Universidad de Barcelona

Instituto de Investigaciones Químicas y Ambientales de Barcelona-CSIC

POLYPHENOLIC FRACTIONS FROM WINE BY-PRODUCTS AS POTENTIAL ANTITUMORAL AND/OR PROTECTIVE AGENTS AGAINST UV DAMAGE

Tesis Doctoral

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2006

Memoria presentada por

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Para optar al grado de

Doctora por la Universidad de Barcelona

Polyphenolic fractions from wine by-products as potential antitumoral and/or protective agents against UV damage

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Programa de Doctorado de Biomedicina (Bienio 2000-2002)

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Barcelona, Enero del 2006

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ABBREVIATIONS

A: absorbance.

ADP: adenosine 5'-diphosphate.

AP: alkaline phosphatase.

ARP: antiradical power.

ATCC: American type culture collection.

ATP: adenosine 5'-triphosphate.

BCA: bicinchoninic acid

BCIP: 5-bromo-4-chloro-3-indolyl-phosphate.

BSA: bovine serum albumin.

CAT: catalase.

DHR: dihydrorhodamine 123.

DISC: death-inducing signalling complex.

DMEM: Dulbecco's modified Eagle Medium.

DMSO: dimethyl sulfoxide.

DPPH: 1,1-diphenyl-2-picrylhydrazyl free radical.

DTT: dithiothreitol.

EC: epicatechin.

ECACC: European collection of cell cultures.

ECG: epicatechin gallato.

EDTA: ethylenediaminetetraacetic acid.

EGC: epigallocatechin.

EGCG: epigallocatechin gallato.

EGTA: ethyleneglycotetraacetic acid.

EPO: eosinophil peroxidase.

ERK: extracellular signal-regulated kinase.

F: flavonols.

FACS: fluorescence-activated cell sorter.

FCM: flow cytometry.

FCS: fetal calf serum.

FITC: fluorescein isothiocyanate.

GA: gallic acid.

GPX: glutathione peroxidase.

GSH: glutathione.

HO-1: heme oxygenase-1.

HRP: horseradish peroxidase.

JNK: c-Jun N-terminal kinase.

MAPKs: mitogen-activated protein kinases.

MAPKKs: MAPK kinases.

MAP3Ks: MAPKK kinases.

mDP: mean degree of polymerization.

MED: minimal erythema dose.

mMW: mean molecular weight.

MTT: 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide.

NAC: N-acetylcysteine.

NADH: nicotinamide adenine dinucleotide.

NADPH: nicotinamide adenine dinucleotide phosphate.

NBT: nitro blue tetrazolium.

NOS: nitric oxide synthase.

OD: optical density.

PBLs: peripheral blood lymphocytes.

PBS: Dulbecco's phosphatebuffer saline.

PI: propidium iodide.

PMSF: phenylmethylsulfonyl fluoride.

PS: phosphatidylserine.

PVDF: polyvinylidene fluoride.

ROS: reactive oxygen species.

RNA: ribonucleic acid.

RP-HPLC: reverse phase-high performance liquid chromatography.

SIDMAP: stable isotope-based dynamic metabolic profiling.

SDS: sodium dodecyl sulfate.

SDS-PAGE: SDS-polyacrylamide gel electrophoresis.

Sem: standard error of the mean.

SOD: superoxide dismutase.

SED: standard erythema dose.

TBE: tris borate EDTA.

TBS: trisbuffered saline.

TBS-T: TBS- tween.

UV: ultraviolet.

1. INTRODUCTION

1.1. CANCER

Cancer is one of the leading causes of death in the prosperous countries of the world, where one person in five die because of this disease. As medical history has shown with other diseases, the lack of treatments is due to our insufficient knowledge of the biological mechanisms involved in the development and progression of cancer. Despite the great advances in the last decades in understanding the molecular and cellular basis of cancer, which has allowed development in clinical treatment, it does not seem that we will be able to control the disease as much as we would like to in the imminent future. To be more precise, the discovery of the increased complexity of cancer has led us to consider it as a group of relatively heterogeneous of diseases more than an unique etiopathology [Alberts et al., 1994; Muñoz, 1997].

During a person's life there are about 10^{16} cell divisions in their body. If cancer were induced by a single genetic change that led to uncontrolled cell proliferation, this enormous number of cell divisions would produce thousand of different cancers in each individual. However, carcinogenesis is a multistage process in which the orderly production, differentiation and replacement of cells in the affected tissue are progressively disrupted, and its incidence in humans increases very steeply with increasing age. The unregulated proliferation of poorly differentiated cells generates a localized lesion, and this eventually develops the ability to invade other tissues. In its simplest form, carcinogenesis can be regarded as having three phases [Johnson, 1998; Murray et al., 1993]:

1. Initiation, in which a single dividing cell acquires a nonfatal, unrepaired mutation.

2. Promotion, in which a colonial population derived from the original cell expands and accumulates new genetic abnormalities.

3. Progression, which signifies the development of malignant tumour.

1.2. INTRACELLULAR OXIDANTS: REACTIVE OXYGEN SPECIES (ROS)

The intrinsic balance between life and death can be influenced by several environmental stresses. Reactive products of oxygen are amongst the most potent and omnipresent threats faced by any living organism.

Small amounts of Reactive Oxygen Species (ROS) such as superoxide anion ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$), and peroxyl

radical ($RO_2^{\bullet}$), are constantly generated in organisms during normal cellular function as a consequence of aerobic metabolism [Chandra et al., 2000; Matés, 2000; Halliwell, 1995]. Although mitochondrial oxidative phosphorylation is one of the major sites of production of these species in eukaryotic cells, they can also be produced by exposure to ionizing or UV radiation, by oxidative chemicals or by release from phagocytic cells in response to bacterial invasion or tumour [Slater et al., 1995; Kong et al., 2000]. Intracellular accumulation of these species has been implicated in many disease processes, including aging and carcinogenesis. Since free radicals are molecules that contain one or more unpaired electrons with anti-parallel spins in their outer orbit, they react easily with biologic structures, particularly polyunsaturated membrane lipids, bio-membranes, DNA, and amino acids. Cells react rapidly to this redox imbalance with a plethora of biological responses, including cell cycle-specific growth arrest, gene transcription, and initiation of signal transduction pathways and repair of damaged DNA. These early events are likely to determine whether a cell will necrose, senesce, apoptose or survive and proliferate [Chandra et al, 2000; Matés et al., 2000; Kong et al., 2000].

1.3. ANTIOXIDANTS

A broad definition of an antioxidant is any substance that, when present at low concentrations compared with those of an oxidizable substrate (including every type of molecule found *in vivo*), significantly delays or prevents oxidation of that substrate [Halliwell, 1995].

The defence provided by antioxidant systems is crucial to the survival of organisms. Detoxification of ROS in the cell is provided by both enzymatic and non-enzymatic systems, which constitute the antioxidant defence systems:

- o Enzymatic systems include extensively studied enzymes such as superoxide dismutase (SOD) and catalase (CAT), which act specifically against ROS; glutathione peroxidase (GPX), D-T diaphorase, thioredoxin reductase, nitric oxide synthase (NOS), heme oxygenase-1 (HO-1), eosinophil peroxidase (EPO) and glutathione-regenerating enzyme systems.

- o Non-enzymatic antioxidants, which are less specific than the enzymatic ones, can also scavenge other radicals, both organic and inorganic. These antioxidants can be classified as water-soluble or lipid-soluble, depending on whether they act primarily in the aqueous phase or in the lipophilic region of cell membranes:

- Hydrophilic antioxidants include ascorbic acid (vitamin C) and urate.
- Lipid-soluble antioxidants include ubiquinols, retinoids, carotenoids and tocopherols (vitamin E).

Plasma proteins, Glutathione (GSH), urate, and others are some of the endogenous antioxidants, while ascorbic acid, carotenoids, retinoids, flavonoids, other polyphenols and tocopherols constitute some of the dietary antioxidants [Middleton et al., 2000; Matés, 2000]. Many studies have reported that the intake of these antioxidants within our diet can carry out an effective protective action toward the oxidative stress created in the body by imbalance between ROS and its endogenous defence mechanism [Wiseman et al., 1996; Prior et al., 1999; Wattenberg, 1992].

Vegetables and fruits, such as tomato, cabbage, onion, tea, bean and citrus fruits are the food products with the largest amounts of natural antioxidants. Grapes and grape products such as wine also contain large amounts of antioxidants, in particular, polyphenols (12.6-22.4 mmol/l), which play a very important role in cardiovascular protection, anti-cancer action, and cellular membrane protection [Miller et el., 1995; Brenna et al., 2001; Kampa et al., 2000]. However, more recent studies have alerted about the possible risks resulting from too high consumption of antioxidants such as polyphenols [Vitaglione et al., 2004; Scalbert et al., 2005] which demonstrate more human studies are needed to provide clear evidence of their protective effects and to determine the optimal levels of intake for better health.

1.3.1. Phenolic compounds

Plant tissues are a rich source of biologically active phenolic compounds containing one or more aromatic acids. The presence of hydroxyl groups and other functional groups gives rise to a rich variety of derivates, which are responsible for much of the flavour, colour and texture of plant foods [Johnson, 1998].

Flavonoids are the largest class of phenolic compounds. Thus, over 4000 structurally unique compounds have been identified in plant sources. They are mainly classified into the following groups [Middleton et al., 2000]:

o Flavanols (Figure 1):

The main flavanols are catechins, which are abundant in tea, red wine and chocolate. Grape and chocolate catechins are mainly (+)-catechin and (-)-epicatechin (EC), whereas tea catechins also have gallocatechins and their galloyl

esters as major components. Both grape skins and seeds contain monomers and oligomeric and polymeric proanthocyanidins, the mean degree of polymerisation being higher for skin flavanols. Red wine contains more flavonoids than grape juice because the winemaking process extracts some of the flavonoids from the seeds and skins of grapes [Yang et al., 2001; Souquet et al., 1996; Prieur et al., 1994].

A   **B**

	R$_1$	R$_2$
Epigallocatechin gallate (EGCG)	OH	gallate
Epicatechin gallate (ECG)	H	gallate
Epigallocatechin (EGC)	OH	OH
Epicatechin (EC)	H	OH

arrows indicate possible polmerization positions

R$_1$= H: procyanidins, OH: prodelphinidins

R$_2$= OH,   (gallates)
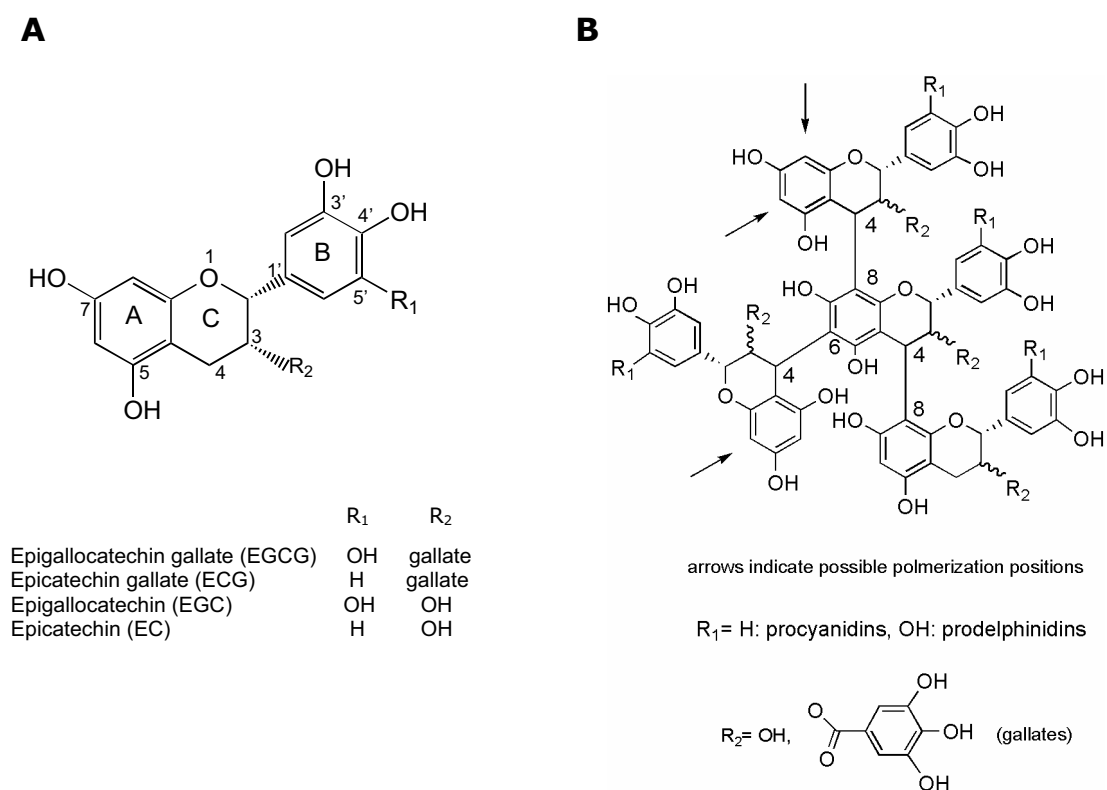
Figure 1. *Flavanols: monomers (**A**) and polymeric proanthocyanidins (**B**)*

o   Flavonols (Figure 2):

Quercetin is the main flavonol in the human diet, present in many fruits, vegetables and beverages such as tea or wine. Quercetin, as well as other flavonols is particularly abundant in grape skins and stems. Quercetin usually occurs as *O*-glycosides, with D-glucose as the most frequent sugar residue. More than 170 different quercetin glycosides have been identified [Yang et al., 2001; Soleas et al.; 1997; Souquet et al., 2000].
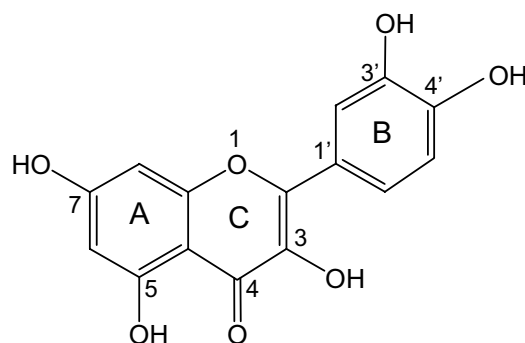
Figure 2. *Quercetin*

o Isoflavones (Figure 3):

Soybeans are the only significant dietary source of isoflavones. The primary isoflavones in soy are genistein and daidzein (approximately 1 mg/g dry bean), which are generally considered as phytoestrogens [Yang et al., 2001].
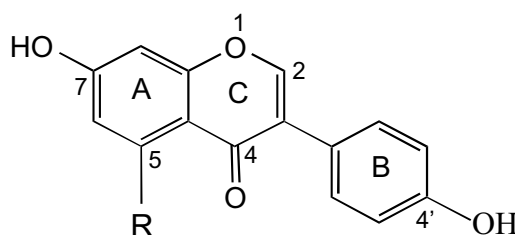
R = OH   Genistein
R = H   Daidzein

Figure 3. *Isoflavones*

1.3.1.1. Absorption and metabolism of flavonoids

In addition to the structural and physico-chemical attributes of the dietary native compound, the absorption, pharmacokinetics, biotransformation, and the relative activities of metabolites are critical determinants of biological effects in organisms. Although existing knowledge of absorption and metabolism is confined to a select group of dietary flavonoids, sufficient evidence supports that most of these compounds are absorbed sufficiently to exert a marked decrease in various parameters of plasma oxidant status [Heim et al., 2002]. Absorption depend on dosage, vehicle of administration,

antecedent diet, sex differences, and microbial population of the colon [Hollman et al., 1999; Erlund et al., 2001; Hollman et al., 1995]. Some modifications that ensue during metabolism are known to include hydroxylation, *O*-methylation, conjugation with thiols (particularly GSH), cleavage of the heterocycle, deglycosylation, and scission of polymeric species into monomeric units [Heim et al., 2002; Williams et al., 2004].

