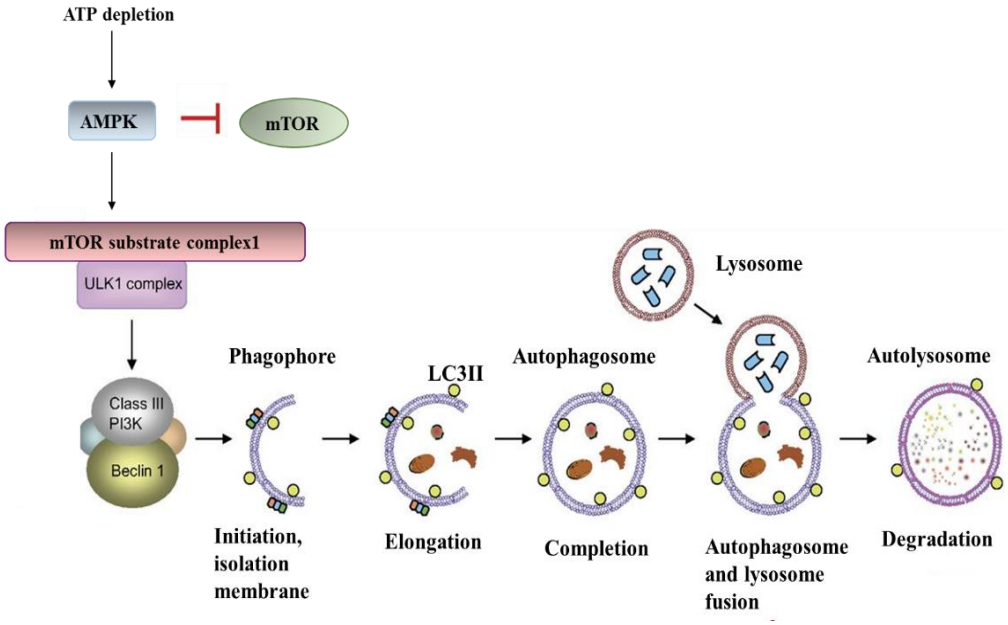


Figure 4.2. *Autophagy induction and regulation mechanisms[23]*.

Once the membrane formation initiated, follows its extension, which requires two ubiquitin-like conjugation systems: ATG5-ATG12 and LC3-ATG8 (microtubule-associated protein light chain 3). The LC3 system is present in two forms: the inactive, free cytosolic form (LC3-I) and the active form conjugated to phosphatidylethanolamine (LC3-II). The function of ATG5-ATG-12 and LC3 systems is the complexation to autophagosome membrane extension, sustaining their growth until the formation of mature vesicle (autophagosome mature) and represents the specific signal, need to address the vesicle to the process of fusion with lysosomes and finally to the degradation of the cargo [24] (**Figure 4.2**).

As mentioned above, the autophagic process is involved in the regulation of cellular homeostasis in response to environmental stimuli. However, new and more complex roles have been attributed to autophagy in pathological conditions, such as cancer. In fact, it should be underlined the dual and contrasting function of autophagic activation in oncogenesis: in early stage of

tumorigenesis, its activation can initially prevent tumor formation, protecting cells; however, in the late stages of carcinogenesis, autophagy may sustain cancer growth by inducing drug resistance. In particular, four functionally different forms of autophagy can be induced by drugs treatment, defined as protective, not protective, cytotoxic or cytostatic autophagy [25] (**Table 4.4**).
The protective form of autophagy can be activated by chemotherapy and confers resistance to treatments, protecting cancer cells. To detect this form, pharmacological inhibitors of autophagy, such as chloroquine (**Figure 4.2**) (inhibition of the proton pump responsible for lysosomal acidification) are experimentally used. After inhibition of the protective autophagy, it is possible to observe an increase of the cytotoxic effects of treatment, generally resulting in apoptotic cell death.

Table 4.4. *Characteristics of the four functional forms of autophagy [25]*.

Forms of autophagy	Characteristics
Cytoprotective	May confer resistance to therapy
	Increased sensitivity to therapy when blocked
Cytotoxic	Promotes cell death when induced
	Reduced sensitivity to therapy when blocked
Cytostatic	Mediates growth inhibition
	Potentially associated with senescence
Not protective	Inhibition does not influence sensitivity to therapy

On the opposite, when the “not protective” form is blocked, the cell sensitivity to therapy is not altered, as in the previous case (**Table 4.4**). The cytostatic form is related to cell growth arrest, while the cytotoxic autophagy is associated with a reduction of cell viability (**Table 4.4**).

4.2 Aims of the Chapter

The aim of this section of the thesis is the evaluation of the potential biological effects of a carotenoid extract obtained from *Cucurbita moschata* in cancer cell lines. The specific objectives are as follows:

-To develop a cellular delivery method (nanoemulsion) to vehicle the carotenoid extract;

-To evaluate the biological effect of carotenoid extract on different malignant cell lines, chosen for their different resistance to anticancer therapies (drugs and radiations);

-To study the mechanism of action triggered by carotenoids extract by the evaluation of: 1. the cellular phenotype; 2. the biochemical regulation of cell process involved in these *in vitro* models.

4.3 Materials and Methods

4.3.1 Chemicals

Phosphate buffer saline (PBS) tablets (Life Technologies); Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies); Fetal bovine serum (FBS) (HyClone); Glutamine (Life Technologies); Penicillin/streptomycin solution (Life Technologies); Not-essential amino acids (EuroClone, Milan, Italy); Tetrahydrofuran (THF); Butylated hydroxytoluene (BHT); Tween 80; Hexane; Ethanol; Trypan Blue (Sigma-Aldrich); Chloroquine (Enzo Life Sciences); CyQuant Detection Kit (Life Technologies); Cell-ID Autophagy Detection Kit (Enzo Life Sciences); Hoechst; DMEM without phenol red (Life Technologies,); β-carotene (Sigma-Aldrich); Quercetin (Sigma-Aldrich); ATRA (all-trans retinoic acid); MES (2-(N-morpholino)ethanesulfonic acid); Anti-LC3 I/II (Cell Signalling); Anti-pAMPK (Thr^{172}) (Cell Signalling); Anti-α-tubulin (Sigma-Aldrich); Horseradish peroxidase-linked secondary antibody (GE Healthcare). All other chemicals used were of research highest purity grade.

4.3.2 Sample preparation

Neapolitan pumpkin, *Cucurbita moschata*, was provided from Farris Company, located in Foggia, Italy. The rind was removed and the peeled flesh was chopped into small pieces and dehydrated to constant weight at 60 °C in the vacuum-drying oven. Simultaneously, seeds were recovered and dehydrated in the vacuum-drying oven. After grinding, aliquots (25 g each) of flesh matrix, milled seeds (co-matrix) or of a blend of oven-dried matrix plus co-matrix (1:1, w/w) were extracted by SC-CO_2, carried out at ISPA-CNR (Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle

Ricerche), Lecce (Italy) as reported by [26]. The oils extracted by S-CO_2 from the pumpkin flesh (matrix), milled seeds (co-matrix) and matrix/co-matrix (1:1, w/w) blend were analyzed by HPLC method at ISPA-CNR as reported [26]. Briefly, each oil sample (0.1 g) was dissolved in 1 ml of ethyl acetate, filtered through a 0.45 μm syringe filter and immediately analyzed by HPLC without extraction and saponification. Quali-quantitative analyses of tocols and carotenoids were carried out using an Agilent 1100 Series HPLC system and were separated using a reverse-phase C30 column (5 μm, 250 x 4.6 mm) with mobile phases consisting of methanol (A), 0.2% ammoniumacetate aqueous solution/methanol (20/80, v/v) (B) and methyl-t-butyl ether (C). The isocratic elution was as follows: 0 min, 95% A and 5% B; 0–12 min, 80% A, 5% B and 15% C; 12–42 min, 30% A, 5% B and 65% C; 42–60 min, 30% A, 5% B and 65% C; 60–62 min, 95% A, and 5% B. The column was re-equilibrated for 10 min between runs. The flow rate was 1.0 ml/min and the column temperature was maintained at 25 °C. The injection volume was 10 μl. Absorbance was measured by diode array at wavelengths of 475 nm for carotenoids and 290 nm for vitamin E.

4.3.3 Preparation and analysis of nanoemulsions from the carotenoid extract

Oil-in-water (O/W) nanoemulsions containing carotenoid extract (**CEN**, **c**arotenoid **e**xtract **n**anoemulsion) were prepared using a high-energy emulsification-evaporation technique, according to [27]. Briefly, the carotenoid extract was mixed in a 1:3 ratio (v/v) with tetrahydrofuran (THF) containing 0.0025% (v/w) butylated hydroxytoluene (BHT) and was added to an aqueous solution containing 0.3% Tween 80. Organic/aqueous phase volume ratio was 1:9. Similar procedure was followed for a standard

preparation of β-carotene nanoemulsions (**βCN**) (2% w/v in THF/BHT). The emulsions were homogenized using an Ultra-Turrax homogenizer (T8, Ika-Werke, Germany) performing three cycles at 10000x*g* for 2 min. To reduce particle size, samples were subjected to sonication (Sonicator, ultrasonic processor XL, Misonic), according to [28], performing three cycles of 30 sec each. The solvent was removed from nanoemulsion under nitrogen vapors. Afterwards, samples were sterilized throughout a 0.2 μm membrane. Emulsion control (**CTRL**) was prepared replacing carotenoid extract with the same quantity of THF in the aqueous solution.

The preparations were characterized by:

- *Granulometric analysis*

Particle size (expressed as "derived diameter") distributions were measured by LALLS (Low Angle Laser Light Scattering Technique). A MasterSizer Model S equipped with Malvern Application Software (Malvern Instruments, Malvern, UK) and fitted with a Small Volume Presentation Unit (MS1) was employed. The refractive index of samples and dispersants was measured using an Optech model RM Abbe refractometer at a temperature of 20 °C and white light [29].

- *β-Carotene concentration*

The concentration of carotenoids incorporated into nanoemulsion was determined by extracting 0.5 ml of nanoemulsion with 6 ml of hexane and absolute ethanol (2:1 v/v) according to Yuan et al. [30]. Samples were vortexed for 1 min, centrifuged at 1200x*g* and the hexane phase containing carotenoids was collected. The extraction was repeated three times. An aliquot was lyophilized for the quantitative determination.

4.3.4 Cell culture

Two different malignant cell lines, Caco-2 and SAOs, derived from human colorectal adenocarcinoma and osteosarcoma, respectively, were employed. For the proliferation assays, the cells were maintained at low confluence in polystyrene Petri plates (Corning, Milan) treated to ensure an optimal adhesion. The culture medium used was DMEM enriched to 10% FBS, 1% L-glutamine, 1% not-essential amino acids, and 1% solution penicillin/streptomycin. The cells were maintained at a density of $1x10^6$/ml, at a temperature of 37 °C, with a percentage of 100% humidity and 5% CO_2. The culture medium and the stimulation with CTRL and CEN were renewed every 48 h to ensure a constant supply of nutrients. Cell count was performed using Trypan blue, a dye able to selectively stain the dead cells permeating through the damaged cytoplasmic membranes. Caco-2 and SAOs cells were incubated at different times (96, 120, 144, 168 h and 48, 72, 120, 168 h, respectively) with CEN, at a concentration of 400 μg/ml and 200 μg/ml (w/v) of carotenoid extract, respectively, and with their respective CTRL. At the end of incubation, cell suspension was stained with Trypan Blue solution (0.04% v/v), diluted in the culture medium (1:1). The cell count was performed using EVE Automatic cell counter (NanoEnTek) and expressed as cell number/ml. Furthermore, a software (EveTM PC Software, NanoEnTek) provided the number of living and dead cells also considering the presence of aggregates or cellular debris.

4.3.5 CyQuant viability assay

CyQuant viability assay was performed to quantify the number of living cells using a nuclear dye that selectively binds to nucleic acids, emitting fluorescence. Cells, Caco-2 and SAOs, were treated for 168 h with CEN, at a

concentration of 400 μg/ml and 200 μg/ml (w/v), respectively, and with their respective CTRL. In particular, to discriminate between protective or not protective autophagy, cells were added with 20 μM chloroquine in the last 48 h of incubation. At the end of incubation, CyQuant mixture, containing the nuclear dye (CyQuant nuclear stain) and the suppressor of basal fluorescence (Background suppressor) were added to the culture medium and incubated for 1 h at 37 °C. Fluorescence was measured at the excitation wavelength of 485 nm and 530 nm emission and the results were expressed as percentage of fluorescence of the untreated control using a microplate reader (Synergy HT-BioTek, Milan).