1.3.1.2. Structural features and antioxidant/pro-oxidant activity of flavonoids

Phenolic antioxidants such as flavonoids can be antioxidative and pro-oxidative depending on the balance between their reduced forms, which act as antioxidants and their oxidized forms (phenoxyl radicals or quinone/quinone methide intermediates), which have pro-oxidant activities [Galati et al., 2004]. The balance between antioxidant and pro-oxidant characteristics of flavonoids have been attributed not only to their structural features, but also to the concentration, suggesting induction of antioxidant defence metabolism by low concentrations and ROS production at high concentrations [Raza et al., 2005]. Some of the structural elements than influence flavonoids behaviour are the following:

- o One or two hydroxyl groups, especially in the B-ring (eg. 3',4' dihydroxylated B-ring catechol group), confer antioxidant capacity capable of readily donating hydrogen to stabilize the radical species [Rice-Evans et al., 1996]. However, pyrogallol structure (three hydroxyl groups) or multiple hydroxyl groups have been found to promote ROS production [Hanasaki et al., 1994; Ohshima et al., 1998].

- o Presence or absence of an unsaturated 2,3-bond in conjugation with a 4-carbonyl group, characteristic of flavonols structure, has been associated with stronger antioxidant activity in a microsomal system compared to those with saturated heterocycles [Cholbi et al., 1991]. On the contrary, there is evidence that the unsaturated 2,3-bond and 4-oxo arrangement of flavones may promote the formation of ROS induced by divalent copper in the presence of oxygen [Cao et al., 1997].

- o Existence of carbohydrate moieties, which decreases the antioxidant properties of flavonoids depending on the position, structure and total number of glycosidic moieties [Plumb et al., 1999; Ioku et al., 1995].

- o Degree of polymerization, which enhances the effectiveness of procyanidins against a variety of radical species [Vennat et al., 1994]. Extensive conjugation

between 3-OH and B-ring catechol groups, together with abundant $\beta_{4\to8}$ linkages, endow a polymer with significant radical scavenging properties by increasing stability of its radical [Castillo et al., 2000].

1.4. CELL CULTURES IN CANCER RESEARCH

Although questions about cell growth and the induction of cancer are ultimately questions about the behaviour of individual cells in a living organism, for practical and ethical reasons, many of the potential antitumoral drugs are first tested in cells growing in culture. Thus, the environment of cultured cells can be manipulated, the type of target cell can be well defined, the changes in cells following treatment with carcinogens or anticarcinogens products can be examined, and the fate of these substances can be determined. Furthermore, cultured cells can be quiescent or growing; they can have, in fact, precisely defined growth parameters. They can also be manipulated genetically. For these reasons, studies of normal cell growth as well as of cancer induction depend heavily on the use of cultured cells [Lodish et al., 1995].

1.5. CELL CYCLE

The cell cycle can be defined as the ordered set of processes by which one cell grows and divides into two daughter cells, and include the period between two mitotic divisions. Cell growth and division is a cornerstone of biology. Without knowing the checks and balances that normally ensure orderly cell division, we cannot devise effective strategies to combat the uncontrolled cell divisions of the cancers that will kill one of five of us.

For many years scientists actively debated whether the cell cycles of different cells run in fundamentally different ways. We know now, however, that the cell cycles of all eukaryotes are governed by the same principles. Thus, the key proteins that regulate the cell cycle have been so well conserved during evolution that human proteins can successfully regulate the cell cycle of simple yeasts even though our ancestors diverged more than a billion years ago [Murray et al, 1993; Muñoz, 1997].

The duration of the cell cycle may vary greatly from one cell type to another. Thus, fly embryos have the shortest known cell cycles, each lasting 8 minutes, while the cell cycle of a mammalian liver cell can last longer than a year. However, we can consider

that fairly rapidly dividing mammalian cells have a cycle time of about 24 hours [Alberts et al., 1994].

1.5.1. Phases of the cell cycle

The cell cycle is divided into two fundamental phases (Figure 4):

- o Interphase, which occupies the majority of the cell cycle, and includes phases G_1, S and G_2.

- o Mitosis, which lasts only about 30 minutes, and ends with the division of the cell.
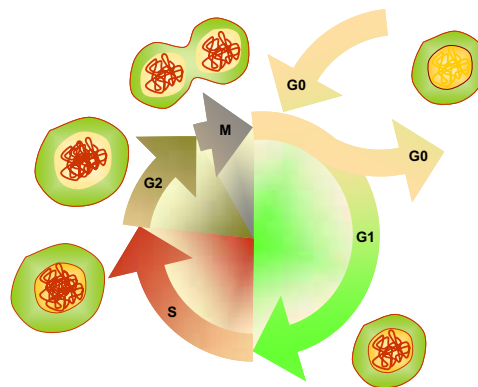
Figure 4. *Phases of the cell cycle*

During interphase the DNA is diffusely distributed within the nucleus, and individual chromosomes cannot be distinguished. Low activity can be detected with a microscope, although important processes are occurring and are referred to collectively as growth. These processes include the synthesis of new ribosomes, membranes, mitochondria, endoplasmic reticulum, and most cellular proteins.

Stepwise processes occur once per cell cycle. For example, chromosome replication is restricted to a specific S phase (for DNA synthesis), which occurs in the middle of interphase, and is preceded by a gap called G_1 and followed by a gap called G_2. G_1 and G_2 provide additional time for growth: if interphase lasted only long enough for DNA replication, the cell would not have time to double its mass before its division. During G_1 the cell monitors its environment and its own size and, when the time is ripe, takes a decisive step that commits it to DNA replication and completion of a division cycle. The G_2 phase provides a safety gap, allowing the cell to ensure that DNA replication is complete before it plunges into mitosis.

After chromosome replication is completed, the cell seems to pause briefly in a state called metaphase, in which the two daughter chromosome remain attached to each other at multiple points along their length and referred to as sister chromatids. The segregation of the duplicated chromosome marks the beginning of anaphase, during which the chromosomes move to the poles of the spindle, where they decondense and re-form intact nuclei. The cell is then pinched in two by a process called cytokinesis, which is traditionally viewed as the end of the mitotic phase, or M phase of the cell cycle.

In a typical animal cell cycle, G_1 last 12 hours, S phase 6 hours, G_2 6 hours, and mitosis about 30 minutes. Although the lengths of all phases of the cycle are variable to some extent, by far the greatest variation, in most of the commonly studied types of cells, occurs in the duration of G_1. Cells in G_1, if they have not yet committed themselves to DNA replication, can pause in their progress around the cycle and enter a specialized resting state, often called G_0, where they can remain for days, weeks, or even years before resuming proliferation [Alberts et al., 1994; Murray et al., 1993].

1.5.2. Cancer and the cell cycle

The decision for a cell to divide is a tightly regulated process that integrates signals from many sources. These signals indicate when division is needed and appropriate. Two sources of these signals are now known, and these arise from the extracellular environment or from intracellular checkpoint controls:

- o Environmental signals help cells to determine when there is a need to divide, that the required nutrients are available, and that there are no overriding reasons not to begin a replicative cycle.

- o Signals from internal checkpoints ensure the preceding steps of the cell cycle have been completed correctly before the next stage of division takes place.

At the centre of these signalling pathways lies a mechanism for integrating these signals, the proteins for basic cell cycle control. All processes that contribute to decisions about proliferation or that are regulated in a cell-cycle-dependent manner must ultimately interface with the basic controls of the cell cycle.

Normally cell cycle runs smoothly. The steps of the cell division occur uneventfully, and even when problems do arise, they are corrected efficiently. However, when major perturbations are introduced by mutation of key regulatory genes, the control of division can be disrupted permanently. Depending on the mutation, different manifestations of

the loss of control are seen. In some circumstances mutations promote a selective growth advantage for affected cells, leading to excessive and inappropriate division. When this occurs in multicellular organisms, uncontrolled division can be the initiating event of a number of diseases, the most devastating of which is cancer [Hutchison et al, 1995].

Although the concept of cancer as a disease of the cell cycle implies that every tumour is defective in one or more aspects of cell cycle control, it clearly does not mean that oncogenesis targets only the cell cycle clock. Development of a full-blown malignancy appears to require also aberrations in the cell death machinery and cell-cell and/or cell-matrix interactions that co-operate with cell cycle defects. The above concept simply regards cell cycle deregulation as an essential step in the process of multistep tumourigenesis [Bartek et al., 1999].

1.6. APOPTOSIS

Apoptosis is a key mechanism in developing organisms, used to rid the body of cells that are in excess, in the way, damaged, or potentially dangerous. For instance, our fingers are separated because embryonic cells that joined them died during development; in aquatic birds these cells live and form the webbing of the hind legs. Apoptosis is also a mechanism used in the thymus to eliminate self-reactive T lymphocytes and avoid autoimmunity. When T cells kill other cells, they activate the latent apoptosis pathway in their targets. Discovered and rediscovered several times by various developmental biologists and cytologists, programmed cell death acquired a number of names over the past two centuries. The term finally adopted is apoptosis, coined by Currie and colleagues in 1972 to describe a common type of programmed cell death that the authors repeatedly observed in various tissues and cell types [Kerr et al., 1972; Lodish et al.,1995; Hengartner, 2000].

Apoptotic cell death is characterized by controlled autodigestion of the cell, and can be separate in two main phases [Muñoz, 1997; Matés et al., 2000]:

o First phase or initiation phase, in which cells respond to a stimulus (or its absence) deciding to begin the death process. These stimuli include hyperthermia, growth-factor or hormone withdrawal, glucocorticoids, oxidative stress, ionising radiation and multiple classes of chemotherapeutic agents.

o Second phase or effector phase, where cells suffer a series of structural changes that lead them to death.

Apoptosis differs from necrosis by distinct morphological and biochemical features, such as chromatin condensation, membrane surface blebbing, oligonucleosomal DNA fragmentation and finally, the breakdown of the cell into a series of smaller units (membrane-bound fragments). These are called apoptotic bodies that in most tissues are phagocytosed by macrophages and their contents recycled without leakage of the contents of the dying cells. As a consequence, apoptotic cells do not induce an inflammatory response *in vivo*. Such events are associated with activation of specific cysteine proteases called caspases and loss of membrane phospholipid asymmetry resulting in phosphatidylserine externalization, which can be specifically recognize by annexin V [Matés et al, 2000; Slater et al, 1995; Muñoz, 1997; Fadok et al, 1992].

1.6.1. Apoptosis mechanisms

Most of the recent advances in the elucidation of apoptosis pathways have come about through the characterization of the effector mechanisms, which have several components. Nowadays, two effector mechanisms associated with caspase activation have been characterized extensively (Figure 5):

o   The extrinsic or death receptor-mediated pathway that is initiated when a death receptor ligand bind to its death receptor, which promotes the recruitment of adapter molecules, leading to the assembly of a protein complex called death-inducing signalling complex (DISC). The clustering of pro-caspases in this complex is sufficient for their initial activation. Subsequently, pro-caspase-8 is activated to caspase-8, which is sufficient to process caspase-3 and initiate the proteolytic cascade that results in apoptotic cellular degradation.

o   The intrinsic or mitochondrial pathway that relies solely on the permeabilization of mitochondrial membranes to release apoptogenic mitochondrial proteins such as Smac/DIABLO or cytochrome *c*, required for caspase activation and apoptosis. Thus, release of cytochrome *c* to the cytosol, causes the formation of the apaf-1/pro-caspase-9 apoptosome which can activate caspase-9, which is able to trigger the downstream caspase cascade, finally leading to apoptosis [Hengartner, 2000; Chinnaiyan et al., 1995; Muzio et al., 1998].
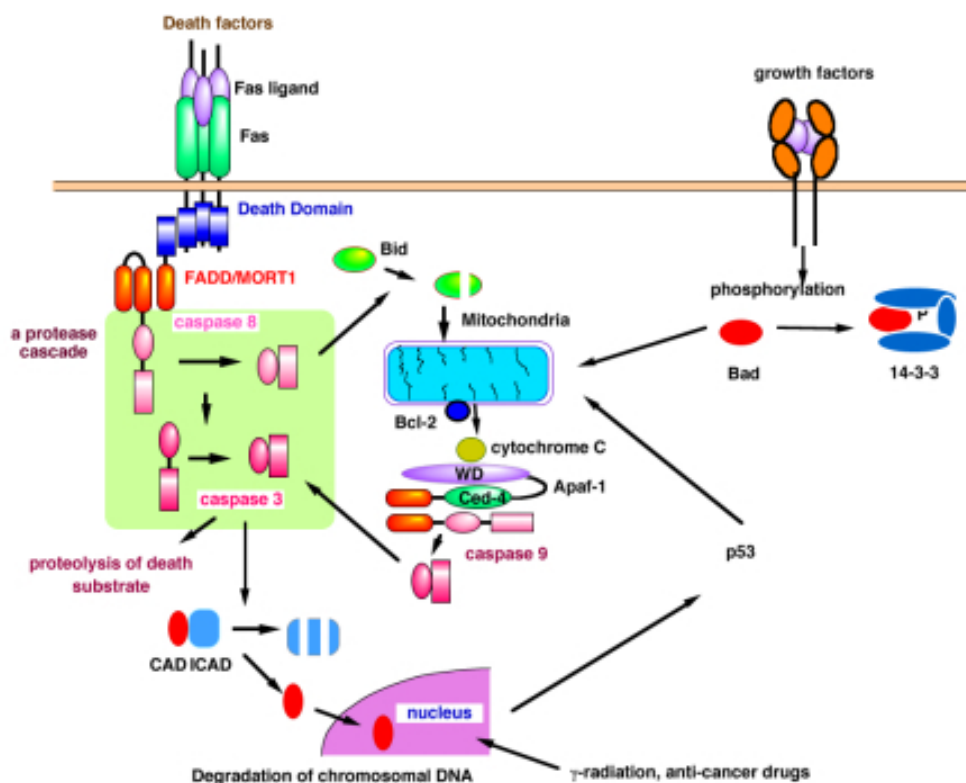
Figure 5. *Extrinsic and intrinsic apoptosis pathways*

Bcl-2 family members are apoptotic regulators that appear to play an important role in the control of both, intrinsic and extrinsic apoptotic pathways. Thus, antiapoptotic Bcl-2 family members (eg., Bcl-2 and Bcl-X$_L$) residing in the outer mitochondrial membrane can be destabilized during conditions of cell stresses by the induction of proapoptotic Bcl-2 family members (eg. Bax, Bad, and Bak), which causes pores in the outer mitochondrial membrane, liberating apoptogenic mitochondrial proteins to activate caspases and induce apoptosis. Additionally, caspase-8 can cleave the pro-apoptotic member of the Bcl-2 family, Bid into the active form tBid, which can also facilitate pore formation in the outer mitochondrial membrane, acting as a link between the extrinsic and intrinsic pathways of apoptosis [Adams et al., 1998; Reed et al., 1998].

1.6.2. Importance of ROS in apoptosis

Although Reactive Oxygen Species (ROS) may mediate growth regulatory pathways, there is emerging evidence that also suggests a role of ROS in apoptosis [Payne et al., 1995; Jacobson et al., 1996]. Evidence in favour of such a role includes the findings that:

o ROS can be detected in many forms of apoptosis.

o Exogenous low-dose ROS induce apoptosis.

o Antioxidants prevent most forms of apoptosis whether or not they appear to be initiated by ROS.

o Mitochondria, a major source of ROS, appear to be critical for the apoptotic process.

o ROS generation has been reported to occur in death receptor-mediated apoptosis [McGowan et al., 1996; Suzuki et al., 1998; Gulbins et al., 1996; Susin et al., 1998].

In spite of these findings, data also exist indicating that ROS are not essential. For example:

o ROS cannot be detected after all apoptosis inducers or with the same inducer in different cell types.

o Antioxidants can induce apoptosis in some systems. For example, the thiol antioxidant N-acetylcysteine (NAC) can prevent apoptosis after treatment with a number of different toxic compounds, but it is also capable of enhancing apoptosis. Thus, while it is clear that oxidative damage is not required to induce apoptosis, it may play a critical role in some systems

One useful concept regarding free radicals and apoptosis is that of the requirement of an "oxidative tonus" for normal cell function. If this tonus is disrupted, either by increasing the reduction or oxidation state of the cell, apoptosis may ensure. If the disruption is too severe, apoptosis may not have a chance to develop before necrosis occurs [Kehrer, 2000].

1.6.3*. Cancer and apoptosis*

The link between apoptosis and cancer was early postulated in 1972 [Kerr et al., 1972]. However, the field of apoptosis was largely ignored until the last decade. Now, it is apparent that aberrant apoptosis is involved in a number of disease states, particularly cancer [Johnstone et al., 2002].

In many cases, apoptotic processes are dependent on tumour suppressor genes such as the *p53* gene. Thus, when this tumour suppressor gene is not expressed or the

activity of the protein that encodes is altered by mutation, cells do not die. Then, DNA replicates and daughter cells with aberrant chromosomes are generated, which result in the loss of control of proliferation and the beginning of cancer [Muñoz, 1997]. An interesting chemopreventive approach entails the use of agents that quickly eliminate premalignant cells by inducing them to undergo apoptosis [Sun et al., 2004].

1.7. GLUCOSE METABOLISM

The oxidation of glucose is known as cellular respiration and involves the following processes: glycolysis, the formation of acetyl coenzyme A, the Krebs Cycle, and the Electron Transport Chain. Glycolysis is the oxidation of glucose to pyruvate and occurs in most cells in the body. It provides some ATP and energy-containing NADH + H$^+$. Because glycolysis does not require oxygen it is known as anaerobic cellular respiration. The formation of acetyl coenzyme A from pyruvate is a transition step between glycolysis and the Krebs cycle that prepares pyruvate for entrance into the cycle. Both the Krebs cycle and the electron transport chain require oxygen to produce ATP, they are therefore referred to as aerobic cellular respiration. Under aerobic conditions, most cells convert pyruvate to acetyl coenzyme A, which links glycolysis with the Krebs cycle. However, under anaerobic conditions, pyruvate is reduced by the addition of two hydrogen atoms to form lactate. The object of anaerobic glycolysis is not to produce lactate but to reoxidize NADH and thus permit continued ATP production from glycolysis. Apart from glycolysis exists a completely different pathway of glucose oxidation called the pentose phosphate pathway which supplies ribose-5-phosphate for nucleotide and nucleic acid synthesis and reducing power in the form of NADPH. The enzymes responsible for catalysing those reactions are most abundant in tissues with high demands for NADPH where it is used for reductive synthesis, and in rapidly dividing cells which require ribose-5-phosphate for DNA synthesis.

The pentose phosphate pathway has two main parts:

o The oxidative phase where glucose-6-phosphate is converted to ribose-5-phosphate and CO_2, during which NADP$^+$ is reduced to NADPH.

o The non-oxidative phase which includes several interconversion steps of C7, C4, and C3 ketoses and aldoses regenerating glucose-6-phosphate, fructose-6-phosphate, and glyceraldehyde-3-phosphate, all three being metabolites of glycolysis.

The two phases of pentose phosphate pathway have different functions and can occur independently although have been accepted as a cycle where the oxidative section would produce ribose-5-phosphate and NADPH, and the non-oxidative section would recycle excess of ribose-5-phophate to glycolysis (Figure 6) [Elliot et al., 1997].
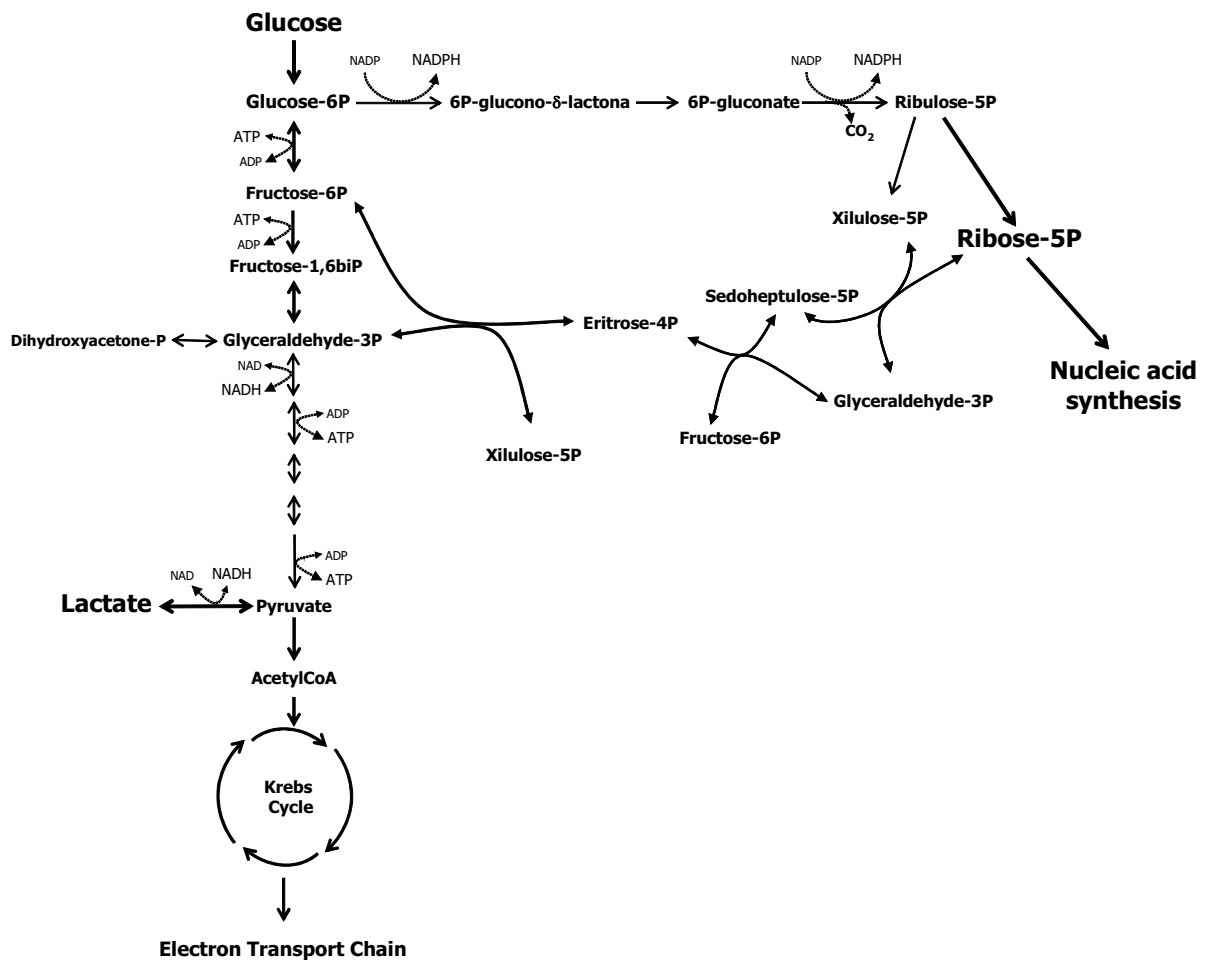
Figure 6. *Glucose metabolism*

1.7.1. Glucose metabolism of tumoral cells

The maximal proliferative capacity of a cell is limited by the activities and abundances of the different rate-limiting enzymes of the essential purine and pyrimidine nucleotide and carbohydrate anabolic pathways. The biochemical schedule of transformed cells has been reprogrammed to support continued proliferation through a metabolic and enzymatic imbalance, which becomes more pronounced with progression to a more

malignant phenotype [Weber, 1983]. Such imbalance implies a shift in the anabolic direction, as manifested by increased activities of rate-limiting enzymes of the synthetic (anabolic) pathways, concomitant with decreased activities of the opposing enzymes of the degradation (catabolic) pathways. Consistent with this concept, an altered nucleotide metabolism has been observed in many cancers leading to an increase in the *de novo* synthesis of the nucleic acid precursor ribose-5-phosphate [Hatse et al., 1999]. Moreover, studies performed in our laboratory have demonstrated that the non-oxidative phase of the pentose phosphate pathway plays a significant role in that tumour cell nucleic acid synthesis [Boros et al., 1997; Cascante et al., 2000]. Other important metabolic pathway altered in tumour cells is glycolysis, which is highly increased as observed in numerous studies [Warburg, 1956; Gatenby, 1995; Mathupala et al., 2001]

1.8. ULTRAVIOLET RADIATION

The sun emits ultraviolet (UV) radiation which covers a small part of the electromagnetic spectrum from 400 to 100 nm. Even in the UV portion of the spectrum the biological effects of the radiation vary enormously with wavelength and for this reason the UV spectrum is further subdivided into three regions:

o UVA: 400- 315 nm
o UVB: 315-280 nm
o UVC: 280-100 nm

UV radiation is a very prominent environmental toxic agent. Both, UVB and UVA lights reach the terrestrial surface and human skin in significant doses. Thus, UVB wavelengths penetrate the epidermis and are nearly fully absorbed in the upper dermis, whereas UVA penetrates to the deeper dermis. However, UVC is absorbed by forming atmospheric ozone and is not present at the Earth's surface [Diffey, 2002; De Grujil, 2000].

Exposure to the solar ultraviolet spectrum that penetrates the Earth's surface (UVA and UVB) causes cellular damage within skin cells. Thus, UV radiation has a variety of adverse effects on human health including sunburn, basal cell and squamous cell carcinoma, melanoma, cataracts, photoaging of the skin, and immune suppression [Soter, 1990; Mukhtar et al., 1996; Krutmann et al., 1995]. Among all of them, sunburn reaction is the most common response of human skin to UV radiation. The term *Minimal Erythema Dose* (MED) is conventionally used for biological evaluation of an individual's skin response to UV radiation. Hence, after exposure of previously non-sun-exposed skin

to sunlight or artificial UV light, erythema is graded visually at 24 hours and the MED is the lowest dose which induces uniform erythema with distinct borders. Although this term is extensively used, Diffey and colleagues have proposed to reserve it solely for observational studies in humans and other animals and use other term to refer to erythemal effective radiant exposures from natural or artificial sources of UV radiation. This term is the *Standard Erythema Dose* (SED), which is equivalent to an erythemal effective radiant exposure of 100 J/cm^2 considering e.g. than the ambient diurnal exposure on a clear sky summer day in Europe is approximately 30-40 SED and than an exposure dose of 4 SED would be expected to produce moderate erythema on unacclimated white skin, but minimal or no erythema on previously exposed skin [CIE Standard, 1998; Diffey et al., 1997; Diffey, 2002].

1.8.1. Biological effects of UV radiation

Even though UVB penetrates in the skin not as much as UVA and its average intensity in sunlight is 100 times less than that UVA, it is far more effective in inducing biological effects in human and experimental animals [De Grujil, 2000].