4.3.6 CytoID Autophagy assay

Autophagy was monitored by using the Cell-ID Autophagy Detection Kit which includes a dye used as a selective marker of pre-autophagosomes, autophagosomes and autolysosomes. Caco-2 and SAOs cells were incubated for 168 h with CEN, at a concentration of 400 μg/ml and 200 μg/ml (w/v), respectively, and with their CTRL in the presence of 20 μM chloroquine, as positive control. After incubation, cells were washed with assay buffer and incubated with the autophagy detection marker and the nuclear dye, Hoechst, diluted 1:500 in DMEM without phenol red, supplemented with 5% FBS for 30 min. Cells were rinsed with assay buffer to eliminate the excess of dye and the fluorescence was determined by a double reading: the fluorophore of autophagosomes was detected at the wavelengths of 495 nm (excitation) and 519 nm (emission), as for FITC (fluorescein isothiocyanate); the Hoechst fluorochrome (which stains nuclei) was detected at the wavelengths of 358/461 nm, such as for DAPI (4',6-diamidin-2-phenylindole). To quantify the autophagic vacuoles, we calculated the ratio of FITC/DAPI fluorescence using

a microplate reader (Synergy HT- BioTek). The autophagic vesicles were observed and photographed using a fluorescence microscopy (Zeiss Axiovert 200, Milan, Italy) and by confocal microscopy (Leica SP8), using the filters for FITC and DAPI.

4.3.7 SDS-PAGE and immunoblotting

Caco-2 and SAOs cells were incubated with CEN, at a concentration of 400 μg/ml and 200 μg/ml (w/v), respectively, and with their respective CTRL, in the presence of 2 μM ATRA, as positive control. At the end of incubation, cells were lysed using a lysis buffer containing protease and phosphatase inhibitors, as reported by [31]. After the measurement of protein concentration [32], the total protein lysates were loaded on a 4%–12% pre-cast gel (Novex Bis-Tris pre-cast gel 4%–12%; Life Technologies, Milan) using MES (2-(N-morpholino) ethanesulfonic acid) buffer and immunoblots were performed following standard procedures. To detect the presence of LC3-II, a specific antibody was used to recognize the two isoforms present (LC3-I/II), characterized by different electrophoretic mobility. To detect activated kinase AMPK, a primary antibody against pAMPK (Thr^{172}) was used. To normalize and quantitate the expression levels of these proteins, an anti-α-tubulin antibody was employed. PVDF membranes were finally incubated with horseradish peroxidase-linked secondary antibody raised against mouse or rabbit and immunoblots developed using the ECL Plus Western Blotting Detection System Kit (GE Healthcare). Band intensities were quantified measuring optical density on a Gel Doc 2000 Apparatus (Bio-Rad Laboratories, Milan, Italy) and Multi-Analyst Software (Bio-Rad Laboratories,).

4.3.8 Chemiluminescence assay

ATP measurement was performed using ATP detention kit (Lonza, Italy) according to [33], by a chemiluminescence assay. In presence of ATP, the luciferase converts the luciferin into the oxidised form, oxyluciferin, emitting a chemiluminescence light, linearly dependent on ATP concentration:

ATP + D-luciferin + O_2 -> oxyluciferin + AMP + PPi + LIGHT

Caco-2 and SAOs cells were incubated for 48 h and 24 h, respectively, with CEN, at a concentration of 400 μg/ml and 200 μg/ml (w/v), respectively, and with their respective CTRL. As positive controls, 50 μM quercetin and 2 μM ATRA were used for Caco-2 and SAOs, respectively. At the end of incubation, cells were treated with ATP detection kit and chemiluminescence was measured after 10 min of incubation using a microplate reader (Synergy HT- BioTek).

4.3.9 Statistical analysis

Data are presented as mean values±standard deviation (SD) and the significance was measured by the use of Student's test of at least five determinations.

4.4 Results and Discussion

4.4.1 Pumpkin S-CO_2 extract composition

The oils extracted by S-CO_2 from the oven-dried pumpkin flesh (matrix), milled pumpkin seeds (co-matrix) and matrix/co-matrix (1:1, w/w) blend, were assayed for their total vitamin E and total carotenoid contents, including the vitamin E homologues (i.e., tocopherol and tocotrienol forms) and carotenoid distribution profiles. The results obtained from ISPA-CNR showed that the oil extracted from the matrix/co-matrix mixture had a biochemical profile in-between the seed and flesh oils (**Table 4.5**).

Table 4.5. *Vitamin E forms composition and carotenoid profile of oils extracted by SC-CO_2 [26].*

Fed material	Vitamin E					Carotenoids					
	α-T3	γ-T3	α-T	γ-T	Total (mg/ 100g oil)	Lutein	β-Criptoxantin	α-Carotene	β-Carotene	13-Cis β-Carotene	Total (mg/ 100g oil)
	% of total					% of total					
Oven-dried matrix	nd	nd	92.1± 0.3	7.9± 0.4	199± 27	4.4±0.2	1.1±0.1	38.5±0.1	46.7±0.1	9.3±0.2	1171±163
Co-matrix	0.6± 0.2	82.3± 5.1	10.9± 0.1	6.3± 0.4	442± 14	4.5±0.2	1.0±0.1	36.1±1.8	58.4±1.6	nd	13.6±1.7
Oven-dried matrix/co-matrix	1.0± 0.1	80.3± 2.4	10.3± 0.9	8.4± 1.4	274± 53	5.0±0.3	0.7±0.0	33.3±0.5	55.5±0.3	5.5±0.4	222±31

Data, expressed on a percentage basis and as mg/100 g oil, are the mean ± SD of three independent replicates (n = 3).
nd, not detectable.
Vitamin E form abbreviations are as follows: α-T3, α-tocotrienol; γ-T3, γ-tocotrienol; α-T, α-tocopherol; γ-T, γ-tocopherol.

In fact, the extracts obtained from the matrix/co-matrix mix were enriched in

α-T3 and γ-T3 beside tocopherols, compared to that obtained from the matrix alone, while γ-tocotrienol (γ-T3) was the constituent mostly contributing (>82%) to total vitamin E content of pumpkin seed oil extracted (**Table 4.5**). As expected, total carotenoid amount in the seed oil was extremely low (13.6 mg/100 g). β-Carotene represented the main carotenoid of pumpkin flesh oil, followed by α -carotene, β -cryptoxanthin according to Seo et al. [34].

4.4.2 Nanoemulsions preparation and characherization

To study the biological effect of carotenoid extract, an appropriate cellular delivery method was developed in order to increase the cellular uptake due to poor solubility of the lipophilic extract in the aqueous media. For this reason, we employed different protocols, including the incorporation in serum bovine serum, the preparation of lipid micelles and finally the use of oil-in-water (o/w) nanoemulsions. Considering the high sensitivity to light, oxygen and heat of the extract, we employed a formulation strategy that allowed to reduce the manipulations during the preparation steps and a better preservation over time. The incorporation of carotenoids in lipid micelles highlighted some critical points, relating to reproducibility and stability of the preparations (data not shown). On the other hand, the preparation of nanoemulsions improved carotenoids dispersion in aqueous media and facilitated their cellular uptake. For the production of o/w nanoemulsions, we used an emulsifier (**Figure 4.3**), able to reduce the interfacial tension between the oil and water phase, employing several protocols involving the use of various emulsifiers, which provided different results relating to the particle size. In particular, Tweens 80 allowed the most stable preparation and the lowest particle size respect to other emulsifier used, such as Tween 20, carbomer and lecithin. The distribution of the particle size diameter of emulsions was determined by Laser

Light Scattering Technique. We obtained a greater reduction of particle size, by the use of ultrasonic technique in addition to the standard protocol of high-energy homogenization method (**Figure 4.3**).

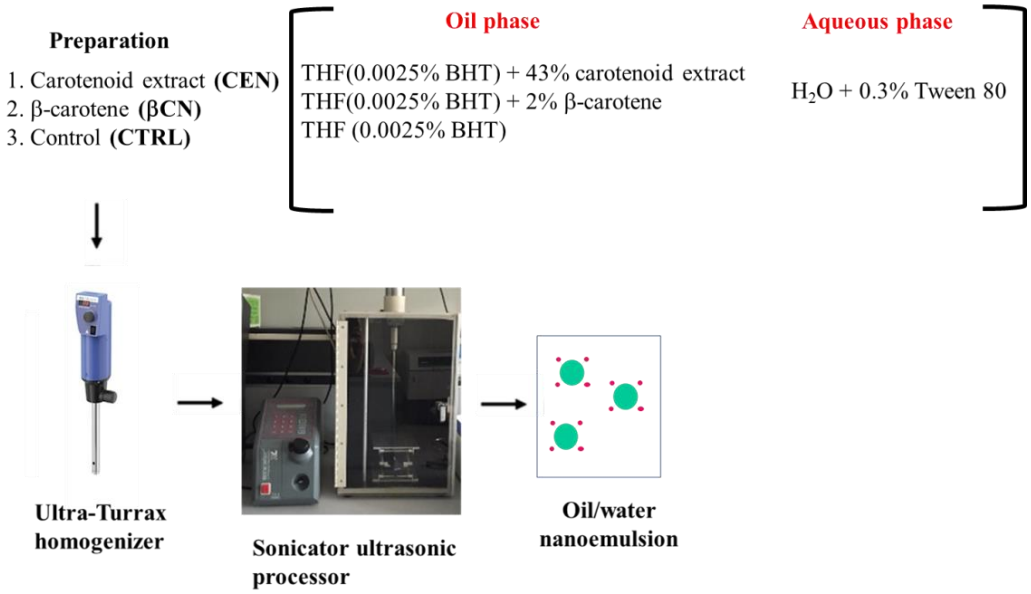


Figure 4.3. *Preparation of oil-in-water (o/w) nanoemulsions.*

As shown in **Figure 4.4**, the particle diameter of CTRL, βCN **(A)** and CEN **(B)** was in the nanometer range. To verify the carotenoid retention in nanoemulsions, we extracted carotenoids from the nanoemulsion to measure the yield, which was of about 12 mg/ml (w/v) in each preparation, showing the high reproducibility of this methods. Furthermore, during the nanoemulsion preparation, we tested several formulations, changing specific parameters (e.g., amounts of carotenoids or emulsifier, the ratio carotenoids/emulsifier). We obtained an optimal incorporation of carotenoids in nanoparticles employing a concentration of 2% β-carotene (w/v in THF/BHT) and 0.3% Tween 80.

To our knowledge, this method of delivery of carotenoids extract is a novelty

in the studies of biological effect of these class of molecules employing cell culture models.