Until recently, DNA damage has been regarded as the major mediator of UV-induced signal transduction in the target cells, mainly because genomic DNA represents the main cellular chromophore with absorption maximum in the UV region (200-290 nm) and thus a direct target for UVB and UVC radiations. Indeed, UV radiation induces a range of direct and indirect modifications to DNA in a wavelength-dependent manner: while UVA induces damage through intermediates such as sensitizer radicals and ROS, UVB and UVC are directly absorbed by DNA. Thus, the most common lesions are formation of 8-hydroxydeoxyguanosine (8-OHdG) and cyclobutane pyrimidine dimmers from UVA and UVB exposures respectively. This UV-induced DNA damage itself induces cellular UV responses leading to cell cycle arrest and repair of the damaged DNA or to cell death. However, evidence that has emerged over the last few years shows that UV radiation also affects a number of cytosolic and cell membrane structures, including the activation of a number of protein kinases, transcription factors and cell membrane receptors to induce a variety of specific biological effects [Griffiths et al., 1998; Assefa et al.,2005].

1.9. MITOGEN-ACTIVATED PROTEIN KINASES (MAPKS)

Mitogen-activated protein kinases (MAPKs) are a large family of serine/threonine kinases whose function and regulation have been conserved during evolution from unicellular organisms such as brewers' yeast to complex organisms including humans [Widmann et al., 1999]. MAPKs are activated by a dual phosphorylation on invariant threonine and tyrosine residues within a specific sequence of three amino acids Thr-X-Tyr (where X varies depending on the MAPK). Their upstream activators, designed as MAPK kinases (MAPKKs), are a highly conserved group of dual specifity threonine/tyrosine kinases which, in turn, are activated through phosphorylation on conserved serine and threonine residues by a somewhat more diverse group of MAPKK kinases (MAP3Ks). One out-come of having many different MAP3Ks is that they can be matched with specific MAPKK-MAPK cassettes, such that cells can respond to different stimuli with the activation of a specific MAPK pathway.

In multicellular organisms, there are three well-characterized subfamilies of MAPKs. These MAPKs include:

- o The extracellular signal-regulated kinases (ERK1 and ERK2).

- o The c-Jun N-terminal kinase/stress-activated protein kinases (JNK1, JNK2 and JNK3).

- o The four p38 kinases (p38α, p38β, p38γ, and p38δ, being p38α the most well characterized and expressed in most cell types).

The ERK subfamily of MAPKs is widely expressed and are involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells. They are activated most acutely by growth and differentiation factors in pathways mediated by receptor tyrosine kinases, heterotrimeric G protein-coupled receptors or cytokine receptors. On the other hand, JNK and p38 MAPKs are mainly implicated in cellular response to a variety of inflammatory or stress signals including cytokines, ionizing or UV radiation, chemotherapeutics, heat shock and hyperosmotic stress (Figure 7).
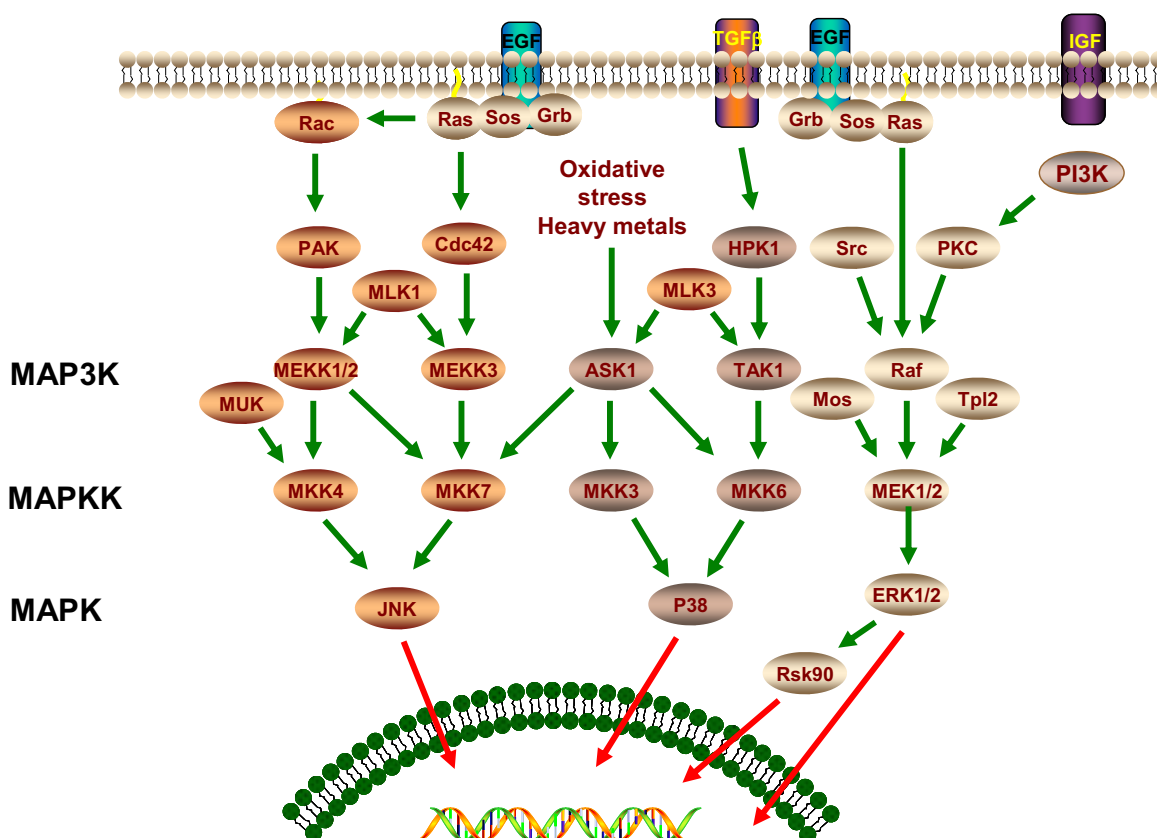
Figure 7. *Mitogen-activated protein kinase pathways*

The transcriptional consequences of the activation of a single MAPK cascade can vary often according to the biological context of the stimulus and the cell type. Moreover, even in the same type of cell, the physiological response associated with a MAPK signalling pathway appears to vary, indicating that mechanisms other than just the selective activation of a MAPK cascade play a role in the transduction of a specific response [Johnson et al., 2002; Lewis et al., 1998; Kyriakis et al., 2001].

1.9.1. UV-induced activation of MAPKs

Activation of MAPKs by UV radiation represents one of the early cellular responses and displays strict dependence on dose, time and wavelength [Assefa et al., 1997; Kabuyama et al., 2001]. UVA, UVB and UVC radiations induce the different MAPKs in various cell types and to a significantly different extent, mainly because of the divergence in their primary targets. Even the same wavelength of UV radiation could have a variable influences on the level of MAPK activation in different cell types or under different experimental conditions [Englaro et al., 1998; Shimizu et al., 1999; Tada et al., 2002].

Several potential mechanisms are involved in UV-induced activation of MAPKs and are mostly independent of nuclear DNA. A number of studies have confirmed that ROS, generated in the cytosol either as a result of the absorption of UV radiation by as yet unidentified endogenous photosensitizers in the presence of molecular oxygen [Foote et al., 1968] or from organelles such as mitochondria [Zamzami et al., 1995], significantly contribute to both UVA- and UVB-induced signal transduction in general and activation of MAPKs in particular. Thus, it has been observed that UVA-induced activation of signal transduction pathways, including the activation of MAPKs cascades and DNA damage, is mainly mediated by increased production of ROS [Cadet et al., 1997]. Additionally, accumulating evidence indicates that UVB radiation-induced activation of MAPKs signal transduction pathways in human keratinocytes also involves ROS to a significant extent, as evidence by the negative effect of antioxidants on this UVB-induced activation [Assefa et al., 1997; Peus et al., 1999].

1.10. PROTECTION AGAINST UV-INDUCED ADVERSE EFFECTS

In last years there has been an alarming increase in the incidence of sunlight-related skin disorders. For example, between 1960 and 1986, there was a 400% increase in melanoma in certain areas of the United States [Glass et al., 1989]. Changes in lifestyle have led to a significant augmentation in the amount of UV radiation that many people receive. Depletion of stratospheric ozone and the expanded use of suntainning devices for cosmetic purposes may contribute to that problem [Longstreth et al., 1998; Swerdlow et al., 1998]. Consequently, there has been a concerted effort to educate the public about how to protect against the harmful effects of overexposure to UV radiation, in addition to a greatly increase in the study of potential chemopreventive agents with capacity to protect against sunlight-related skin disorders. Among the agents that have been identified as having potential chemopreventive activities in humans are antioxidants, which are used as diet supplements and/or on topical application. Thus, several studies have reported the protective effects of antioxidants as polyphenols against UV-induced skin cancer development [Lu et al., 2000; Elmets et al., 2001; Katiyar et al., 1995].

2. OBJECTIVES

This study is part of a project supported by the Ministerio de Ciencia y Tecnología, into the programme Plan Nacional I+D+D, named "Extraction and purification of bioactive polyphenols obtained from wine by-products, potentially useful as food or demoprotective antioxidants". The main objective of this project is to purify and characterize different polyphenolic fractions, obtained from the wine by-product resulting from pressing destemmed Parellada white grapes (*Vitis vinifera*), with the aim of using them as potential anticarcinogens in food and dermatology industries.

The general aim of this work is to find compounds or mixtures with application as cancer chemopreventive agents. To this end the specific goals of this work are:

o   Evaluate and compare the possible antitumoral properties of polyphenolic fractions by analysing their effect on cancer cells at cellular and metabolic levels.

o   In the particular case of skin cancer: evaluate and compare the possible capacity of polyphenolic fractions to protect against cellular damage induced by ultraviolet radiation.

3. MATERIALS AND METHODS

3.1. <u>CELL CULTURE</u>

3.1.1. CELL LINES

The cell lines used in this study were the following:

- o Hepa-1c1c7: adherent cells derived from a mouse hepatoma and obtained from the European Collection of Cell Cultures (ECACC) [Health Protection Agency, Wiltshire (UK)].

- o SK-Mel-28 and A-375: adherent cells derived from human melanomas purchased by the American Type Culture Collection (ATCC) [LGC Promochem, Barcelona (Spain)].

- o Jurkat: non-adherent human leukemia cells which were delivered by the ATCC.

- o HaCaT: non-tumoral adherent cell line of human keratinocytes which were a gift from Dr. Lluis Ruiz [Advancell, Barcelona (Spain)].

Adherent cells Hepa-1c1c7, SK-Mel-28 and A-375 were routinely cultured in Dulbecco's modified Eagle medium (DMEM) containing glucose (25 mM) and L-glutamine (4mM) [Sigma, St. Louis, MO (USA)] and supplemented with 10% heat-inactivated fetal calf serum (FCS) [PAA Laboratories GmbH, Pasching, (Austria)] and 0.1% antibiotic (10,000 U/ml penicillin, 100 μg/ml streptomycin) [Gibco-BRL, Eggenstein (Germany)].

Non-adherent leukemia Jurkat cells were usually seeded in RPMI 1640 medium containing glucose 10 mM [Sigma] and supplemented with 10% FCS, L-glutamine 4mM and antibiotic at the same concentrations indicated for the adherent cells. However, the metabolic profile studies of Jurkat cells were carried out using a RPMI 1640 medium without glucose [Biological Industries, Kibbutz Beit Haemet (Israel)] supplemented with FCS, L-glutamine and antibiotic as indicated above and 10 mM of glucose (50% as D-glucose and the other 50% as $[1,2-^{13}C_2]$ D-glucose, with >99% purity and 99% isotope enrichment for each position [Isotec, Inc., Miamisburg, OH]).

Adherent human keratinocytes HaCaT were cultured in Dulbecco's modified Eagle medium (DMEM) containing glucose 25 mM [Cambrex Bioscience, Verviers, (Belgium)] and supplemented with L-glutamine 2mM, Hepes 10mM and 0.2% antibiotic.

All of these cell lines were free of mycoplasm infection as shown by the EZ-PCR mycoplasm test kit [Biological Industries] and were maintained at 37 ºC in a humidified atmosphere with 5% CO_2.

3.1.2. PRIMARY CULTURE

o Peripheral blood lymphocytes (PBLs): lymphocytes isolated from buffy coat cells of fresh heparinized blood of healthy donors, which were a generous gift from the blood bank at the Clinic Hospital, [Barcelona (Spain)].

Isolation of peripheral blood mononuclear cells was carried out through the Ficoll gradient method [Boyum, 1968] and further purification of lymphocytes from them was performed by removal of the adherent mononuclear cells by 2-3 hours of cell suspension incubation at 37ºC in a humidified atmosphere with 5% CO_2. Isolated PBLs were immediately used for the experiment, being cultured under the same conditions as leukemia Jurkat cells, or frozen until use as explained later in section **3.1.4.**

3.1.3. CELL SUBCULTURE

3.1.3.1. Adherent cells

For all the adherent cells, a maximal of 10 sub-cultures were performed. Thus, nearly confluent cells were sub-cultured by removing the culture medium and washing 1-2 times with PBS. Immediately after, cells were detached by incubating with trypsin-EDTA solution C (0.05% trypsin −0.02% EDTA) [Biological Industries] for Hepa-1c1c7, A-375 and SKMel-28 cells and trypsin-EDTA solution (0.25% Trypsin, 1mM EDTA) [GibcoBRL] for HaCaT cells at 37ºC in a humidified incubator with 5% CO_2 for 3-5 minutes. Trypsin was inactivated by adding standard culture medium. Next, cells were collected by centrifugation and sub-cultured or seeded to perform an experiment.

3.1.3.2. Non-adherent cells

o Leukemia Jurkat cells: since they grew in suspension it was not necessary to detach them. Thus, these cells were subcultivated by diluting in culture medium depending on requirements and maintained in cultured for a maximal of 2 months.

o PBLs: as they are a primary culture, it was not necessary to subcultivate them.

3.1.4. CELL PRESERVATION AND DEFROST

- o Cell lines Hepa-1c1c7, A-375, SK-Mel-28 and leukemia Jurkat cells were frozen in standard culture medium with 10% DMSO.

- o Keratinocytes HaCaT were frozen in serum with 10% DMSO.

- o Isolated PBLs were immediately used for experiment or frozen in serum with 10% DMSO and 10% PBS or culture medium.

All of the cell lines and primary PBLs were frozen at -80 ºC by using a cryo 1ºC freezing container previous to their preservation at -196ºC in liquid N_2. To quickly defrost them, criotubes were immersed in a water bath at 37ºC. Afterwards, all the cell lines were diluted in 10-20 ml of culture medium and incubated at 37ºC in a humidified incubator with 5% CO2, except for the primary PBLs that were previously washed twice in 40-50 ml of 20% FCS solution in PBS, and once in 40-50 ml of culture medium.

3.2. <u>NATURAL PRODUCTS</u>

The natural products analysed in this study included several polyphenolic fractions which differ in polyphenolic composition and were obtained at the Institute for Chemical and Environmental Research-CSIC by extraction and fractionation of the by-product resulting from pressing de-stemmed Parellada white grapes (*Vitis vinifera*).

3.2.1. EXTRACTION AND FRACTIONATION

Briefly, the crude fraction **OW**, soluble in both ethyl acetate and water, was obtained from the grape by-product as described by Torres et al. [Torres et al., 2001]. Moreover, the fractions **I** and **II** were generated by preparative RP-HPLC chromatography performed on a Waters [Milford, (USA)] Prep LC 4000 pumping system equipped with a PrepPack 1000 module fitted with a PrepPack cartridge (300 x 47 mm i.d.) filled with VYDAC [The Separations Group, Hesperia, (USA)] C_{18}, 300Å pore size, 15-20 µm particle size stationary phase. The lyophilised crude fraction **OW** (2 g) was loaded and the cartridge washed with 0.1% (v/v) aqueous trifluoroacetic acid at a flow rate of 100 ml/min. Then, the fractions were eluted with mixtures of CH_3CN in the starting buffer: fraction **I** with 12% CH_3CN (2 l) and fraction **II** with 24% CH_3CN (1.6 l). Afterwards, part of the fractions **OW**, **I** and **II** were separately fractionated using Toyopearl HW-40 F

stationary phase by two steps, MeOH (250 ml) and water/acetone (2:3) (250 ml) at flow rates of 12 ml/min and 3 ml/min respectively, following elution protocols described in the literature [Sun, 1999; Labarbe et al., 1999]. Thus, the fractions **III** and **IV** were obtained from the fraction **OW**, **V** and **VI** from the fraction **I** and **VII** and **VIII** from the fraction **II**. Alternatively, the fraction **OW** was more extensively fractionated with MeOH and water/acetone (1:1) to generate the fractions **IX**, **X** and **XI** [Torres et al., 2002]. The fractions were analysed by RP-HPLC on a Smart System [Amersham Biosciences, Uppsala, (Sweden)]. The following scheme resumes this process and indicates the qualitative polyphenolic composition of each fraction:
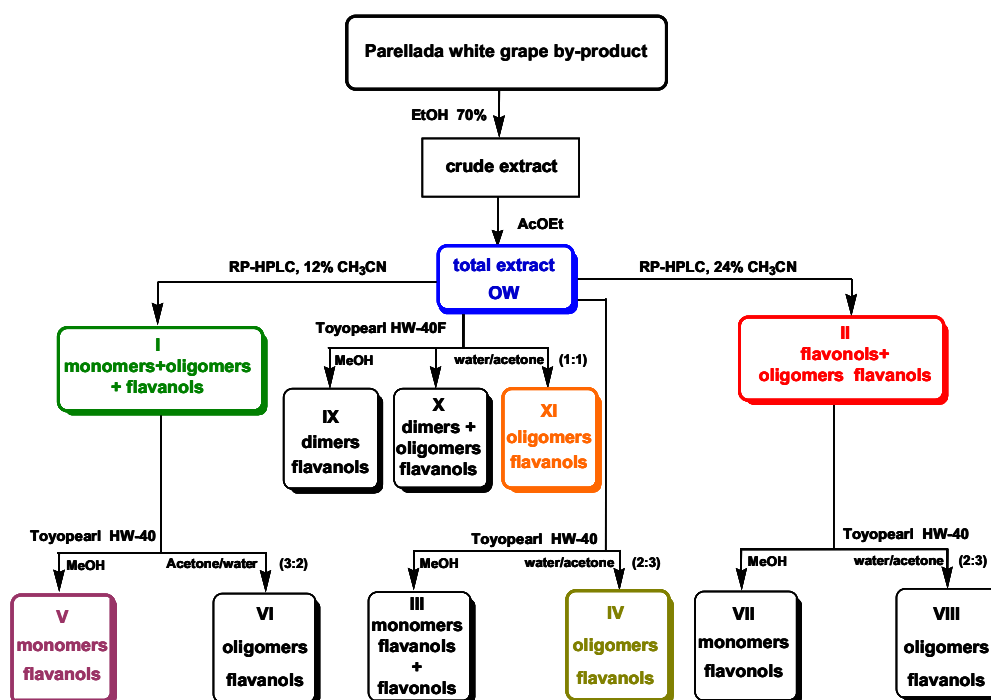
Scheme 1. *Extraction and fractionation of polyphenols from Parellada white grape by-product and composition of the obtained fractions.*

The polyphenolic fractions generated were lyophilised to give slightly coloured fluffy solids and stored at -20ºC in silica gel.

3.2.2. CHEMICAL CHARACTERIZATION AND ANTIRADICAL POWER OF THE POLYPHENOLIC FRACTIONS

The characterization was performed at the Institute for Chemical and Environmental Research-CSIC. Thus, the mean degree of polymerization (mDP), mean molecular weight

(mMW) and galloylation (molar percentage of galloyl ester containing species) of the procyanidin oligomers within the fractions were estimated by thiolysis and HPLC essentially as described [Rigaud, 1991; Prieur et al., 1994] except for cysteamine was used instead of toluene-α-thiol [Torres et al, 2002]. Moreover, the free radical scavenging potency of the crude and the fractions was evaluated by the DPPH radical method as described [Blois, 1958; Brandwilliams et al., 1995; Torres et al., 2001] and expressed as ED_{50} (amount of sample able to scavenge half the amount of the initial radical) (Table 1).

Fraction	mDP	mMW	galloylation (%)	ARP (1/ED_{50}) x 10^3
OW	1.7	552	15	19
I	1.4	422	7	26
II	3.0	1005	31	22
III	1.0	----	<1	10
IV	2.7	880	25	27
V	1.0	290	<1	19
VI	2.4	751	16	24
VII	1.0	----	0	7
VIII	3.4	1160	34	27
IX	2.0	624	12	23
X	2.2	759	30	30
XI	3.7	1232	31	28

Table 1. *Size, composition and antiradical power of polyphenolic fractions from Parellada white grape by-product*

The polyphenolic fractions analysed in this study were the total fraction **OW** and the derived fractions **I**, **II**, **IV**, **V** and **XI** which differ in polyphenolic composition and percentage of galloylation but not so much in antiradical power as it is shown above.

Stock dilutions of these fractions were prepared in PBS at a final concentration of 5 mg/ml, sterilized by filtration through 0.2 µm pore size filters, aliquoted and immediately used for studies of protection or stored at -20ºC for no more than 1 month for studies at cellular or metabolic levels.

3.3.   ANTITUMORAL   EFFECT   OF   THE   POLYPHENOLIC FRACTIONS OW, I AND II AT CELLULAR AND METABOLIC LEVELS

The antitumoral effect of polyphenolic fractions at cellular and metabolic levels was evaluated on several tumour cell lines and a primary culture of lymphocytes. The fractions analysed in these studies were the total fraction **OW** and the derived fractions **I** and **II**, which differ in percentage of galloylation and polyphenolic composition(Table 1 and Scheme 1):

o Fraction **OW** composed of gallic acid (GA); flavanol monomers such as (+)-catechin (Cat), (−)-epicatechin (EC); glycosylated flavonols (F) and procyanidin oligomers.

o Fraction **I** contained monomers and small procyanidin oligomers and a low content of gallate esters.

o Fraction **II** included flavonols and procyanidin oligomers of high molecular weight, having a high percentage of galloylation.

3.3.1. ANALYSIS OF CELL VIABILITY

To analyse the effect of the total polyphenolic fraction **OW** and the derived fractions **I** and **II** on the different tumoral cell lines (Hepa-1c1c7, A-375, SK-Mel-28 and Jurkat cells) and their possible cytotoxicity to primary PBLs, different assays of cell viability were carried out after 72 hours of treatment with the polyphenolic fractions, as follows:

o The method used for the tumoral cell lines was the MTT assay as described later in section ***3.3.1.1.1.*** This method derived from the colorimetric assay described by Mosmann in 1983. MTT is a pale yellow tetrazolium salt that is cleaved in active mitochondria of living cells, producing a dark blue formazan product when it accepts electrons from reductor agents or determined coenzymes (such as NADH and NADPH), which can be quantified by a colorimetric assay [Mosmann, 1983; Pauwels et al., 1988].

o The method used for primary PBLs consisted of counting cells in suspension in a Coulter Multisizer Accucomp which allows counting particles between 0.75 and 335 µm in diameter (PBLs are around 10-15 µm in diameter) [Alberts et al.,

1994] in combination with flow cytometric analysis of dead cells dyed with propidium iodide (PI).

3.3.1.1. Screening of adherent cells.

To establish the optimum number of adherent cells necessary to carry out reliable studies of cell viability, we previously performed a cell screening to test the range of cell number in which optical density obeyed Beer's law (where absorbance is directly proportional to the path length *l* through the medium and the concentration *c* of the absorbing species, being ε the constant of proportionality: $A= \varepsilon bc$) [Skoog et al., 1996].

3.3.1.1.1. Protocol

The screening of adherent tumoral cells was carried out by a variation of the colorimetric method described by Mosmann [Mosmann, 1983] and two different MTT treatments were tested as described below. Thus, after trypsinization and centrifugation at 500 x *g* for 5 minutes at room temperature, cells were counted using a Neubauer chamber. Once this number was known, a 10^5 cells/ml dilution was prepared. Starting from this solution and according to the Table 2, samples containing a 200 µl cell suspension at different cell concentrations (from 500 to 20000 cells) were plated in 96-well flat-bottomed microtitre plates:

Cell number	Vol. dil. 10^5 cells/ml (µl)	Vol. culture medium (µl)	Total vol. (µl)
500	5	195	200
1000	10	190	200
1500	15	185	200
2000	20	180	200
3000	30	170	200
4000	40	160	200
5000	50	150	200
6000	60	140	200
7000	70	130	200
8000	80	120	200
9000	90	110	200
10000	100	100	200
15000	150	50	200
20000	200	0	200

Table 2. *Culturing scheme of dilution from the initial solution 10^5 cells/ml.*

To allow cell attachment, after 24 hours of incubation at 37ºC in a humidified incubator with 5% CO_2, medium was replaced by fresh one and incubated 72 additional hours. Afterwards, the medium was aspirated and MTT, dissolved in PBS and sterilized by passing through a filter with pores of 0.2 μm, was added to the wells. In this way, two different MTT treatments were tested. In the first one, MTT was added at a final concentration of 0.2 mg/ml and incubated at 37ºC in a humidified incubator with 5% CO_2 for 4 hours. However, in the second one, final concentration of MTT was 0.5 mg/ml and incubation time 1 hour. Subsequently, MTT-medium solution was removed and 100 μl of DMSO solution was added to all wells of the two assays to dissolve the blue formazan crystals. The absorbance was determined at 550 nm in an ELISA plate reader (Merck ELISA System MIOS version 3.2.) and related to the cell number. Ideal absorbance was considered between 1 and 1.5.

3.3.1.2. Cell viability

The effect of the total polyphenolic fraction **OW** and the derived fractions **I** and **II** on the cell viability was studied after 72 hours of treatment. To determine the respective IC_{50} values (concentration of fraction that causes 50% inhibition of cell growth) for each polyphenolic fraction and type of cell, different fraction dilutions, prepared from a 5 mg/ml stock in PBS, were used.

3.3.1.2.1. Adherent tumoral cells

As explained above for the cell screening assay, after trypsinization and centrifugation, cells were counted and seeded in 96-well flat-bottomed microtitre plates, considering the necessary number of cells previously determined in the cell screening. The number of cells per well was 1,200 for A-375 and 4,000 for both SK-Mel-28 and Hepa-1c1c7. The final volume was 200 μl per well for all the cell lines. After 24 hours, the culture medium was replaced by fresh medium in the control wells and fresh medium with the polyphenolic fractions in the rest of the wells. Thus, different polyphenolic fraction dilutions on a scale of 1 μg/ml to 200 μg/ml (depending on the cell line), prepared from the 5 mg/ml stock, were added to wells, up to a final volume of 200 μl per well. 72 hours after adding the fraction, the medium was aspirated and the MTT solution was added to all the wells to a final concentration of 0.5 mg/ml. After 1 hour of incubation at 37ºC in a humidified incubator with 5% CO_2, solution was aspirated again and 100 μl DMSO was added to all the wells. Immediately after, the optical density was measured on an ELISA plate reader at 550 nm. Absorbance was proportional to the

number of cells. Results were expressed as a percentage of untreated control cell viability [Matito et al., 2003].

3.3.1.2.2. Non-adherent tumoral cells.

In the case of leukemia Jurkat cells, the same assay described for adherent tumoral cells was carried out by culturing 20,000 cells/well in 96-well flat-bottomed microtitre plates, as previously performed at our laboratory [Comín-Anduix, 2002]. The experiment was essentially as described for adherent cells, except for the following changes:

- Polyphenolic fractions were added the same day as cells were cultured, since these are cells than do not need to attach to the plates, and the final volume was 180 µl in each well.

- After 72 hours of incubation, the MTT solution was added to all the wells at a final concentration of 0.75 mg/ml without previous aspiration of the medium. Once the cells were incubated for 1 hour under the same conditions as adherent cells, plates were centrifuged at 500 x *g* for 5 minutes at room temperature. MTT solution plus medium were carefully aspirated with a hypodermic syringe to avoid loosing cells, prior to the addition of DMSO.

3.3.1.2.3. Primary peripheral blood lymphocytes

As mentioned before, the potential cytotoxicity of the total polyphenolic fraction **OW** and the derived fractions **I** and **II** was studied by a method different from the used for tumoral cell lines.