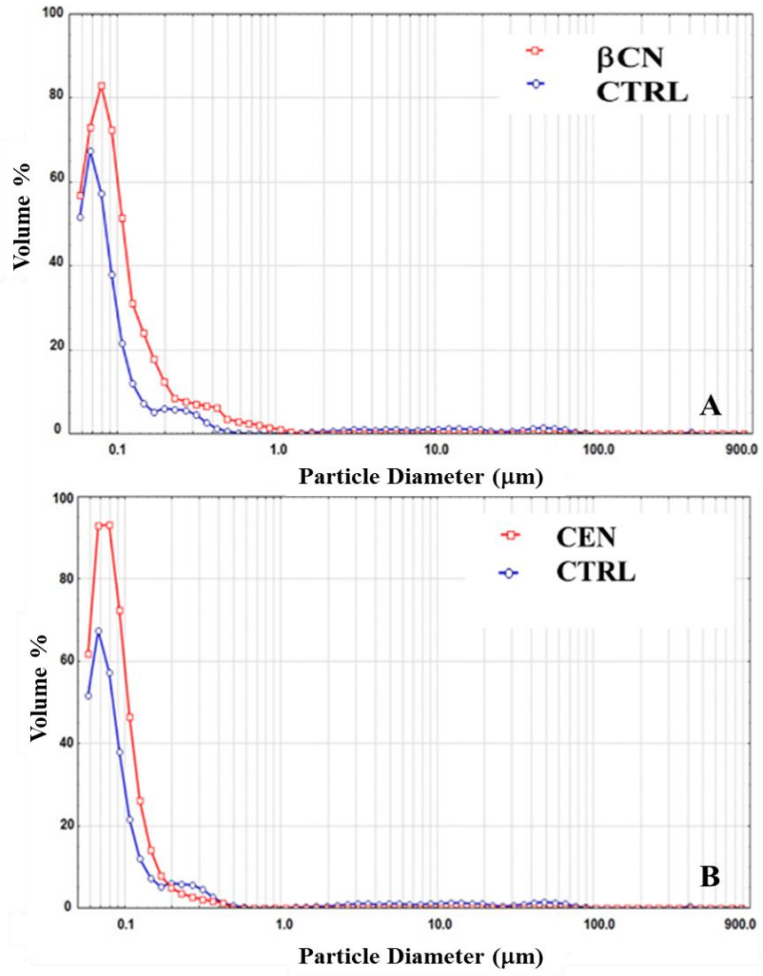


Figure 4.4. *Volume distribution % in graphical form of β-carotene nanoemulsion* ***(A)*** *and carotenoid extract nanoemulsion* ***(B)****. Particle size, expressed as "derived diameter" distributions, was measured by LALLS (Low Angle Laser Light Scattering Technique) as described in "Materials and Methods". The refractive index of samples and dispersants was measured using an Optech model RM Abbe refractometer at a temperature of 20 °C and white light.*

4.4.3 Antiproliferative effect of CEN on Caco-2 and SAOs cell lines

We evaluated the effects of the three oils extracted and we decided to employ for our study the preparation from pulp/seed (1:1, w/w) blend, which showed the higher biological activity (data not shown).

We firstly tested CEN biological activity on several malignant cell lines, chosen for their different sensitivity to anticancer drugs (BH-3 mimetics, death ligands) [35, 36] or γ–radiations [13]. In a preliminary screening, we tested the effect of CEN on the proliferation of cell lines derived from solid tumors of epithelial (Caco-2) [37], or mesenchymal origin (SAOs, U2Os, HepG2) [38], or derived from chronic lymphocytic leukemia cell line (HG3). However, since HG3 and HepG2 did not produce reproducible results, probably due to their sensitivity to nanoemulsions reagents, we employed Caco-2 and SAOs cell lines as a suitable model since their low toxicity to CTRL nanoemulsions preparation. In particular, Caco-2, derived from a human colorectal adenocarcinoma is able to simulate the carotenoids path *in vivo* after oral absorption. In fact, *in vivo*, after oral ingestion, carotenoids are solubilized in mixed micelles, formed by lipids emulsified with pancreatic juice. Dietary carotenoids solubilized in mixed micelles are taken up into the intestinal epithelia for the incorporation into chylomicrons. The transfer of carotenoids to intestinal cells are mediated by simple diffusion dependent on the concentration gradient across the cellular membrane and/or facilitated diffusion through scavenger receptors. Previous work indicated that when carotenoids solubilized in mixed micelles were incubated with human intestinal Caco-2 cells, the absorption is simulated and a positive relationship was found between carotenoids hydrophobicity and the amount taken up by the cells [39]. In addition, Caco-2 cells represent an ideal cellular model for their ability to spontaneously differentiate *in vitro* upon reaching the confluence or following the exposure with differentiating agents derived from

diet such as butyric acid and carotenoids [37]. Similarly, SAOs cell line, derived from a human osteosarcoma, is able to differentiate *in vitro* following treatment with ATRA, the physiologic derivative of β-carotene [38]. SAOs also show high resistance to apoptotic stimuli and γ-radiation [13, 35, 36].

To evaluate the effect of carotenoid extract on cellular proliferation, we firstly performed a dose-response experiment to determine and select the ideal concentration of CEN, which corresponds to the lower toxic effect of the vehicle (CTRL). Therefore, Caco-2 and SAOs cells were incubated at different times with CEN, employing the minimum active concentration of 400 μg/ml and 200 μg/ml of carotenoid extract (w/v), respectively. Nanoemulsions without carotenoids (CTRL) were employed as a control, using the same volumes applied for CEN. The effect on cell viability (Trypan blue method) is reported in **Figure 4.5** and shows how incubation of Caco-2 with CEN significantly *slowed* cell proliferation at 96, 120, 144, 168 h of incubation, compared to control experimental points (untreated and CTRL) (**Figure 4.5, A**). Similarly effect was measured at 120 and 168 h for SAOs cells after CEN treatment compared to controls, as reported in **Figure 4.5, B**. It is interesting to note that the antiproliferative effect of CEN cannot be attribute solely to the presence of β-carotene, since when we assayed this compound at the same concentration present in CEN, we did not measure any significant effect on cell growth; it behaved as CTRL (data not shown).

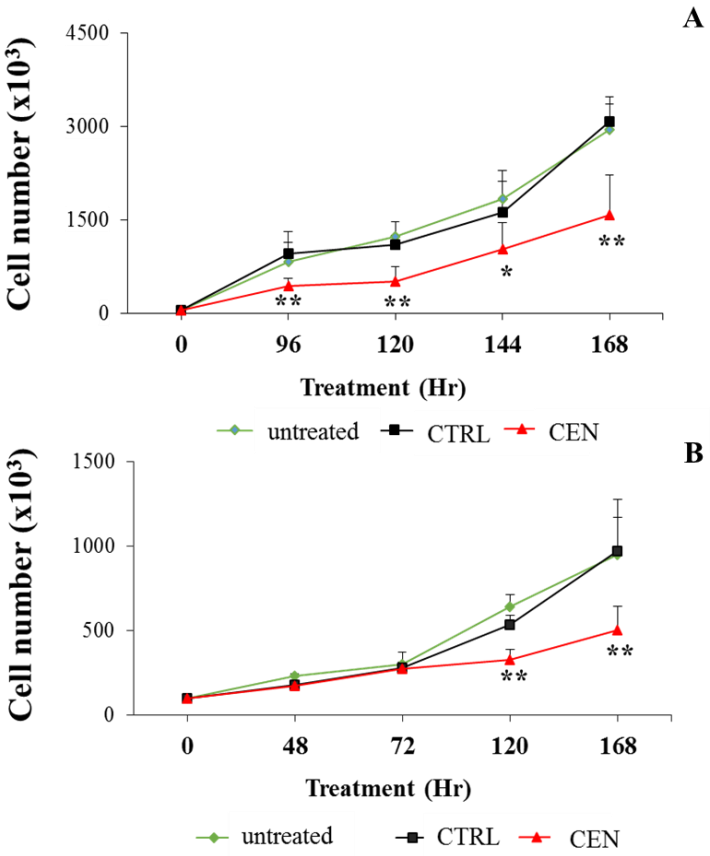


Figure 4.5. *Cell growth of Caco-2 and SAOs cell lines.* ***(A)*** *Caco-2 cells were mantained at 37 °C at a density of 3x10^4/ml of DMEM and treated with CTRL and CEN (400 µg/ml, w/v) every 48 h. Cell count was performed at all times indicated.* ***(B)*** *SAOs cells were mantained at 37 °C at a density of 6.7x10^4/ml of DMEM and treated with CTRL and CEN (200 µg/ml, w/v) every 48 h. Cell count was performed at the indicated times. Cell viability was evaluated using Trypan blue method as described in "Materials and Methods". The bars in the graphs indicate SD; symbols indicate significance: *p<0.05 and **p<0.01 respect to CTRL (nanoemulsions prepared in the absence of carotenoid extract).*

The decreased rate of cell growth was also confirmed by CyQuant viability

assay, a methoth which uses a nuclear stain that selectively binds to nucleic acids, especially to DNA, emitting fluorescence directly proportional to the cell number. The representative micrographs of the cell nuclei performed by fluorescence microscopy, after CyQuant assay, report the slowdown of Caco-2 (**Figure 4.6, C**) and SAOs (**Figure 4.6, F**) cell proliferation at 168 h from treatment with CEN, compared to CTRL (**Figure 4.6, B** and **E**) and untreated points (**Figure 4.6, A** and **D**).

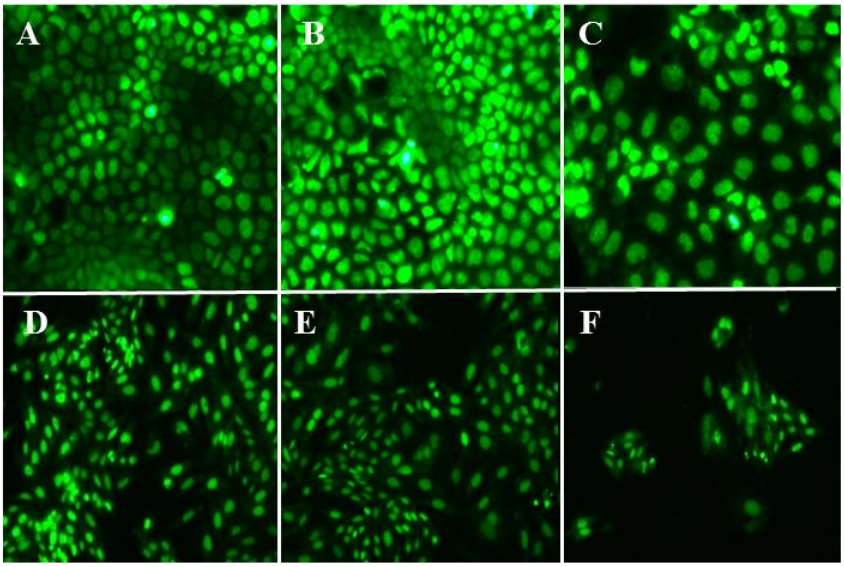


Figure 4.6. *Micrographs of cell nuclei of Caco-2* ***(A, B, C)*** *and SAOs* ***(D, E, F)*** *after the indicated treatments (optical microscope Axiovert 200 Zeiss; FITC 200X magnification). Representative images of untreated Caco-2 cells* ***(A)****, treated with CTRL* ***(B)*** *and CEN* ***(C)****. Representative images of untreated SAOs cells* ***(D)****, treated with CTRL* ***(E)*** *and CEN* ***(F)****.*

The quantitative measurement of this effect is reported in **Figure 4.7**, confirming that the treatment with CEN significantly reduced the cell proliferation, expressed as percentage of fluorescence, in Caco-2 (**Figure 4.7, A**) and SAOs (**Figure 4.7, B**).