First of all, PBLs were defrosted as explained in section **3.1.4.** Secondly, cells were counted in a Multisizer Coulter [Beckman Coulter, Fullerton, CA, (USA)]. After counting, 5 x 10^5 cells/well were cultivated in 12 well flat-bottomed microtitre plates and polyphenolic fractions were added to all the wells, except for the controls, at final concentrations between 5µg/ml and 1000 µg/ml, prepared from the 5mg/ml stock, in a final volume of 2 ml. After 72 hours, cells were counted again on the Coulter. Since this system does not distinguish between alive and dead cells, PI was added to 1 ml of the samples at a final concentration of 20 µg/ml and analysed by FACS (Flourescence-actived Cell Sorter) at 488 nm in an Epics XL flow cytometer. Thus, the number of dead cells calculated through this analysis was subtracted from the number of cells obtained by counting in the Coulter Multisizer, to estimate the real live cell number. The results were expressed as a percentage of untreated control cell proliferation.

3.3.2. CELL CYCLE ANALYSIS

Cell cycle status was assessed by flow cytometry, which measures the number of cells in one of the three stages of the cell cycle [Lodish et al., 1995]. The analysis was carried out 72 hours after treatment with the total polyphenolic fraction **OW** or the derived fractions **I** and **II** at concentrations equal to their respective IC_{50} values and at a higher dose (IC_{80} or concentration causing 80% of cell growth inhibition) in the different tumoral cell lines (Hepa-1c1c7, A-375, SK-Mel-28 and Jurkat cells).

3.3.2.1. Adherent cells

Human melanomas A-375 and SK-Mel-28 and mouse hepatoma Hepa-1c1c7 were seeded in 6-well flat-bottomed microtitre plates containing 2 ml of cell suspension in culture medium. Because all these cells were attached to the plates, the correct number of cells to seed was determined by the relationship number of cells/area, considering the number of cells that were cultured in 96-well plates. Therefore, the quotient between areas ($A_{6 \text{ wells}}/A_{96 \text{ wells}}$= 9.03/0.31=29.1) is the factor by which the number of cells seeded in 96-well flat-bottomed microtitre plates must be multiplied in order to be cultured in 6-well plates. Hence, 116,400 cells were seeded for SK-Mel-28 and Hepa-1c1c7, whereas 35,000 cells were cultured for A-375. First, plates were incubated 24 hours at 37ºC in a humidified incubator with 5% CO_2. Second, the medium was removed and fresh medium with polyphenolic fraction was added at their respective IC_{50} values and at a higher dose (IC_{80}) and incubated for 72 hours. Finally, the cells were trypsinized and collected by centrifugation at 500 x *g* at room temperature. The supernatant solution was aspirated and the pellet was resuspended in 1 ml of TBS buffer. Immediately after, samples were transferred to eppendorf tubes and 50 µl of 1mg/ml PI stock solution plus 1 ml of Vindelov/PI solution was added (final concentration of PI 50 µg/ml). Samples were kept at 4ºC for an optimum time of 1 hour. After this time, FACS analysis was performed at 488 nm in an Epics XL flow cytometer and the percentage of cell cycle distribution was calculated using Multicycle program [Matito et al., 2003].

3.3.2.2. Non-adherent cells.

Jurkat cells were cultured in 6-well flat-bottomed microtitre plates containing 2 ml of cell suspension with the polyphenolic fractions at their respective IC_{50} values and at a higher dose (IC_{80}). Since these cells do not attach to the plates, the number of cells to

seed in 6-well plates was 10^5 cells/ml, taken from the data on 96-well plates (20,000 cells/200µl). After 72 hours in a humidified incubator at 37ºC with 5% CO_2, samples were placed in eppendorf tubes and centrifuged at 500 x *g* at room temperature. The rest of the process was performed under the same conditions as indicated above for the adherent cells.

3.3.3. APOPTOSIS

To examine the process that triggered cell death in the tumoral cell lines treated with the total polyphenolic fraction **OW** or the derived fractions **I** and **II**, detection of a common type of programmed cell like apoptosis was carried out. Additionally, the possible mechanism responsible for this programmed cell death was investigated.

3.3.3.1. Apoptosis detection

Like the cell cycle analysis, detection of apoptosis was carried out 72 hours after treatment with the same three polyphenolic fractions (total fraction **OW** or derived fractions **I** and **II**) in the same tumoral cell lines (Hepa-1c1c7, A-375, SK-Mel-28 and Jurkat cells). Detection was performed at concentrations equal to their respective IC_{50} and IC_{80} values, using an annexin V binding assay [Annexin V/FITC Kit; Bender MedSystems, Vienna, (Austria)] combined with PI labelling and analyzing by FACS. Using this technique we could distinguish between cells in early apoptosis (annexin V positive and PI negative) and cells in late apoptosis/necrosis (annexin V and PI positive) [Fadok et al, 1992; Matito et al., 2003].

3.3.3.1.1. Adherent cells

Human melanomas A-375 and SK-Mel-28 and mouse hepatoma Hepa-1c1c7 were cultured, treated with the different polyphenolic fractions, trypsinized and centrifuged following the same instructions described in section **3.3.2.1.** After centrifugation, the supernatant solution was eliminated and the pellet was resuspended in 500 µl of binding buffer. Then, the cells were counted using a Neubauer chamber. A volume corresponding to 0.8 million cells was separated in each case and the eppendorf tubes refilled up to 500 µl with binding buffer. Samples were centrifuged again in the same conditions, the supernatant solution was removed and the pellet was resuspended with 95 µl of binding buffer. Then, 3 µl of Annexin V-FITC was added and incubated for an optimum period of 30 minutes at room temperature in the dark. After this time, 800 µl of binding buffer was

added to each sample in addition to 20 µl of 1 mg/ml PI stock solution 1 minute before the analysis, which was performed at 488 nm by FACS analysis in an Epics XL flow cytometer.

3.3.3.1.2. Non-adherent cells

Leukemia Jurkat cells were cultured and treated with the different polyphenolic fractions following the same instructions described in section **3.3.2.2.** After centrifugation at 500 x *g* in a Jouan 3.11 centrifuge for 5 minutes at room temperature, the process was as described above in section **3.3.3.1.1.**

3.3.3.2. Analysis of caspase activation and release of cytochrome *c* in leukemia Jurkat cells

To elucidate the mechanisms of apoptosis induction in leukemia Jurkat cells, the most affected tumoral cell line, we investigated the effect of the total polyphenolic fraction **OW** and the derived fractions **I** or **II** on initiator caspases-9 and -8 and effecter capase-3, as well as the release of cytochrome *c*. Thus, 10^6 Jurkat cells were cultured in T25 culture flasks in 10 ml of medium (10^5 cells/ml as for the rest of the studies) with the corresponding polyphenolic fractions at their respective IC_{50} values. Furthermore, Jurkat cells treated 3 hours with staurosporine at 0.5 µM were used as an internal positive control of caspase activation and presence of cytosolic cytochrome *c*.

3.3.3.2.1. Protein extraction for the detection of caspases

After 6 hours of treatment with the polyphenolic fractions in a humidified incubator at 37ºC with 5% CO_2, cells were collected by centrifugation at 1,000 x *g*, the cell pellet was washed once with cold PBS and resuspended with 100-200 µl of lysis buffer containing 20 mM Tris/acetate pH 7.5, 270 mM sucrose, 1 mM EDTA, 1 mM EGTA pH 8.8, 1% TRITON X-100 and protease inhibitors (1 mM ortovanadate, 1 mM sodium glycerophosphate, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 5 mM β-mercaptoethanol, 1 mM benzamidine, 34.8 µg/ml PMSF and 5 µg/ml leupeptine). Afterwards, the cells were sonicated and incubated for 20 minutes on ice. Finally, lysates were centrifuged for 10 minutes at 14,000 rpm in an Eppendorf microfuge at 4ºC and the pellets discarded.

3.3.3.2.2. Protein extraction for the detection of cytosolic cytochrome c

Culture, treatment and collection of Jurkat cells were performed as explained in the previous section except that protein extraction was carried out in a different way, since cytosolic extracts were necessary to analyze release of cytochrome *c* from the mitochondria. Thus, the extraction process was performed as follows: the cell pellet was washed once with lysis buffer containing 20 mM Hepes pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1mM EDTA, 1mM EGTA pH 8.8, 250 mM sucrose and protease inhibitors (1mM DTT, 0.1 mM PMSF, 1 µg/ml pepstatin A, 2 µg/ml leupeptin and 10 µg/ml aprotinin). After centrifugation at 1,000 x *g* at 4ºC, cells were suspended in 150 µl of lysis buffer, incubated 30 minutes on ice and plestled with 15 strokes using a homogenizer [Sigma]. Homogenates were centrifuged at 25,000 x *g* for 30 minutes at 4ºC. Supernatants were collected and further centrifuged under the same conditions to obtain the cytosolic fraction.

3.3.3.2.3. Determination of protein concentration.

To determine the protein concentration of both extracts (total extracts for analysis of caspases and cytosolic extracts for study of cytochrome *c* release) obtained from Jurkat cells treated with the polyphenolic fractions, the BCA assay protein kit [Pierce,Rockford, IL, (USA)] was performed. Firstly, a BSA (Bovine Serum Albumin) standard curve with dilutions from 125 to 2000 µg/ml was made in triplicate adding the volumes indicated Table 3. Second, 10 µl of each standard or sample was placed in a 96-well flat-bottomed microtitre plate and 200 µl of BCA protein kit (50 volumes of reactive A for each volume of reactive B) were added to all the wells, with milliQ water as blank. After 30 min of shaking incubation at 37 ºC, absorbance at 550 nm was measured on an ELISA plate reader [Sunrise Tecan, Barcelona, (Spain)]. Protein concentration was obtained by extrapolation in the standard curve.

Final concentration BSA (µg/ml)	Vol. Stock BSA (2 mg/ml) (µl)	Vol. MiliQ water (µl)
125	3.13	46.87
250	6.25	43.75
500	12.5	37.5
750	18.75	31.25
1000	25	25
1500	37.5	12.5
2000	50	0

Table 3. *Standard curve for the BCA protein assay*

3.3.3.2.4. Samples preparation.

Once the protein concentration was known, samples with identical amounts of protein were prepared in loading buffer containing 50 mM Tris pH 6.8, 2% (w/v) SDS, 10 mM DTT, 10 % (v/v) glycerol and 0.2% (w/v) bromophenol blue, and adding milliQ water to obtain equal volumes for all the samples. Depending on the antibody and the sample, the quantity of protein to analyze was different:

o   50 µg for caspase-9 detection.

o   20 µg for caspases-8 and -3 detection.

o   10 µg for cytochrome *c* detection.

o   10 µg for detection of any of the caspases in the internal positive control.

After preparing the samples, they were boiled for 5 minutes at 100ºC and centrifuged (short spinning at 14,000 rpm in an Eppendorf microfuge) for properly collection of the sample in the tube.

3.3.3.2.5. Electrophoresis

Protein samples were resolved by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) using 4% and 15%acrylamide stacking and resolving gels respectively. The amounts of reagents necessary to make one gel are detailed in Table 4:

	15 % Resolving Gel	4 % Stacking Gel
Solution 1	5 ml	----
Solution 2	5 ml	360 μl
Solution 3	------	1,5 ml
MiliQ water	-----	1,2 ml
TEMED	14 μl	7,5 μl
APS 10%	65 μl	30 μl
Total Volume	**10 ml**	**3 ml**

Solution 1: Tris-HCl 0.75 M, pH 8.8, 0.29% SDS

Solution 2: 30% Acrylamide/Bis solution

Solution 3: Tris-HCl 0.25 M, pH 6.8, 0.2% SDS

APS 10%: ammonium persulfate 10% (w/v)

Table 4. *Solutions composition and volumes necessary to prepare the resolving and the stacking gels.*

When the gels were polymerised using a Mini- PROTEAN 3 Electrophoresis Cell system [Bio-Rad Laboratories, CA, (USA)], protein samples were loaded by using a Hamilton syringe, including a molecular weight standard of low range [Biorad] prepared from the stock by diluting 1:20 in miliQ water with loading buffer. The bands of low molecular weight standards:
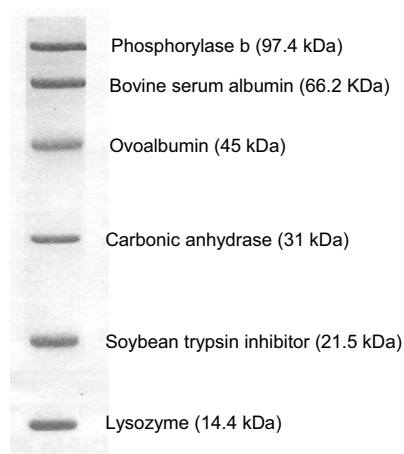
After loading, the electrophoresis was carried out at 35 mA of intensity per gel for about 45 min or until the loading dye reached the bottom of the resolving gel layer.

3.3.3.2.6. Electrotransference and blocking

Once the electrophoresis had been completed, the gel was removed and the stacking gel was eliminated. Resolving gel was used to transfer proteins to PVDF membranes [Biorad] by making a humid electrotransference with the Mini Trans-Blot Cell System [Biorad] at 60 V for 2 hours at 4ºC. When proteins had been transferred to the membranes, they were dried and stained with Ponceau S red staining solution [Sigma] to check equal amount of loaded protein for all the samples.

After Ponceau staining, membranes were washed with TBS buffer (132mM Na Cl; 20mM Tris pH 7.5) and blocked for 1 hour at room temperature in a solution of TBS-Tween (0.1%) (TBS-T) with 5% (w/v) BSA or dry milk, depending on the antibody:

o BSA for later incubation with anti-caspase-8 or anti-cytochrome *c*.

o Dry milk for later incubation with anti-caspase-9 or anti-cleaved caspase-3.

3.3.3.2.7. Antibodies incubation and reprobing

Blocked membranes were incubated with an appropriate dilution of the corresponding primary antibody in TBS-T and BSA or dry milk. Incubation dilution, time and temperature were as follows:

o Polyclonals anti-caspase-9 and anti-cleaved-caspase-3 [Cell Signalling, Beverly, MA (USA)] were used in TBS-T with 5% (w/v) dry milk at 1:1000 dilutions and incubated overnight at 4ºC.

o Polyclonal anti-caspase-8 and monoclonal cytochrome *c* [BD Biosciences /Pharminger Erembodegem, (Belgium)] were used in TBS-T with 5% (w/v) BSA at 1:3000 dilutions and incubated 2 hours at room temperature.