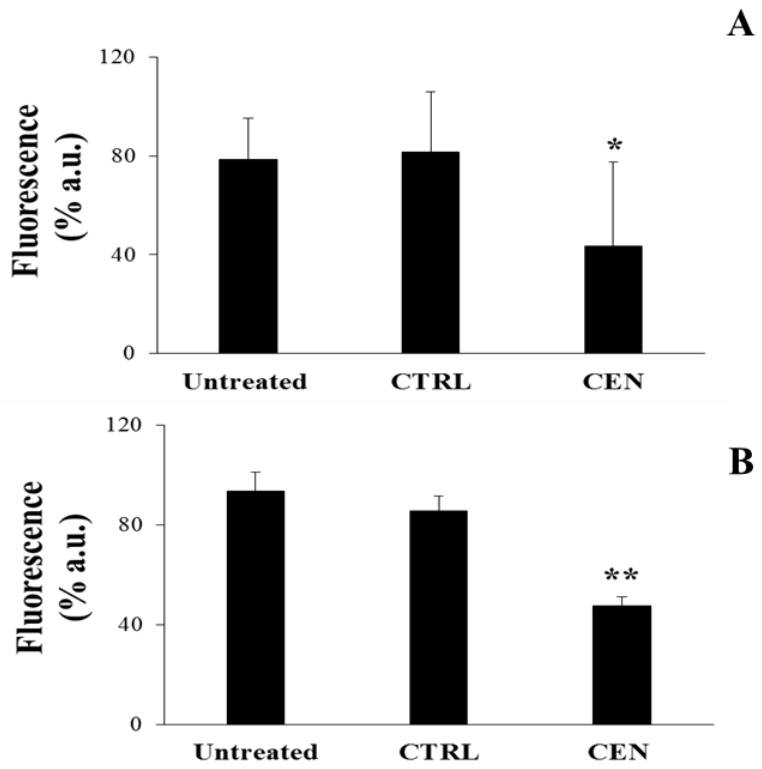


Figure 4.7. *Cell viability of Caco-2* ***(A)*** *and SAOs* ***(B)*** *cell lines treated with CTRL and CEN. Cells were mantained at 37 °C at a density of 2.5x10^3/ml of DMEM and treated with CTRL and CEN (400 μg/ml for Caco-2 and 200 μg/ml for SAOs) every 48 h. The CyQuant viability assay was performed after 168 h of treatment, as described in "Materials and Methods". The bars in the graphs indicate SD; symbols indicate significance: *p<0.05 and **p<0.01 respect to CTRL (nanoemulsions prepared in the absence of carotenoid extract).*

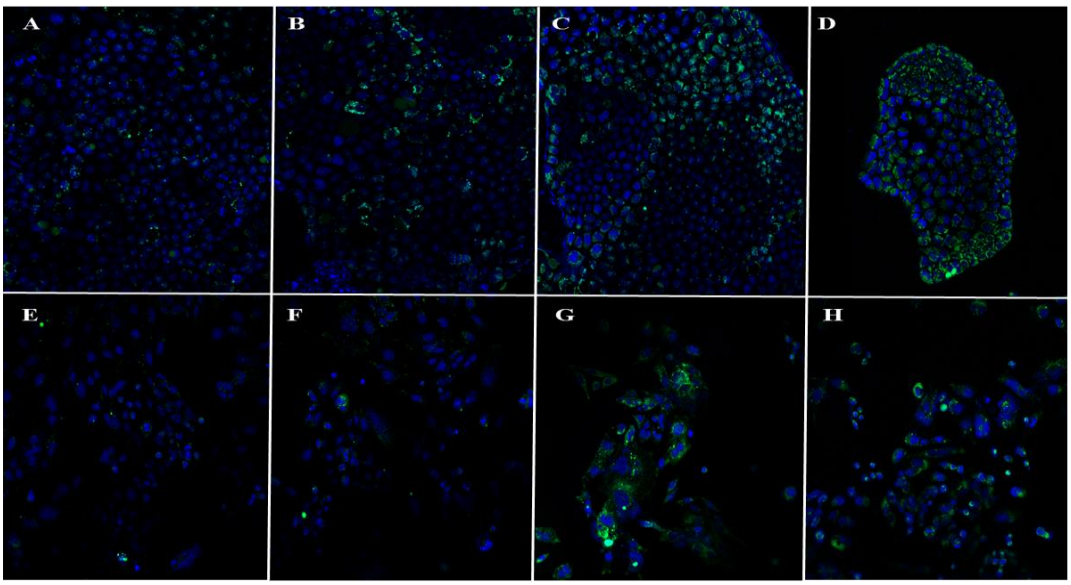
4.4.4 Activation of autophagy by carotenoid extract

We hypothesized that the reduced cell proliferation in the absence of any clear sign of cell death induced by CEN could be related to the activation of an

autophagic process. This hypothesis also emerged from the microscopical observation of CEN-treated cells which evidenced the presence of intracellular vacuoles. To verify this hypothesis, we used multiple assays to detect autophagy and to avoid false-positive results.

The autophagosome were specifically stained with Cyto-ID Green autophagy dye, a fluorescent reagent, after Caco-2 and SAOs cell treatment with CEN, at a concentration of 400 μg/ml and 200 μg/ml (w/v), respectively, and with their respective CTRL. As positive control, we employed chloroquine, a reagent well known due for its ability to block autophagic flux and cause an increase in autophagosome accumulation in the cytoplasm [40]. As reported in **Figure 4.8**, an increse of autophagosomes after treatment with CEN was clearly visible with fluorescence microscopy (green fluorecence), in Caco-2 (**Figure 4.8, C panel 1; 4.8, A panel 2**) and SAOs cell lines (**Figure 4.8, G panel 1; 4.8, B panel 2**) respect to untreated (**Figure 4.8, A** and **E,** respectively) and CTRL (**Figure 4.8, B** and **F,** respectively). The chloroquine treated Caco-2 and SAOs cells are reported in **Figure 4.8, D** and **H**, respectively.

1)



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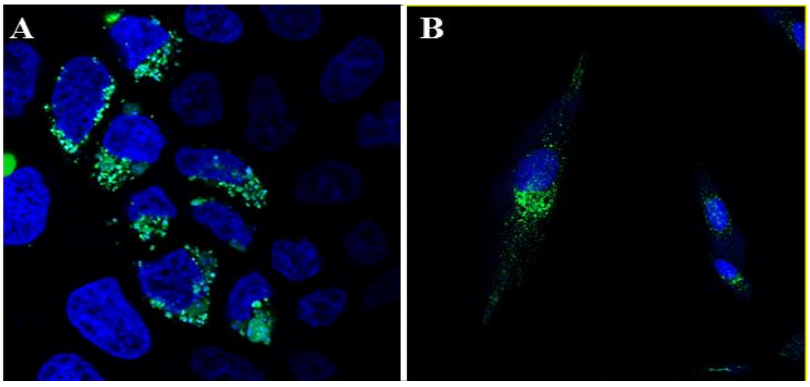

Figure 4.8. *Micrographs of autophagic vacuoles in Caco-2 and SAOs. The photographs were taken with a fluorescence invertoscope (Axiovert 200 Zeiss) and a confocal microscope (LEICA SP8) with a total magnification of 200X* ***(panel 1)*** *and 400X* ***(panel 2)*** *to highlight the autophagic vacuoles (green) and cell nuclei (blue). Representative images of cells Caco-2* ***(A)****, treated with CTRL* ***(B)*** *and CEN* ***(C panel 1; A panel 2)*** *and chloroquine* ***(D)****. Representative images of cells SAOs* ***(E)****, treated with CTRL* ***(F)*** *and CEN* ***(G panel 1; B panel 2)*** *and chloroquine* ***(H)****.*

The result of the CytoID autophagy assay was quantified as described in Methods section by measuring the fluorescence of autophagosomes (FITC) and by normalizing it respect to the fluorescence emitted by cell nuclei (DAPI). Data in **Figure 4.9** report the activation of autophagy by CEN, through a significant increase of autophagic vacuoles, expressed as ratio FITC/DAPI, in Caco-2 **(Figure 4.9, A)** and SAOs **(Figure 4.9, B)**, respect to controls.

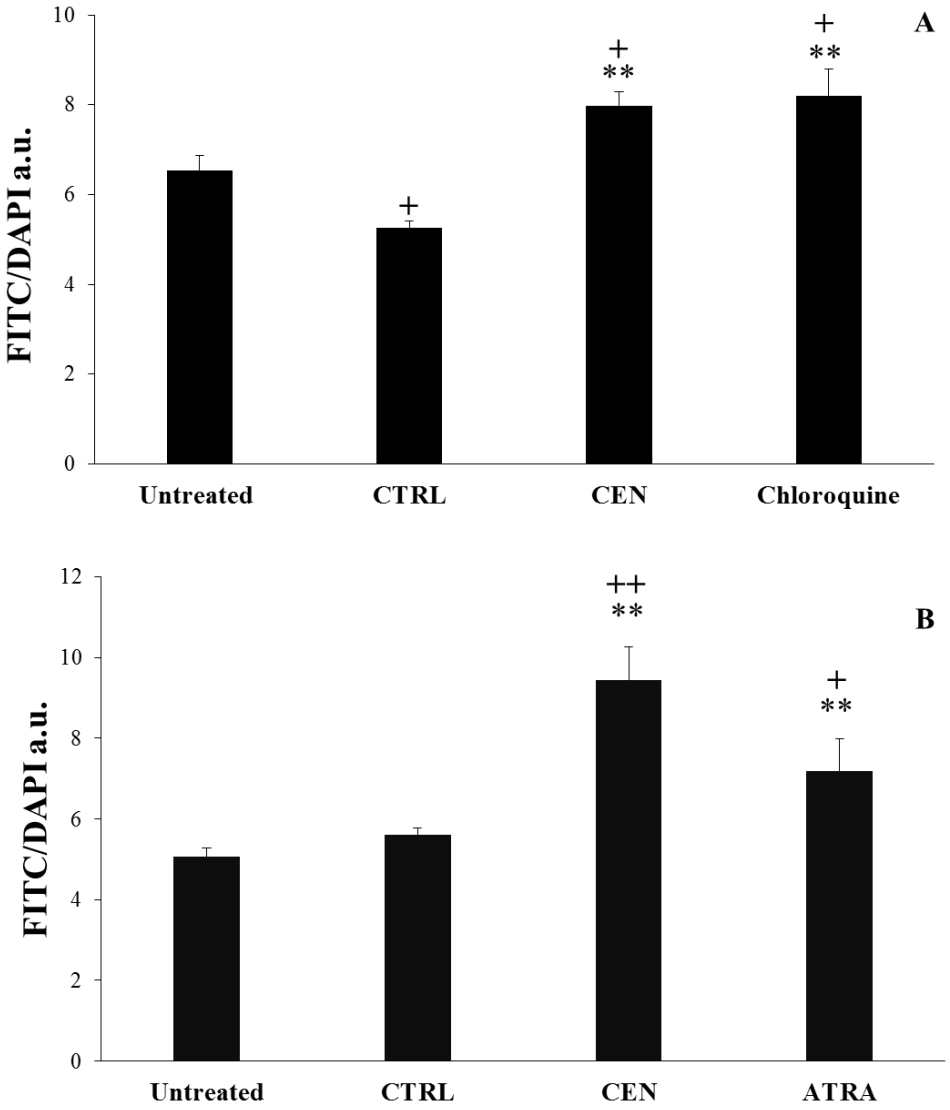

Figure 4.9. *Autopahgosome quantification with Cyto-ID in Caco-2* ***(A)*** *and SAOs* ***(B)****. The cells were mantained at 37 °C at a density of 2.5x10^3/ml of DMEM and treated with CTRL and CEN (400 μg/ml for Caco-2 and 200 μg/ml for SAOs) every 48 h. Cyto ID authophagy assay was performed as reported in "Materials and Methods". The bars in the graphs indicate SD; symbols indicate significance: **p<0.01 respect to CTRL (nanoemulsions prepared in the absence of carotenoid extract), +p<0.05 and ++p<0.01 respect to untreated-cells.*

To further confirm the activation of autophagy after treatment with CEN, we used the molecular marker LC3-II, the lipidated isoform of LC3 protein, essential in the autophagosome membrane formation. The immunoblots and the corresponding densitometric analysis (numbers on the bottom of the panels) are reported in **Figure 4.10**, which shows increased expression of LC3-II band at 168 h of incubation with CEN on Caco-2 (1.25 fold) **(Figure 4.10, A)** and in SAOs (4 fold) **(Figure 4.10, B)**. The increase of LC3-II was also detectable in both cell lines at earlier times (96-120 h; data not shown).