After incubation with primary antibody, membranes were washed in TBS-T three times for 5 minutes each, and incubated with the appropriate secondary antibody diluted in TBS-T and 2% (w/v) dry milk at 1:3000 dilution for 1hour at room temperature:

o HRP-conjugated goat anti-rabbit [Amersham Biosciences AB, Uppsala, (Sweden)] for polyclonal primary antibodies.

o HRP-conjugated rabbit anti-mouse [DAKO, Copenhagen, (Denmark)] for monoclonal primary antibodies.

After secondary antibody incubation, membranes were washed again three times for 5 minutes in TBS-T and once in TBS before protein detection.

To verify an identical protein load, after protein detection (explained in subsequent section **3.3.3.2.8**), membranes were stripped with Re-Blot Plus, strong antibody stripping solution [Chemicon, Hampshire, (UK)] by incubating with a dilution 1:10 of that solution in miliQ water for 20 minutes at room temperature, followed by two washing steps with the blocking solution. Afterwards, membranes were incubated with polyclonal anti-α-actin [Sigma] in TBS-T with 5% (w/v) BSA at 1:1000 dilution for 1 hour at room temperature, washed with TBS-T, incubated with the appropriate secondary antibody and washed again as explained above.

3.3.3.2.8. Protein detection

Caspases-8, -9 and -3, as well as cytochrome *c* and α-actin were visualized by enhanced chemiluminiscence using an ECL kit [Biological Industries] as substrate for the horseradish peroxidase and developing on film.

3.3.4. THE METABOLIC PROFILE OF LEUKEMIA JURKAT CELLS

To track and measure the substrate carbon flux in the pentose cycle and in glycolysis, as well as nucleic acid synthesis rate of leukemia Jurkat cells treated with the total polyphenolic fraction **OW** or the derived fractions **I** and **II**, the mass isotopomer distribution analysis (MIDA) approach was used. It consisted of the simultaneous measurement of substrate redistribution within and among major metabolic pathways of macromolecule synthesis and energy production under various physiologic conditions [Boros et al., 2002].

To perform the experiments, 3×10^6 Jurkat cells were seeded in T75 culture flasks in 30 ml of culture medium containing glucose 10 mM (50% as [1,2-$^{13}C_2$] D-glucose, verified previously to cell incubations) and the corresponding fraction. Since we were interested in the metabolic changes induced by the polyphenolic fractions at initial stages, where cell death was not as much as fifty per cent, the study was carried out at a concentration of 40 µg/ml (about half of their respective IC_{50} value after 72h of treatment). After 24h and 48h of treatment, the cells were centrifuged at 500 x *g* for 5 minutes at room temperature, the medium was separated and the pellet was washed with PBS. Then, both medium and pellet were stored at -20ºC for later metabolite isolations.

3.3.4.1. Metabolite isolations

3.3.4.1.1. Glucose isolation

To verify that 50% of glucose was as [1,2-$^{13}C_2$] D-glucose, 400 µl of the original medium were passed through a mixed DOWEX 50-DOWEX 1 ionic exchange column earlier prepared. Then, 6 ml of milliQ water were passed through the column and the volume was collected into a glass tube. Finally, the remaining liquid was eliminated by air pressure for about 12 hours and the dried sample was stored until determination.

3.3.4.1.2. RNA ribose isolation

RNA ribose was isolated by acid hydrolysis of cellular RNA from the pellet of Jurkat cells treated or not with the polyphenolic fractions as indicated above in section **3.3.4.** The procedure was carried out as follows:

First, 1 ml of Trizol was added to the cold pellets and they were dissolved by sonication (30 seconds). Second, the homogenates were transferred to free RNase eppendorfs filled with 200 µl of chloroform. After shaking, samples were centrifuged at 14,000 rpm for 30 minutes in an Eppendorf microfuge at 4ºC. The non-coloured upper phase, containing chloroform and RNA, was collected and added to 1 ml of cold 2-propanol. To precipitate the DNA, the mixture was placed to repose for 1-2 hours. Then, samples were centrifuged under the same conditions indicated above; supernatants were discarded and 1 ml of 75% cold ethanol was added to all of the pellets to further purify the RNA. Thus, after shaking, samples were centrifuged again and the supernatant was discarded. The pellets were re-suspended in 10 µl of miliQ water and passed to a glass tube with 2 ml of HCl 2N. Afterwards, an acid hydrolysis of cellular RNA was carried out by heating at 100 ºC for 2 hours followed by cooling at room temperature. Finally, tubes were stored at - 80ºC or immediately dried eliminating the remaining liquid by air pressure.

3.3.4.1.3. Lactate isolation

Lactate from the culture media of Jurkat cells, which had been cultured and treated as explained in section **3.3.4.**, was extracted by ethyl acetate. In more detail, 1ml of culture medium was acidified with 2-3 drops of HCl, followed by addition of 1ml of ethyl acetate plus a little amount of sodium chloride and generous shaken for 1 min. After 2 min of rest, samples were centrifuged at 4,000 rpm for 10 min in a Jouan B3.11

centrifuge. The upper organic phase was transferred to another tube and subjected to slight N_2 pressure about 10-15 min to eliminate the remaining ethyl acetate.

3.3.4.2. Isotopomers quantification in glucose, ribose and lactate

Isolated glucose, ribose and lactate from leukemia Jurkat cells treated with the polyphenolic fractions were sent to the Stable Isotope Research Laboratoty [Los Angeles Biomedical Research Institute (LABiomed)], where isotopomer distribution in each sample was quantified by Gas Chromatography/Mass Spectrometry analysis, according to standard published protocols [Lee et al., 1998].

3.3.4.3. Results analysis

Measurement of ^{13}C label distribution in the studied metabolites let us determine the different mass isotopomers m0 (without any ^{13}C labels), m1 (with one ^{13}C label), m2 (with two ^{13}C labels), etc, which are reported as molar fractions. Additionally, the total label incorporation was represented by SIGmn (Σm_n= 1*m1 + 2*m2+ 3*m3….).

3.3.4.3.1. Stable isotope incorporation into RNA ribose

Jurkat cell nucleic acid synthesis rates were measured by quantification of $[1,2-^{13}C_2]$ D-glucose incorporation into RNA ribose. The SIGmn value, which represents the molar enrichment of ^{13}C in ribose synthesized from $[1,2-^{13}C_2]$ D-glucose was calculated as 1*m1 + 2*m2 + 3*m3 + 4*m4.

3.3.4.3.2. Stable isotope incorporation into lactate

Substrate carbon flux in the pentose cycle and in glycolysis was measured by ^{13}C label distribution in lactate. The total label incorporation in lactate was represented by SIGmn (m1+ 2*m2+ 3*m3). The amount of lactate with two labels (m2) is directly originated from glucose by glycolisis, whereas lactate with one label (m1) originates from glucose metabolised by direct oxidation via the oxidative steps of the pentose phosphate pathway and then recycled to glycolysis via the non-oxidative pentose cycle as indicated in the following scheme:

m2
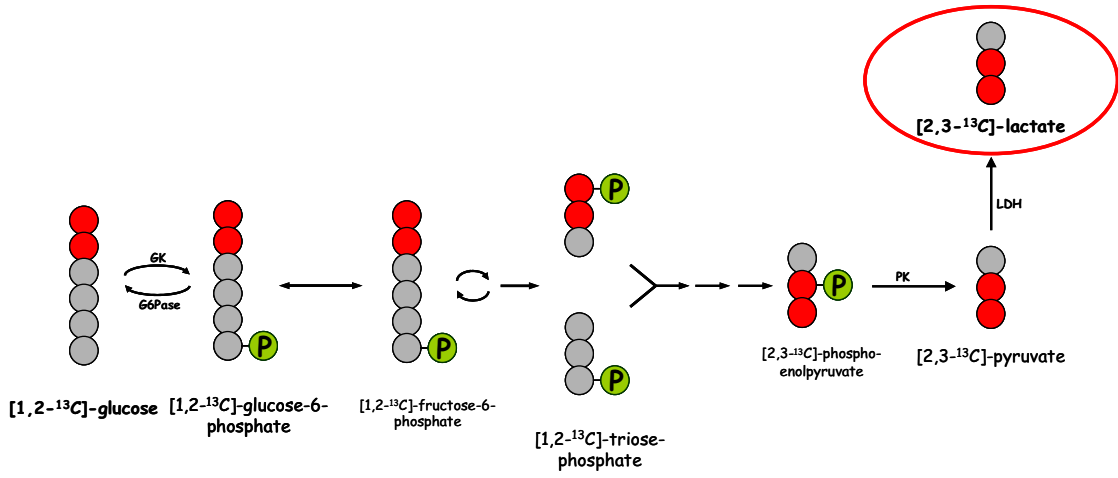
m1
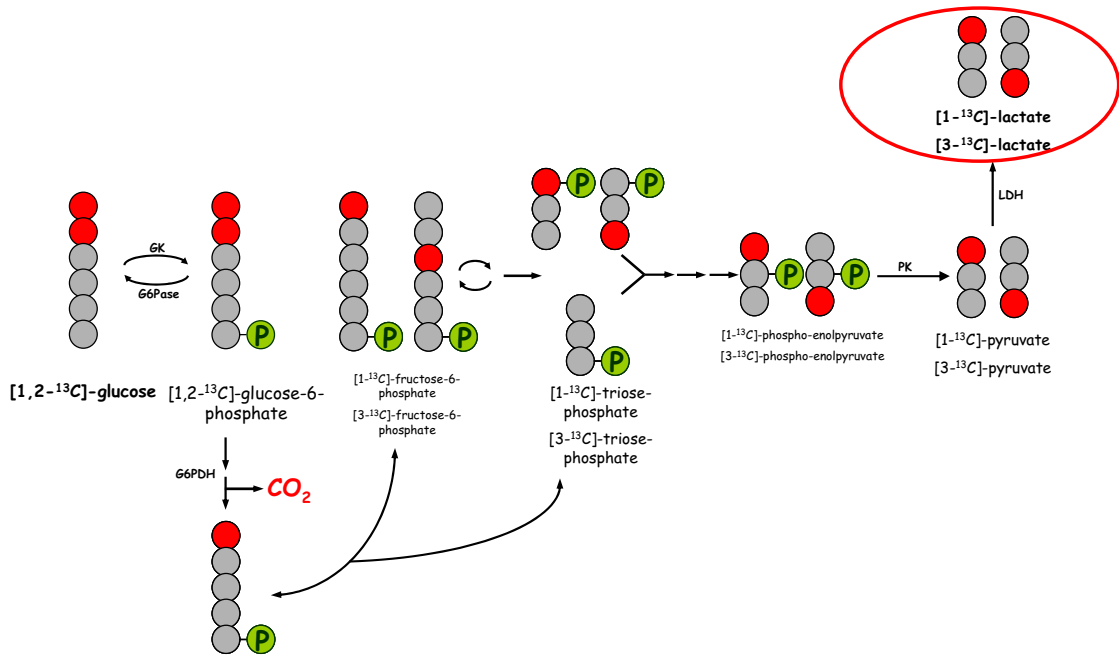
Scheme 2. *Formation of lactate m2 or m1 from [1,2-$^{13}C_2$] D-glucose.*

3.4. <u>PROTECTIVE EFFECT OF THE POLYPHENOLIC FRACTIONS OW, IV, V AND XI AGAINST UV DAMAGE</u>

The possible protective capacity of polyphenolic fractions against UV-induced cell damage was analysed in human keratinocytes HaCaT. The polyphenolic fractions tested were the total fraction **OW** and the derived fractions **IV**, **V** and **XI** (Scheme 1), which have different percentage of galloylation and polyphenolic composition (Table 1):

o Fraction **OW** contained GA, flavanol monomers such as Cat and EC, glycosylated flavonols and procyanidin oligomers.

o Fraction **IV** included procyanidin oligomers with a considerable galloylation percentage.

o Fraction **V** contained only flavanol monomers without gallate groups.

o Fraction **XI** with a similar composition to the fraction **IV** but including procyanidin oligomers of higher molecular weight and gallate content.

3.4.1. ANALYSIS OF DNA FRAGMENTATION IN HUMAN KERATINOCYTES HaCaT IRRADIATED WITH UVB LIGHT

To study if the total polyphenolic fraction **OW** protected against the DNA fragmentation caused by UVB radiation, the most effective radiation to cause DNA damage leading to the development of skin cancer [De Grujil, 2000], human keratinocytes HaCaT were pre-treated with different fraction concentrations, UVB radiated and post-incubated different periods of time.

3.4.1.1. Screening of human keratinocytes HaCaT

To determine the correct number of cells to be used later in the DNA fragmentation assay, cell screening of HaCaT keratinocytes was performed from 1,000 to 20,000 cells as described in section ***3.3.1.1.1***. except that once the cells were attached to the plates (24 hours after seeding), the medium was removed and cells were incubated only 24 hours with fresh one, previous to the MTT assay (1hour of incubation with MTT at a final concentration of 0.5 mg/ml).

3.4.1.2. Screening of the total fraction OW

Because the aim of this experiment was to study the possible protective effect of the total polyphenolic fraction **OW**, a screening of concentrations of that fraction was necessary since the optimal protective concentration must not alter viability of HaCaT cells. This assay was carried out as explained for the cell screening in the previous (section **3.4.1.1.**), except that in this case 10,000 cells were seeded per well (the optimum cell number determined in the cell screening) and cells were incubated 24 hours with the fraction **OW** at concentrations from 1 to 10 µg/ml.

3.4.1.3. Analysis of DNA fragmentation and possible protective effect of the total fraction OW

Once the correct cell number to seed in 96-well plates was determined, 2,000,000 cells were cultured in 100 cm^2 plates in 10 ml of medium [keeps the relationship of areas * number of cells in 96-well microtitre plates; $(A_{100}/A_{96\ wells})$*10,000 cells]. As explained for other assays, cells were allowed to attach to the plate by incubating for 24 hours. Then, cells were pre-treated with the total fraction **OW** at concentrations of 5 and 10 µg/ml for 24 hours and UVB radiated.