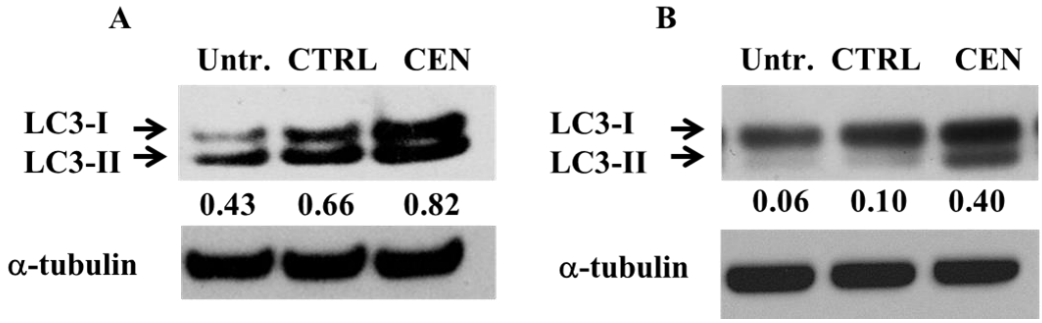

Figure 4.10. *Immunoblots of LC3-I, LC3-II expression in Caco-2* ***(A)*** *and SAOs* ***(B)*** *cells treated for 168 h with CTRL and CEN (400 μg/ml for Caco-2 and 200 μg/ml for SAOs) as reported in "Materials and Methods". The numbers, below the bands indicate the densitometric analysis values, resulted by the ratio of the lipidated isoform LC3-II and α-tubulin.*

4.4.5 CEN activates "not protective" autophagy

Data obtained from CytoID assays and immunoblotting confirmed the involvement of autophagy in reducing cell proliferation after CEN treatment. Therefore, we further investigated this phenomenon trying to classify the effect of CEN on autophagic induction in one of the four forms of autophagy described above (**Table 4.4**), which are differently associated with cancer cell

growth in response to stress induced by chemotherapy. In the present study, we can easily exclude cytotoxic autophagy, characterized by cell death, as well as cytostatic autophagy where a classical cell cycle arrest can be detected. Therefore, we tried to discriminate between the protective or not-protective forms of autophagy, using chloroquine as one of the most used pharmacological inhibitor of autophagic flux [41]. The rational of the experiment has been the following: in the presence of "protective" autophagy, we expect that the inhibition of the autophagic flux caused by chloroquine should result in an increased cytotoxicity of the treatment under investigation (in our case, CEN+chloroquine treated cells compared to CEN mono-treatment) generally associated to apoptotic cell death; alternatively, in the presence of a "not protective" autophagy, following chloroquine inhibition, we should not detect any significant changes in the cytotoxic effect of CEN+chloroquine versus CEN alone. As shown in **Figure 4.11**, the co-treatment chloroquine+CEN did not reduce significantly cell viability compared to chloroquine or CEN mono-treatments. Therefore, we concluded that the activation of autophagy in Caco-2 and SAOs cells did not protect cells from the effect of CEN on cellular viability, inducing a not protective autophagic phenotype.

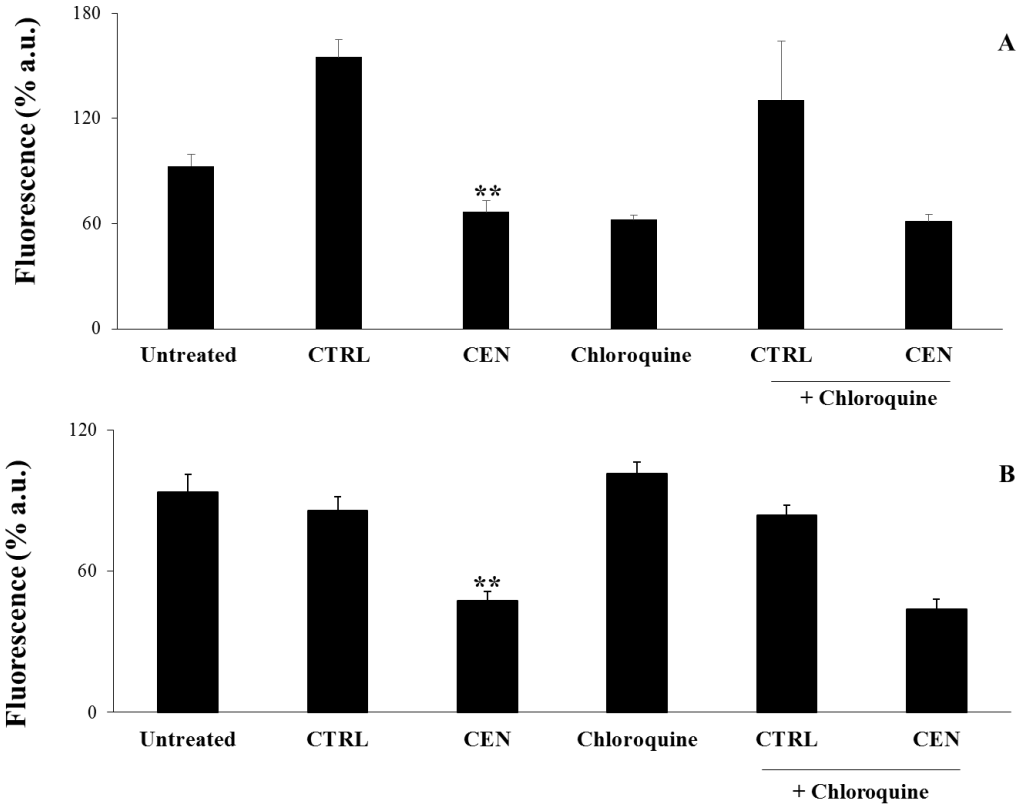

Figure 4.11. *Activaction of a not protective autophagy by CEN in Caco-2* ***(A)*** *and SAOs* ***(B)****. The cells were mantained at 37 °C at a density of 2.5x10*^{3}*/ml of DMEM and treated with CTRL and CEN (400 μg/ml for Caco-2 and 200 μg/ml for SAOs) every 48 h and 20 μM chloroquine was added in the last 48 h of incubation. The CyQuant viability assay was performed after 144 h of incubation as described in "Materials and Methods". The bars in the graphs indicate SD; symbols indicate significance: *p<0.05 and **p<0.01 respect to CTRL (nanoemulsions prepared in the absence of carotenoid extract); no significant differences between CEN+Chloroquine vs CEN.*

The paradoxical role of autophagy is revealed especially in studies of tumor biology and its complex function is strongly dependent on the cellular context. As reported above, the autophagy has two primary and opposing functions in

tumor cells in response to stress induced by chemotherapy or radiation [25]. Initially, the induction of a "protective" autophagy by chemotherapy in tumor cells was considered to be a positive response from scientists. Therefore, it has been examined that the modulation of this process could provided for new era in the enhancement of antitumor drug and radiation effectiveness. In fact, since the protective autophagy may protect cancer cells from environmental insults and confers resistance to chemotherapy, several clinical trials have focused on improving the response to treatment, combining chloroquine or hydroxychloroquine with various conventional treatment modalities [25]. Even if in positive outcomes of clinical trials could potentially represent a revolution in cancer therapy based on the inhibition of autophagy, the data on preclinical animal models are still insufficient [25]. Furthermore, from a pharmacokinetic point of view, it is not known whether chloroquine or hydroxychloroquine may achieve such concentrations to effectively inhibit autophagy in normal clinical regimens [25].

For these reasons, it has been discussed that the possibility of chemotherapeutic drugs and radiation to promote a "not protective" form of autophagy could be a positive phenomenon, especially if it is related to the cell cycle block (cytostatic autophagy) and/or the activation of cellular differentiation. In the past years it was introduced the concept of "differentiation therapy" in tumor biology by inducing the cancer cells to differentiate, in order to inhibit their proliferation and reduce their invasivity. As reported above, one of the greatest achievements in this field was reached by the discovery that ATRA was able to induce, in most cases, the complete remission of the disease in patients with APL, by inducing a process of cellular differentiation [12].

It has also been showed that the activation of the differentiation in osteosarcoma cell lines by ATRA involves the activation of the autophagic

process [13], presumably for his key role in the recycling of macromolecules and cell organelles essential to osteoid formation in mature osteoblasts [42].

In support of this view, it should be considered the role of autophagy in osteoblasts function, involved in mineralization and bone homeostasis [42]. In fact, it has been demonstrated the involvement of autophagy as important mediator of bone cell function in normal physiology and in pathology [43]. In particular, the relevance of the autophagy, related to the differentiation process, is also reported by Vessoni et al. [44] in some stem cell populations. Moreover, it has been showed that genetic or pharmacological autophagy inhibition suppresses mesenchymal stem cell differentiation to osteoblasts [45].

The role of not protective autophagy in cellular differentitation is demonstrated also in several intestinal cell lines (Caco-2, HT-29) [46]. In particular, it has been reported that a cytoplasmic heterotrimeric G_{i3} protein regulates autophagy in these human colon cancer cell lines and their state of differentiation [46]. Since the interpretation of our data suggest a *slow down* effect on cell proliferation, it is reasonable to assume that not protective autophagy induced by CEN could be related to cellular differentiation in SAOs and Caco-2 cell lines. For these reasons, further studies are needed to evaluate an induction of differentiated phenotype associated with retarded proliferation after incubation of Caco-2 and SAOs cells with CEN, using biochemical and morphological markers such as alkaline phosphatase, particularly expressed in these two experimental models [47].

4.4.6 Biochemical regulation of autophagy by carotenoid extract

Energetic depletion is an important modulator of autophagy. Therefore, we investigated whether autophagy induced by CEN was related to an initial alteration of energetic metabolism. To this aim, intracellular ATP was measured as an index of functional integrity of metabolically active cells. As illustrated in **Figure 4.12**, the ATP intracellular levels, expressed as chemiluminescence value, were significantly reduced after 48 h and 24 h of incubation with CEN in Caco-2 cells **(Figure 4.12, A)** and SAOs **(Figure 4.12, B)**, respectively.

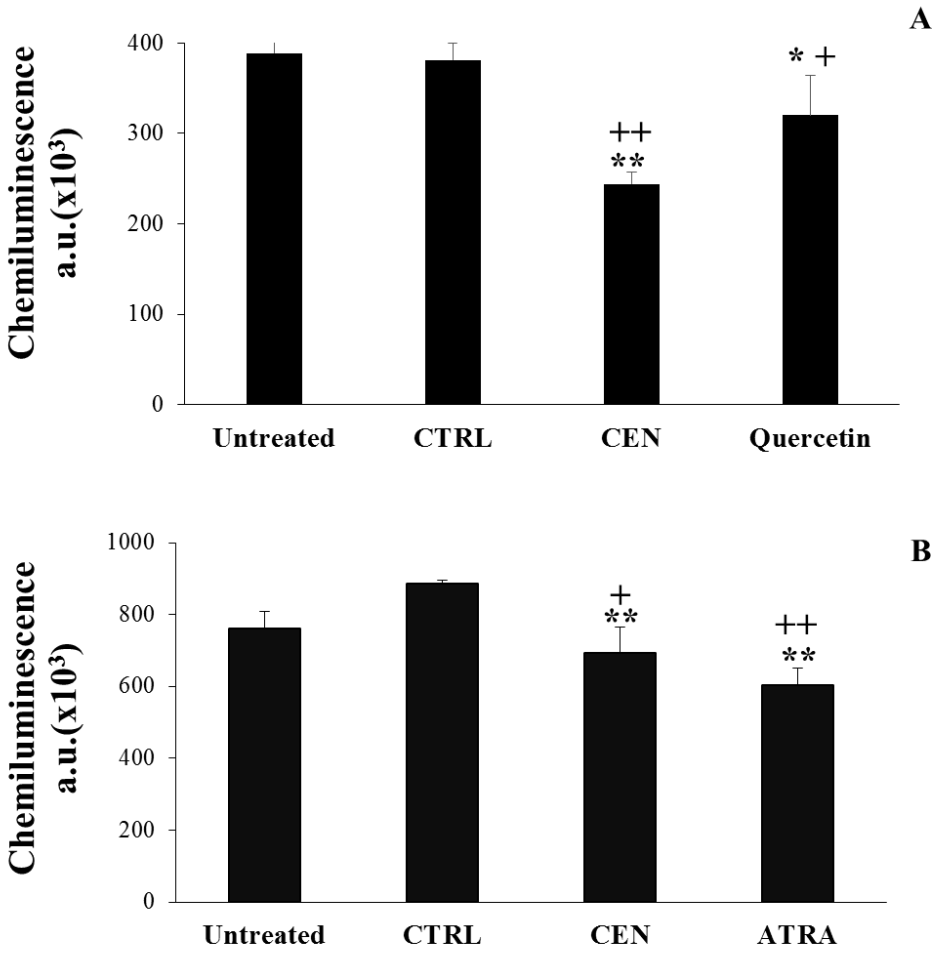

Figure 4.12. *ATP levels of Caco-2* ***(A)*** *and SAOs* ***(B)*** *after treatment with CEN. The cells were mantained at 37 °C at a density of 2.5x10^3/ml of DMEM and treated with CTRL and CEN (400 μg/ml for Caco-2 and 200 μg/ml for SAOs). After 24-48h h, ATP intracellular levels were measured as reported in "Materials and Methods". The bars in the graphs indicate SD; symbols indicate significance:*p<0.05 and ** p<0.01 respect to CTRL (nanoemulsions prepared in the absence of carotenoid extract). and +p<0.05and ++p<0.01 respect to untreated-cells.*

Starting from data showed in **Figure 4.12**, we verified if the decrease of ATP could activate kinase AMPK, a metabolic modulator of glucose and lipid metabolism in response to the alterations of nutrients and intracellular ATP levels. AMPK is an αβγ heterotrimer with the threonine-172 phosphorylation on the catalytic α-subunit essential for its fully activation [22]. In particular, the γ-subunit includes the four domains of allosteric regulation by AMP/ATP, which act as an allosteric activators and inhibitors, respectively, by binding at exchangeable sites CBS1 and CBS3, two pairs of motifs in tandem related to cystathionine-b-synthase sequences (**Figure 4.13**). Therefore, the activation of AMPK is induced by changes in the AMP/ADP/ATP binding on the CBS domains of γ-subunits. In details, an energetic imbalance induces the formation of ATP and AMP from the adenylate kinase by the reaction 2ADP -> ATP + AMP. Considering that a 10% variation of ATP levels results in a more than 8-fold change in AMP concentrations, AMP replaces ATP in the γ-subunit. This allosteric change triggers a myristoyl switch, freeing Thr172 which becomes available for phosphorylation by upstream kinases.

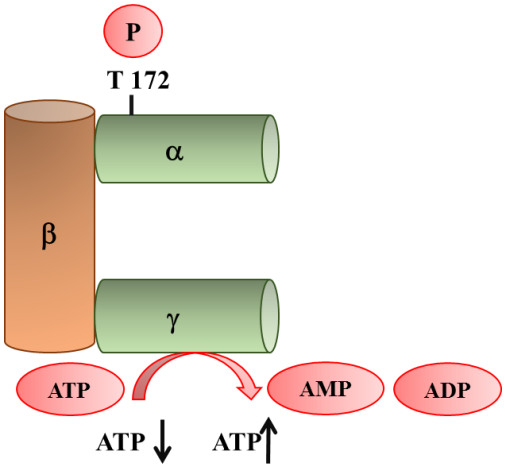

Figure 4.13. *AMPK allosteric regulation by AMP, ADP and ATP [22].*

AMPK is required for the autophagy induction and is involved in the initial stage of autophagosomes nucleation. We hypothesized that autophagy could be activated by a reduction of ATP induced by CEN, through the involvement of AMPK. To confirm this possibility, we evaluated the expression levels of the phosphorylated and active form of protein, pAMPK (Thr^{172}), which increased twice after CEN treatment compared to controls, as resulted by densitometric analysis in **Figure 4.14**.

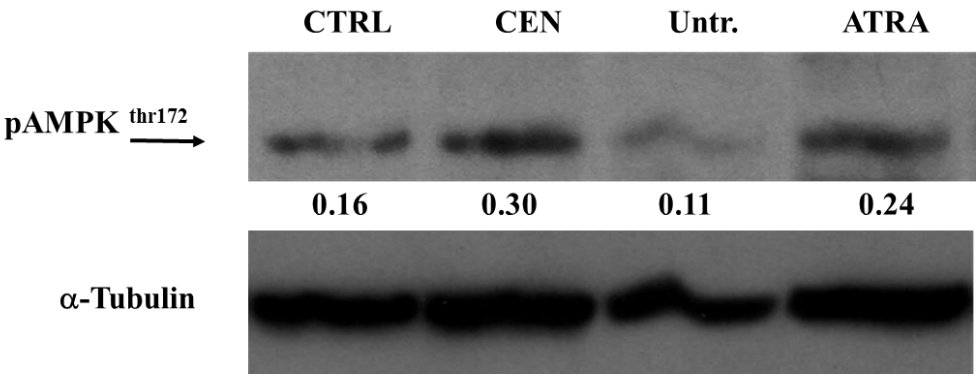

Figure 4.14. *Immunoblots of pAMPK in SAOs cells treated for 24 h with CTRL and CEN (200 μg/ml of carotenoid extract) as reported in "Materials and Methods". 2 μM ATRA was used as positive control. The numbers, below*

the bands indicate the densitometric analysis values, resulted by the ratio of pAMPK and α-tubulin.

Therefore, to support our hypothesis that autophagy induced by CEN could be linked to cellular differentiation, it was shown by [48] that AMPK is activated during osteoblast differentiation. In particular, it has been suggested that early catabolic changes are important to provide the energy source for osteoblasts and the activation of AMPK and autophagy is a significant event to induce osteoblasts to progress to the final stages of differentiation [48]. In this study the authors demonstrate that the induction of autophagy is required at the beginning of the differentiation and IGF-I/insulin-like growth factor binding protein 2 (IGFBP-2) activate important components for autophagosome formation. As reported in **Figure 4.15**, IGF-I and IGFBP-2 stimulate AMPK activation, resulting in activation of autophagy, which is required for optimal osteoblast differentiation [48].

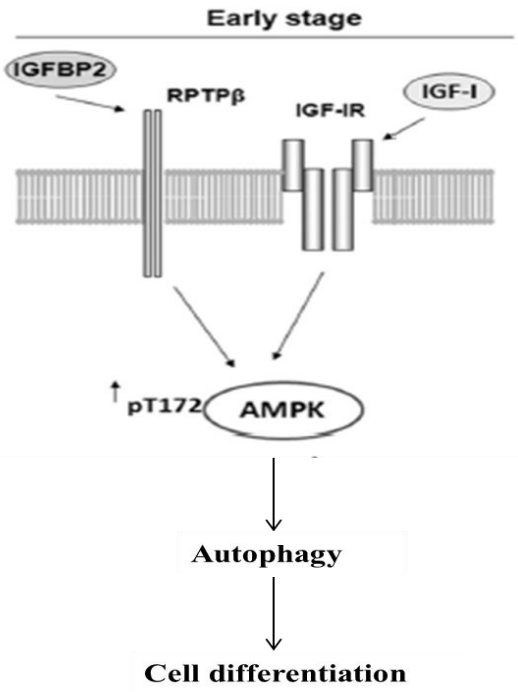

Figure 4.15. *Regulation of AMPK and autophagy required for osteoblast differentiation [48].*

4.5 Conclusions

In the present study, we investigated the biological effect of a carotenoid extract from *Cucurbita moschata* on different transformed human cell lines. The first important novelty here reported is the potential impact of the nanoemulsion technology employed as a promising system to improve solubility of the carotenoids extracts, enhancing their stability and preventing their degradation. Further studies are in progress to improve this technique. The results show that CEN slowdowns cancer cell growth and this effect could be related to an alteration in energy metabolism and induction of not protective form of autophagy through the activation of kinase AMPK. To our knowledge, the data reported in this Chapter represent one of the very few example present in the Literature where a carotenoid-derived preparation interfered with the autophagic process in malignant cell lines. This preliminary pre-clinical study represents an important indication that carotenoid extract from *Cucurbita moschata* can be tested as therapeutic adjuvant in association with chemotherapeutic or γ–radiation treatments. Data from our group on ATRA reinforces this hypothesis [13]. In this context, the next pre-clinical step should be the recurrence to a suitable animal models to test the chemopreventive and/or chemotherapeutic efficacy of our carotenoid nonoemulsions.

4.6 References

1. Russo, M., et al., *Phytochemicals in Cancer Prevention and Therapy: Truth or Dare?* Toxins 2010. **2**(4): p. 517-51.
2. Wang, Y., et al., *Lycopene, tomato products and prostate cancer-specific mortality among men diagnosed with nonmetastatic prostate cancer in the Cancer Prevention Study II Nutrition Cohort.* Int J Cancer, 2016. **138**(12): p. 2846-55.
3. Kirsh, V.A., et al., *A prospective study of lycopene and tomato product intake and risk of prostate cancer.* Cancer Epidemiol Biomarkers Prev, 2006. **15**(1): p. 92-8.
4. Holzapfel, N.P., et al., *The potential role of lycopene for the prevention and therapy of prostate cancer: from molecular mechanisms to clinical evidence.* Int J Mol Sci, 2013. **14**(7): p. 14620-46.
5. Wang, Y., et al., *Effect of Carotene and Lycopene on the Risk of Prostate Cancer: A Systematic Review and Dose-Response Meta-Analysis of Observational Studies.* PLoS One, 2015. **10**(9): p. e0137427.
6. *The alpha-tocopherol, beta-carotene lung cancer prevention study: design, methods, participant characteristics, and compliance. The ATBC Cancer Prevention Study Group.* Ann Epidemiol, 1994. **4**(1): p. 1-10.
7. [No authors listed], *The alpha-tocopherol, beta-carotene lung cancer prevention study: design, methods, participant characteristics, and compliance. The ATBC Cancer Prevention Study Group.* Ann Epidemiol, 1994. **4**(1): p. 1-10.
8. Omenn, G.S., et al., *The Carotene and Retinol Efficacy Trial (CARET) to prevent lung cancer in high-risk populations: pilot study with asbestos-exposed workers.* Cancer Epidemiol Biomarkers Prev, 1993. **2**(4): p. 381-7.
9. Tanaka, T., M. Shnimizu, and H. Moriwaki, *Cancer chemoprevention by carotenoids.* Molecules, 2012. **17**(3): p. 3202-42.
10. Schenk, T., S. Stengel, and A. Zelent, *Unlocking the potential of retinoic acid in anticancer therapy.* Br J Cancer, 2014. **111**(11): p. 2039-45.
11. Niranjana, R., et al., *Carotenoids modulate the hallmarks of cancer cells.* NAO, 2015. **18 Pt B**: p. 968–85.
12. Breitman, T.R., S.E. Selonick, and S.J. Collins, *Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid.* Proc Natl Acad Sci U S A, 1980. **77**(5): p. 2936-40.
13. Russo, M., et al., *Radio-sensitizing effects of all trans retinoic acid (ATRA) on human chronic lymphocytic leukemia and osteosarcoma cell lines.* Eur J Cancer 2016. **61**(1): p. S163.
14. Palozza, P., et al., *Modulation of apoptotic signalling by carotenoids in cancer cells.* Arch Biochem Biophys, 2004. **430**(1): p. 104-9.

15. Palozza, P., et al., *Mechanism of activation of caspase cascade during beta-carotene-induced apoptosis in human tumor cells.* Nutr Cancer, 2003. **47**(1): p. 76-87.
16. Kim, K.N., et al., *Fucoxanthin induces apoptosis in human leukemia HL-60 cells through a ROS-mediated Bcl-xL pathway.* Toxicol In Vitro, 2010. **24**(6): p. 1648-54.
17. Anguiano, J., et al., *Chemical modulation of chaperone-mediated autophagy by retinoic acid derivatives.* Nat Chem Biol, 2013. **9**(6): p. 374-82.
18. Shen, M., et al., *Protective effect of astaxanthin on liver fibrosis through modulation of TGF-beta1 expression and autophagy*. Mediators Inflamm, 2014. **2014**: p. 954502.
19. Hou, L.L., et al., *Essential role of autophagy in fucoxanthin-induced cytotoxicity to human epithelial cervical cancer HeLa cells.* Acta Pharmacol Sin, 2013. **34**(11): p. 1403-10.
20. Gump, J.M. and A. Thorburn, *Autophagy and apoptosis: what is the connection?* Trends Cell Biol, 2011. **21**(7): p. 387-92.
21. Ohsumi, Y., *Historical landmarks of autophagy research.* Cell Res, 2014. **24**(1): p. 9-23.
22. Russo, G.L., M. Russo, and P. Ungaro, *AMP-activated protein kinase: a target for old drugs against diabetes and cancer.* Biochem Pharmacol, 2013. **86**(3): p. 339-50.
23. Nakahira, K. and A.M. Choi, *Autophagy: a potential therapeutic target in lung diseases.* Am J Physiol Lung Cell Mol Physiol, 2013. **305**(2): p. L93-107.
24. Choi, A.M., S.W. Ryter, and B. Levine, *Autophagy in human health and disease.* N Engl J Med, 2013. **368**(7): p. 651-62.
25. Gewirtz, D.A., *The four faces of autophagy: implications for cancer therapy.* Cancer Res, 2014. **74**(3): p. 647-51.
26. Durante, M., et al., *Effect of drying and co-matrix addition on the yield and quality of supercritical CO(2) extracted pumpkin (Cucurbita moschata Duch.) oil.* Food Chem, 2014. **148**: p. 314-20.
27. Silva, H.D., et al., *Nanoemulsions of b-carotene using a high-energy emulsification–evaporation technique.* J. Food Eng. , 2011. **102**: p. 130-35.
28. Kentish, S., et al., *The use of ultrasonics for nanoemulsion preparation.* Innov. food sci. & emerg. technol. , 2008. **9**: p. 170–75.
29. Paolucci, M., G. Fasulo, and M.G. Volpe, *Employment of marine polysaccharides to manufacture functional biocomposites for aquaculture feeding applications.* Mar Drugs, 2015. **13**(5): p. 2680-93.
30. Yuan, Y., et al., *Characterization and stability evaluation of b-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions.* Food Res Int, 2008. **41**: p. 61–8.

31. Spagnuolo, C., et al., *Quercetin downregulates Mcl-1 by acting on mRNA stability and protein degradation.* Br J Cancer, 2011. **105**(2): p. 221-30.
32. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
33. Crouch, S.P., et al., *The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity.* J Immunol Methods, 1993. **160**(1): p. 81-8.
34. Seo, J.S., et al., *Extraction and chromatography of carotenoids from pumpkin.* J Chromatogr A, 2005. **1073**(1-2): p. 371-5.
35. Russo, M., et al., *ABT-737 resistance in B-cells isolated from chronic lymphocytic leukemia patients and leukemia cell lines is overcome by the pleiotropic kinase inhibitor quercetin through Mcl-1 down-regulation.* Biochem Pharmacol, 2013. **85**(7): p. 927-36.
36. Russo, M., et al., *Quercetin enhances CD95- and TRAIL-induced apoptosis in leukemia cell lines.* Leukemia, 2007. **21**(5): p. 1130-3.
37. Russo, G.L., et al., *Protective effects of butyric acid in colon cancer.* Adv Exp Med Biol, 1999. **472**: p. 131-47.
38. Orimo, H. and T. Shimada, *Regulation of the human tissue-nonspecific alkaline phosphatase gene expression by all-trans-retinoic acid in SaOS-2 osteosarcoma cell line.* Bone, 2005. **36**(5): p. 866-76.
39. Yonekura, L. and A. Nagao, *Intestinal absorption of dietary carotenoids.* Mol Nutr Food Res, 2007. **51**(1): p. 107-15.
40. Klionsky, D.J., et al., *Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition).* Autophagy, 2016. **12**(1): p. 1-222.
41. Rubinsztein, D.C., P. Codogno, and B. Levine, *Autophagy modulation as a potential therapeutic target for diverse diseases.* Nat Rev Drug Discov, 2012. **11**(9): p. 709-30.
42. Nollet, M., et al., *Autophagy in osteoblasts is involved in mineralization and bone homeostasis.* Autophagy, 2014. **10**(11): p. 1965-77.
43. Hocking, L.J., C. Whitehouse, and M.H. Helfrich, *Autophagy: a new player in skeletal maintenance?* J Bone Miner Res, 2012. **27**(7): p. 1439-47.
44. Vessoni, A.T., A.R. Muotri, and O.K. Okamoto, *Autophagy in stem cell maintenance and differentiation.* Stem Cells Dev, 2012. **21**(4): p. 513-20.
45. Pantovic, A., et al., *Coordinated time-dependent modulation of AMPK/Akt/mTOR signaling and autophagy controls osteogenic differentiation of human mesenchymal stem cells.* Bone, 2013. **52**(1): p. 524-31.

46. Pattingre, S., et al., *The G-protein regulator AGS3 controls an early event during macroautophagy in human intestinal HT-29 cells.* J Biol Chem, 2003. **278**(23): p. 20995-1002.
47. Rodan, S.B., et al., *Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties.* Cancer Res, 1987. **47**(18): p. 4961-6.
48. Xi, G., C.J. Rosen, and D.R. Clemmons, *IGF-I and IGFBP-2 Stimulate AMPK Activation and Autophagy, Which Are Required for Osteoblast Differentiation.* Endocrinology, 2016. **157**(1): p. 268-81.

Chapter V

Conclusions

5.1 Conclusions

The present thesis reports data on the biological properties of bioactive extracts prepared from Italian cultivars/products and assayed on different *in vitro* models to test their ability to ameliorate conditions of cellular oxidative stress often associated with the occurrence of degenerative pathologies, such as cancer and neurodegenerative diseases. The two classes of biomolecules investigated, polyphenols and carotenoids, are largely present in the three different foods and beverage considered: red wine, elderberries and pumpkin. Although the strategy to test vegetable extracts enriched with a specific class of biological compounds in preclinical cellular models is not novel and many examples are abundantly present in the literature, we selected this approach for two main reasons. Firstly, the measure of the biological response of an extract potentially includes possible additive/synergistic effects of more than a single compound and may result in enhanced functional outcomes. This possibility can reflect the observation that many bioactive molecules are present in the extract at relatively low concentrations, not enough to generate, per se, any measurable effect, but, when associated with others, can contribute to improve a given pathological or pre-pathological condition. The rationale of this hypothesis resides in the observation that, generally, biological extracts, like those tested in the present project, contain compounds which are structurally and functionally similar. To express this concept with a figurative example, a single, strongly active compound, added at high (pharmacological) concentrations to a sick cell/tissue may represent a “gun bullet”, compared to the “hundreds pellets” of a hunting rifle cartridge which mimic the mixture of molecules in a bioactive extract, each of them present at very low concentrations.

The second reason that brought us to focus the present work on the use of polyphenolic and carotenoid extracts is related to the possibility to design and

develop prototypes of functional foods enriched with these bioactive components, an outcome partially obtained, as discussed in the following paragraphs, although not included in the main frame of this thesis since the results are still preliminary. Work is in progress to complete these tasks which represent the expected continuation of the present project.

As stated above, the evaluation of the biological activity of the selected extracts using different pre-clinical experimental models highlights the potential applicative interest of the present work both in the nutraceutical and pharmaceutical sectors. To this regard, we would like to stress the rationale behind the cellular models employed in the present thesis, as summarized in the cartoon below (**Figure 5.1**).

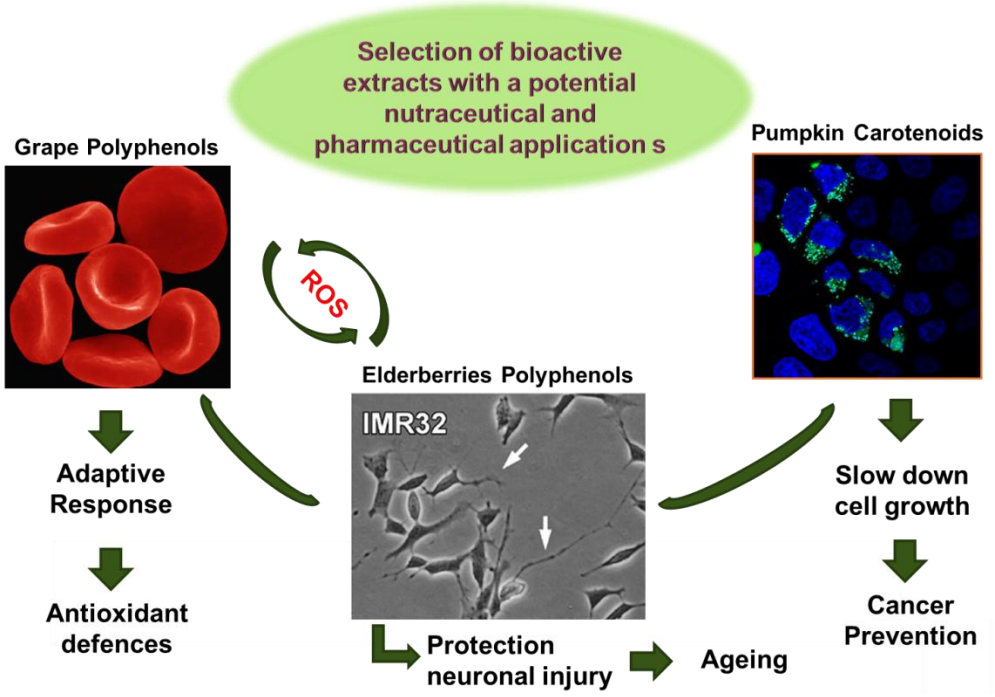

Figure 5.1. *Scheme summarizing the employed experimental models.*

We used human erythrocytes as a "physiological" model of cellular structures where oxidative damage occurring during the life cycle, especially in ageing,

may be ameliorated by grape polyphenols present in red wines. The same class of bioactive compounds can protect against neuronal injury caused by ROS in IMR32 cells, resembling the neurodegenerative damage occurring in elderly. Finally, a different class of antioxidant compounds, carotenoids from pumpkin, showed a very peculiar way to protect cells from cancer, another degenerative disease whose occurrence is highly related to lifestyle (including diet) and ageing.

In details, our data indicate the existence of novel mechanisms triggered by red wine polyphenols and resulting in the protection of RBCs by plasma oxidizing species. These compounds, in fact, are able to increase RBCs antioxidant defences by activating PMRS. The importance of this system is closely related to the increased oxidative stress in plasma, that occurs during aging. For this reason, the strengthening of RBCs antioxidant systems, including GSH and GSH-dependent enzymes, exerted by red wine-derived polyphenols, provides a mechanism to counteract the oxidative injuries, that constantly damage RBCs due to their biological role of oxygen transporters. Moreover, we hypothesized that red wine polyphenols trigger a slight, but significant increase of intracellular ROS, which induces a cellular adaptive response, resulting in increasing RBCs antioxidant defences. The corollary of our conclusion is that red wine polyphenols must act as weak pro-oxidants and undergo autoxidation induced by the ferryl myoglobin. This is not totally new, in fact, when polyphenols lose an electron or act as a reducing agents, become radicals and their oxidized intermediates can also behave as pro-oxidants. Interaction between polyphenols and transition metal ions can result in pro-oxidants formation, oxidized intermediates or oxidation products, such as semiquinones and quinones [1]. These species are extremely reactive and lead to ROS production, such as $O_2^{\cdot -}$ and H_2O_2 inside the cell (**Figure 5.2**) [1].

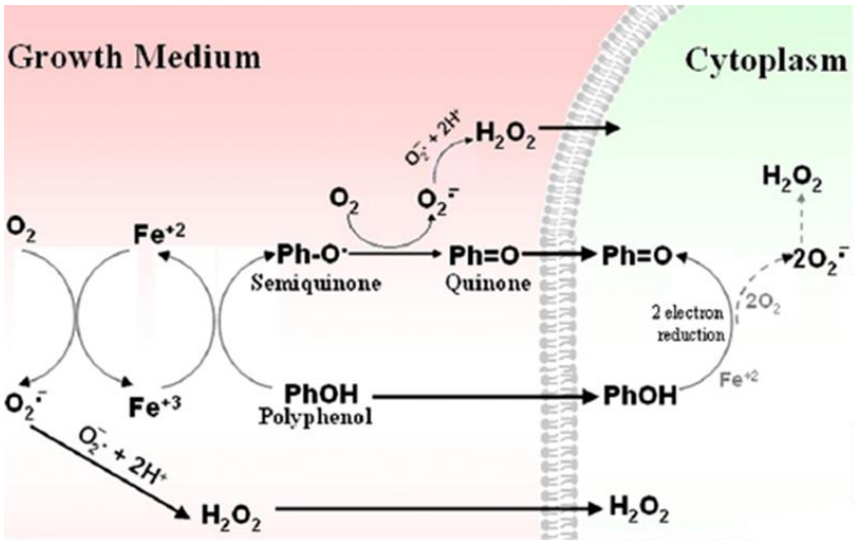

Figure 5.2. *Formation of semiquinone and quinone by polyphenols [1].*

The presence of ROS at low concentrations seems to be involved in normal cellular function, as well as disease prevention [2]. These circumstantial evidence are in favour of our hypothesis that red wine polyphenols generate low concentration of H_2O_2, which induce the cellular adaptation to oxidative stress. This aspect highlights the preventive role of polyphenols, which, through this mechanism of cellular adaptation against oxidative stress, can prevent the onset of degenerative and age-depending diseases related to oxidative damage, including neurodegeneration. In fact, we also described, in an *in vitro* neurodegenerative model, the protective role of a polyphenolic extract from *S. nigra* berries against oxidative damage induced by neurotoxic agents. Also in this case, we produced experimental evidence that polyphenols exert their neuroprotective effect by slightly increasing ROS levels, which, in turns, induced a cellular adaptive response. The data obtained provide an initial indication for future clinical trials addressed to prove the preventive role of this natural extract against neurodegenerative disease. However, we cannot ignore the large body of data which limit the clinical relevance of polyphenols

due to their difficulty to cross the BBB, which is required to explicate the potential neuroprotective effects. Moreover, oral administration of polyphenols implies their biotransformation and metabolism which largely influences their bioavailability. As reported in the "Introduction" of Chapter I, phenolic compounds are metabolized after intestinal uptake before being delivered to different tissues or organs by blood circulation to exert their biological effects. Once absorbed, polyphenols are conjugated to glucuronide, sulphate and methyl groups in the gut mucosa and inner tissues [3]. Future studies are needed to evaluate the biological activities of the conjugated derivatives and microbial metabolites of polyphenols, or to explore the possibility that these derivatives, after their cellular uptake, are converted back to the original aglycone, a possibility verified in several experimental conditions [4]. If proven, this achievement could contribute to the development of a dietary strategy to maximize the health benefits of phenolic compounds and their metabolites.

To this respect, considering the protective role of polyphenols against oxidative damage, we and other colleagues hypothesized that the intake of grape polyphenols could exert a beneficial effect in the plasma of healthy subjects. Therefore, in collaboration with Dr. Giacco of the Institute of Food Science, National Research Council in Avellino, we are participating to a clinical trial, approved by the Ethics Committee, within the frame of a PRIN (Research Projects of National Interest) project and aimed to evaluate the protective role of a juice enriched with grape polyphenols on healthy subjects (*data in preparation*). In particular, we measure the activity of antioxidant enzymes, such as CAT and SOD, and PMRS activity in blood samples drawn from the experimental versus the control group (subjects who drank juice without polyphenols) within 24 h after the intake. We detect that an increase of intracellular GSH concentration and the activitation of antioxidant enzymes,

such as SOD, after 360 min of juice assumption compared to the control group (*work in progress*).

As depicted in **Figure 5.1**, a specific workpackage of this project (Chapter IV) investigated the potentiality of a carotenoid-enriched extract from pumpkin to find applicative outcomes in the biomedical area. This possibility is supported by the large body of scientific evidence, widely cited in Chapter 1 and in the Introduction of Chapter 4, that fruits and vegetable contains dietary chemopreventive agents, including vitamin A derivatives. To support this view, we evaluated the functional effects of a pumpkin carotenoid extract on the proliferation of malignant cell lines, studying the molecular targets affected by carotenoids. We ended up with the observation that pumpkin carotenoids do not directly kill cancer cells, but slow down their proliferation. In two different cell lines, Caco-2 and SAOs, derived from human epithelial colorectal adenocarcinoma and osteosarcoma, respectively, the carotenoid-enriched extract induced an alteration of the energetic metabolism which resulted in a decreases intracellular concentration of ATP and the activation of an autophagic process. Probably, the "not-protective" autophagy detected in our experiments directly or indirectly induces cellular differentiation in malignant cells, a possibility that deserves further investigations.

A novelty of the present work, verified for both wine-derived polyphenols and pumpkin carotenoids is the observation that single compounds present in these extracts, and well-known in the literature for their broad biological activities are not responsible for the observed phenomena. In fact, in **Figure 2.14** of Chapter II, we reported that few of the most representative phenolic compounds tested on erythrocytes at the concentrations usually present in red wines were not as effective as when assayed in the whole extract, suggesting the possible existence of a synergistic mechanisms. Similarly, β-carotene from pumpkin, assayed as individual compound did not show any effects on cell proliferation at the concentration present in the extract, suggesting, also in this

case, a synergism among different components, or the presence of not yet identified bioactive molecules.

Considering the potential therapeutic applications of carotenoids in cancer cure and prevention, we cannot ignore the failure or null effects of several clinical trials mentioned in the above chapters which highlight the need to clarify important aspects of their molecular mechanism of action. We hope that our study may contribute to improve the knowledge on how this class of compounds, poorly studied at molecular level because of their low solubility can be effective against cancer and suggest pharmaceutical applications.

Despite the large rumour present on the media about the potential health effects of functional food and/or nutraceutical containing bioactive extracts, it is important to make a clear distinction between commercial interests and robust scientific data to support conclusions on the disease preventive efficacy of these molecules. Key issues, as metabolism and bioavailability, may strongly influence the positive cause-effect relation between intake of carotenoids-enriched food and disease prevention. For this reason, technological processing of carotenoids is crucial to ensure their applicative outcomes on an industrial scale, through the design of formulations, which allow to preserve their physical and chemical stability and effectiveness. The difficulty in developing an ideal formulation concerns the limits in manipulation of these molecules, which are sensitive to light, high temperature, oxygen, all conditions resulting in reduced stability, formation of degradation products and loss of biological activity [5]. For example, during preparation, processing, and storage of nutraceutical products, for the presence of double bonds in their structure, carotenoids are susceptible to different degradation reactions. The more frequent reactions that may occur are oxidation and isomerization. Oxidation may occur either by a spontaneous autoxidation in presence of oxygen, or by photoxidation, which involves singlet oxygen, while isomerization includes trans-isomers, more common and

stable in foods, and cis-isomers, usually formed during food processing. This chemical transformations influence the phenotypic aspects of product, but also reduce the functional activity of carotenoids. As reported in the "Introduction", Chapter I, low bioavailability of carotenoids limits their efficacy since the amount that reaches the bloodstream is very poor. Considering the lipophilic nature of these compounds, the formulation strategies are related to the use of a delivery vehicle able to increase their solubility in the aqueous fluids. Therefore, several advanced technologies are considerably expanding. Among these techniques, the formation of micelles and liposomes, microscopic spherical lipid vesicles, allows the incorporation of the lipophilic molecules in an internal core surrounded by a phospholipid mono- or bilayer, respectively [6]. Furthermore, microencapsulation is valid technique, that enhances the stability of carotenoids by physical isolation through packaging wall compounds, also called shell or coating. The formation of wall with appropriate polymers can protect bioactive molecules from oxygen and chemical and enzymatic degradations and increase the solubility/dissolution rate in water [7]. These materials are often biodegradable polymers, which protect core material and modify the rate of release of carotenoids in a specific target of the body [7]. Among this, spray-drying is a microencapsulation technique particularly indicated for heat sensitive components such as carotenoids, and widely used by pharmaceutical and food industry. Therefore, we suppose that the transformation in a stable dry powders form by spray-drying is a convenient way to increase the shelf-life and improve the physical chemical behaviour of carotenoid extract through the use of an appropriate polymers [8]. For this reason, during the last part of this project, we performed preliminary experiments aimed to develop a protocol for spray-drying process employing the carotenoid extract (*work in progress*) hoping to obtain a stable and high bioavailable formulations to be included in prototypes of functional foods, a challenge already explored in the course of

the present project. To this aim, we already designed and produced prototypes of bakery products (breads, cookies, **Figure 5.3**) made with flour rich in carotenoids obtained from the pumpkin. In the continuation of this work, these prototypes will be the subject of a study approved by the Ethics Committee, aimed to demonstrate the effects of bread enriched-carotenoids on blood glucose/insulin/lipid metabolism, markers of oxidative stress and subclinical inflammation in overweight subjects after 3-weeks of assumption (*work in progress*).

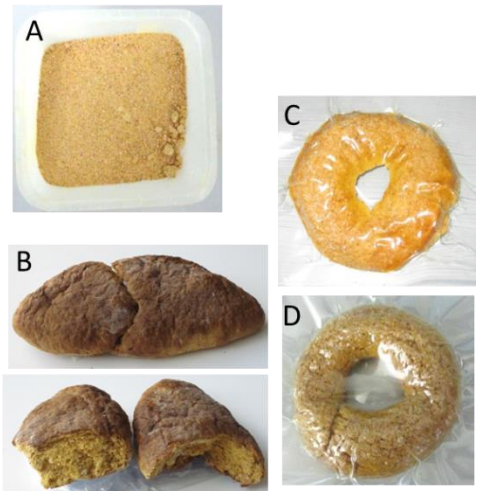

Figure 5.3. *Flour **(A)** obtained from the long pumpkin of Naples and derived prototypes of bakery products: bread **(B)** and cookies (**C** and **D**).*

The data reported in this thesis have been published [9] [10] or presented as posters or communications at national and international conferences [11] [12] [13] [14] [15].

5.2 References

1. Erlank, H., et al., *Polyphenols activate Nrf2 in astrocytes via H2O2, semiquinones, and quinones.* Free Radic Biol Med, 2011. **51**(12): p. 2319-27.
2. Benhar, M., D. Engelberg, and A. Levitzki, *ROS, stress-activated kinases and stress signaling in cancer.* EMBO Rep, 2002. **3**(5): p. 420-5.
3. Scalbert, A., et al., *Absorption and metabolism of polyphenols in the gut and impact on health.* Biomed Pharmacother, 2002. **56**(6): p. 276-82.
4. Russo, G.L., M. Russo, and C. Spagnuolo, *The pleiotropic flavonoid quercetin: from its metabolism to the inhibition of protein kinases in chronic lymphocytic leukemia.* Food Funct, 2014. **5**(10): p. 2393-401.
5. Silva, H.D., et al., *Nanoemulsions of b-carotene using a high-energy emulsification–evaporation technique.* J. Food Eng. , 2011. **102**: p. 130-35.
6. John P. Gleeson, J.P., S.M. Sinéad M. Ryan, and D.J. David J. Brayden, *Oral delivery strategies for nutraceuticals: Delivery vehicles and absorption enhancers.* Trends Food Sci Tech, 2016. **53**: p. 90-101.
7. Sansone, F., et al., *Maltodextrin/pectin microparticles by spray drying as carrier for nutraceutical extracts.* J Food Eng, 2011. **105**(3): p. 468-76.
8. Sansone, F., et al., *Hesperidin gastroresistant microparticles by spray-drying: preparation, characterization, and dissolution profiles.* AAPS PharmSciTech, 2009. **10**(2): p. 391-401.
9. Tedesco, I., et al., *Red wine activates plasma membrane redox system in human erythrocytes.* Free Radic Res, 2016. **50**(5): p. 557-69.
10. Spagnuolo, C., et al., *Neuroprotective Role of Natural Polyphenols.* Curr Top Med Chem, 2016. **16**(17): p. 1943-50.
11. Russo, M., et al., *Effetti antiproliferativi ed attivazione autofagica indotti dai carotenoidi della zucca Cucurbita moschata in linee cellulari tumorali umane*. 2016, XXXVII Congresso Nazionale 2016 della Società Italiana di Nutrizione Umana: Bologna-Italy.
12. Moccia, S., et al., *Risposta antiossidante adattativa indotta dai polifenoli del vino rosso in eritrociti umani* 2016 XXXVII Congresso Nazionale 2016 della Società Italiana di Nutrizione Umana: Bologna-Italy.
13. Spagnuolo, C., et al., *Effetto neuroprotettivo dei polifenoli del sambuco (Sambucus nigra)*. 2016, XXXVII Congresso Nazionale 2016 della Società Italiana di Nutrizione Umana: Bologna-Italy.

14. Spagnuolo, C., et al., *Neuroprotective Role of Natural Polyphenols: a Look to the Potentiality of Elderberry Extracts*. 2016, 10th World Congress on Polyphenols Applications Porto-Portugal.
15. Tedesco, I., et al., *Antioxidant properties of methanolic extracts obtained from blackberries and elderberry*. 2013, 3rd lnternational Conference on Foodomics: Cesena-Italy.

Dipartimento Farmacia
Via Giovanni Paolo II
84084 Fisciano, Salerno