3.4.1.3.1. UVB radiation and post-incubation

After incubation with the fraction **OW**, plates without cover were placed in a BIO-SUN system [Vilber Lourmat, Torcy, (France)] and cells with an 80-100% confluence were UVB radiated (312 nm) at 0.3 J/cm^2. Since the radiation time for that dose lasted 5 minutes, it was necessary to perform the irradiations in PBS or culture medium. Thus, a number of different conditions were tested:

- o Cells washed once with PBS and irradiated in 2ml of fresh PBS.

- o Cells irradiated in 2ml of fresh culture medium.

- o Cells irradiated in 2ml of incubation culture medium with or without the polyphenolic fraction **OW**.

Once the radiation was completed, PBS or culture medium was removed and cells were post-incubated in fresh culture medium with or without the fraction **OW** for 12, 18 or 24 hours at 37ºC in a humidified incubator with 5% CO_2, testing two culture mediums:

o Standard culture medium supplemented as described in section **3.1.1.** for HaCaT cells.

o Medium supplemented as the standard except for concentration of FCS, which was 5% instead of 10%.

3.4.1.3.2. DNA extraction

Once post-incubations were completed, the culture medium was discarded and the cells attached to the plates were washed and scraped with 2 ml of PBS. Afterwards, cells were collected in an appropriate tube (eppendorfs for irradiated cells and 15 ml plastic tubes for controls) and extraction of their genomic DNA was performed by using a REALPURE kit [Durviz, Valencia (Spain)] as indicated in the commercial protocol (Table 5):

o Cell lysis:

Collected cells (about 10 millions for the controls and 3-5 millions for the irradiated cells) were precipitated at 500 x g for 3 minutes (controls) or 14,000 x g for 10 seconds (irradiated cells) at room temperature and the supernatant was eliminated, remaining in the tube 200-400 µl (controls) or 20-40 µl (irradiated cells) of residual liquid. After re-suspending the cells by gently shaking, they were lysed by pipetting in lysis solution and incubated 1 hour at 55 ºC with 100 µg/ml proteinase K.

o RNase treatment:

It was optional but we preferred to do so. Thus, RNase was added to the lysate, mixed by inverting and incubated 15-60 minutes at 37º C.

o Protein precipitation:

Protein precipitation solution was added and strongly shaken by vortex for 20-30 seconds, followed by centrifugation at 2500 x g for 10 min. (controls) or 14,000 x g for 5 min. (irradiated cells).

o DNA precipitation:

The supernatant containing DNA was recovered, placed in a tube with isopropanol and mixed by inversion, followed by 3 minutes of centrifugation at the conditions indicated for the protein precipitation. Afterwards, supernatant was eliminated and

DNA pellet was washed with 70% ethanol, followed by another centrifugation of 2 min. Finally, the supernatant was carefully removed and allowed to DNA drying for about 15-30 min.

o   DNA hydration:

Hydration solution was added and incubated overnight at room temperature for an appropriated DNA hydration. DNA samples were kept at 4ºC for a rapid use or at -20ºC for longer store.

	3-5 millions	10 millions
Lysis solution	0.6 ml	1.5 ml
Proteinase K	3 µl	7.5 µl
Rnase	3 µl	7.5 µl
Protein precipitation solution	0.36 ml	0.9 ml
Isopropanol	0.6 ml	1.5 ml
Hydration DNA solution	30-100 µl	50-150 µl

Table 5. *Reactive volumes necessary for DNA extraction from control and irradiated cells*

3.4.1.3.3. Purity and DNA dose

Verification of the purity of previously extracted DNA was performed by spectrophotometer measurement of absorbance at 260 and 280 nm. Thus, values between 1.8 and 2 for the relation A_{260}/A_{280} were indicative of uncontaminated DNA. Once it was confirmed, the amount of DNA present in the samples was calculated considering 1 unit of A_{260} is equal to 50 µg/ml of DNA:

DNA (µg/ml) = A_{260} value x 50 µg/ml x Dilution factor

(sample dilution for the spectrophotometer measure)

3.4.1.3.4. Determination of DNA fragmentation

The analysis of DNA fragmentation was performed as follows:
First, 1% agarose gels were prepared in fresh TBE 0.5X buffer (45 mM Tris, 45 mM borate and 10 mM EDTA pH 8), by heating 0.5 g agarose in a total volume of 50 ml. Once agarose had been completely dissolved, it was left some minutes to cool down and ethidium bromide was added at a final concentration of 0.4 µg/ml. Second, agarose was

allowed to solidify in ReadyAgarose Gel System [Bio-Rad] and the wells were loaded with equal volumes of samples containing 5-10 µg of the respective DNA, blue/orange loading dye [Promega, Madison, WI, (USA)] and miliQ water. Finally, electrophoresis was carried out at 80 V for about 45 minutes.

As standard DNA ladder, the 1Kb DNA ladder provided by Promega was used in that study. DNA was visualized under UV light and photographed [Vilber Lourmat].

3.4.2. DETERMINATION OF UV CYTOTOXICITY IN HUMAN QUIESCENT KERATINOCYTES HaCaT

Analysis of both UVA and UVB cytotoxicity and the possible protective effect of the total polyphenolic fraction **OW** and the derived fractions **IV, V** and **XI** were performed in human quiescent keratinocytes HaCaT using the MTT assay to determine cell viability.

3.4.2.1. Screening of human quiescent keratinocytes HaCaT

As explained for the other assays, the number of HaCaT cells to culture for UV and/or polyphenolic fractions cytotoxicity evaluation was determined by cell screening as explained in section ***3.3.1.1.1.***, except for the following changes:
- Range of cell screening was shorter, from 7000 to 12500 cells.
- After allowing attachment of the cells by 24 hours of incubation in standard culture medium, it was changed by medium without serum and incubated 48 hours to obtained quiescent cells with low levels of active MAPKs.
- Serum-free medium of quiescent HaCaT cells was replaced by fresh one and incubated for 6 additional hours (fractions incubation time for posterior MAPKs assays) or 6 plus 24 hours (fractions incubation plus post-incubation time for later UV cytotoxicity evaluation assays) previous to the MTT assay (1 hour of incubation with MTT at a final concentration of 0.5 mg/ml).

3.4.2.2. Screening of polyphenolic fractions

As explained in section **3.4.1.2.**, and to estimate the fraction concentrations not affecting cell viability, experiments driven to determine the possible protective effect of the polyphenolic fractions in study (total fraction **OW** and derived fractions **IV, V** and **XI**) require a previous screening of fractions concentration. In that case, once the optimum culture number of quiescent HaCaT cells was decided as 7,000, the concentrations tested

for all the fractions were 5, 10 and 20 µg/ml, being the pre-treatment of 6 hours followed by 24 hours of post-incubation in standard serum-free medium without polyphenolic fractions. Like for the rest of screenings, 1hour of incubation with MTT at a final concentration of 0.5 mg/ml was performed to find out the number of living cells.

3.4.2.3. Determination of UV-induced cell death

Cytoxicity of UV radiation was evaluated by seeding 7,000 HaCaT cells in 96-well flat-bottomed microtitre plates (number of cells determine in cell screening as the correct number to get 80-100% confluence prior to irradiation process). As explained above for the cell screening (section **3.4.2.1.**) cells were allowed to attach and deprived of serum for 48 hours, followed by 6 hours of incubation with fresh serum-free medium previous to UV radiation.

3.4.2.3.1. UVB radiation

To irradiate cells with UVB light, they were washed with PBS; plates without cover were placed in the BIO-SUN system and irradiated at 312 nm and doses of 0.03 or 0.05 J/cm^2 (less than 1 minute of irradiation). Because of the short exposure time to UV, cells were usually irradiated without PBS, although similar results were obtained when irradiated in PBS. Furthermore, parallel to irradiation, control non irradiated cells were treated in the same way.

3.4.2.3.2. UVA radiation

Like for UVB, cells were washed with PBS but in this case 50 µl of PBS was added to all the wells before irradiation in the BIO-SUN system at 365 nm and doses of 10, 20 or 30 J/cm^2, since irradiation times were about 30 min, 1 or 2 hours respectively. Like for UVB radiation, control non irradiated cells were in PBS for the corresponding time.

3.4.2.3.3. Post-incubation and analysis of cell viability

Once the irradiation was completed, 200 µl of fresh serum-free medium was added to all the wells and the cells were post-incubated 24 hours at 37ºC in a humidified incubator with 5% CO_2. Afterwards, MTT assay was carried out as explained for the screening (section **3.4.2.1.**) and number of living cells determined by measuring absorbance on an

ELISA plate reader and expressing results as a percentage of untreated control cell viability.

3.4.2.4. Protection of polyphenolic fractions

To test a possible protective effect of the total fraction **OW** or the derived fractions **IV, V** and **XI** against the cytotoxicity caused by UV radiation, experiments performed in a similar way than those explained in section **3.4.2.3.** were carried out by treating HaCaT cells for 6 hours with the polyphenolic fractions at concentrations previously proved as innocuous for these cells (5, 10 and 20 µg/ml for the fractions **OW** and **V**, and 5 µg/ml for the fractions **IV** and **XI**), followed by irradiations of 0.05 J/cm^2 for UVB or 20 J/cm^2 for UVA, and post-incubations of 24 hours.

3.4.3. ANALYSIS OF MAPKs ACTIVATION INDUCED BY UV RADIATION IN HUMAN QUIESCENT KERATINOCYTES HaCaT

UV-induced activation of mitogen-activated protein kinases (ERK1/2, p38 and JNK1/2) and the possible protective effect of the total fraction **OW** and the derived fractions **IV, V** and **XI** were analysed. It was carried out by western blot detection of phospho-active forms of MAPKs in human quiescent keratinocytes HaCaT irradiated with UVB or UVA lights. Furthermore, analysis of total MAPKs was performed in order to verify the total amount of protein (phospho- plus non-phospho forms) remain identical.

3.4.3.1. Culture and pre-treatment of human keratinocytes HaCaT

Human keratinocytes HaCaT were cultured in fully supplemented culture medium considering the results obtained for the cell screening of quiescent cells (explained in section **3.4.2.1.**). Thus, 500,000 cells were seeded in 60 cm^2 plates in a total volume of 5 ml (keep the relationship of areas * number of cells in 96-well microtitre plates; $(A_{60}/A_{96\ wells})$ * 7,000 cells).

Once the cells were attached to the plates, after 24 hours of incubation at 37ºC in a humidified incubator with 5% CO_2, medium was changed by a standard one without serum and incubated for 48 hours to obtained quiescent cells with low levels of activated MAPKs. Afterwards, the medium was replaced by fresh serum-free medium with or without polyphenolic fractions at concentrations of 5, 10 and 20 µg/ml for the total fraction **OW** and 5 µg/ml for the derived fractions **V**, **IV** and **XI** (optimum concentrations

determined by the screening of polyphenolic fractions explained in section **3.4.2.3.**), and incubated 6 hours more prior to UV radiation.

3.4.3.2. Irradiation and post-incubation of human quiescent keratinocytes HaCaT

When the 6 hours pre-treatments were concluded, the cells were washed with PBS and irradiated at the same conditions used for the study of protection against UV cytotoxicity as indicated in sections **3.4.2.3.1.** and **3.4.2.3.2.**, always considering parallel control non-irradiated cells.

To properly fix a time of post-incubation necessary for activation of MAPKs, timing from 30 minutes to 2 hours was carried out.

3.4.3.3. Detection of active and total MAPKs

Active MAPKs (phospho-ERK1/2, -p38 and -JNK1/2), as well as total MAPKs (ERK2, p38 and JNK1/2) were detected by western blot analysis of total protein extract from HaCaT cells pre-treated with the polyphenolic fractions, irradiated and post-incubated as explained above. Explained more in detail:

3.4.3.3.1. Protein extraction

Cells attached to plates were washed with PBS and lysed by direct addition to plate of 300 µl of lysis buffer containing 81.5 mM Tris pH 6.8, 2% (w/v) SDS and protease inhibitors (10µg/ml leupeptin, aprotinin and PMSF and 1 µg/ml ortovanadate). Lysates were collected by scrapping, sonicated, and stored at -20ºC or used immediately.

3.4.3.3.2. Determination of protein concentration

Since lysis buffer used for MAPKs detection contained more than 0.1% SDS (concentration that cause interference in BCA protein assay), the protein concentration of extracts was determined by the Lowry method.

Firstly, a BSA (Bovine Serum Albumin) standard curve with dilutions from 0 to 80 µg/ml was made in triplicate adding the volumes indicated in Table 6. Secondly, 1,250 µl of solution A was added to the standards and samples, gently shaken and incubated 10 min at room temperature. Afterwards, 125 µl of solution B was added, shaken and incubated 30 min at room temperature. Finally, 1 ml of sample was read in a

spectrophotometer at 750 nm. The protein concentration was obtained by extrapolation in the standard curve.

Final concentration BSA (µg/ml)	Vol. StockBSA (0.1 mg/ml) (µl)	Vol. Lysis buffer (µl)	Vol. MiliQ water (µl)
0	0	X	250-X
10	25	X	225-X
20	50	X	200-X
40	100	X	150-X
80	200	X	50-X
Sample	X	0	250-X

Reagents

Solution 1: 2% Na_2CO_3 in 0.1N de NaOH

Solution 2: 0.5% $CuSO_4$

Solution 3: 1% sodium and potassium tartare

Phenol reactive: Folin-Ciocalteu reactive

Work solutions

Solution A: mixture of solutions **1**, **2** and **3** in 48:1:1 proportions

Solution B: dilution 1:1 of phenol reactive and miliQ water

Table 6. *Solutions composition and volumes necessary to protein determination by the Lowry method.*

3.4.3.3.3. Samples preparation

Samples were made as explained in section ***3.3.3.2.4.*** except for the amount of total protein to analyze, which was 25 µg/ml for MAPKs detection.

3.4.3.3.4. Electrophoresis

As explained in ***3.3.3.2.5****.* section, protein samples were resolved by SDS-PAGE, but in this case using 4% and 10% acrylamide stacking and resolving gels respectively. The amounts of reactive necessary to make one gel are detailed in Table 7, using the same solutions indicated in Table 4:

	10 % Resolving Gel	4 % Stacking Gel
Solution 1	5 ml	----
Solution 2	3,4 ml	360 μl
Solution 3	------	1,5 ml
MiliQ water	1,6 ml	1,2 ml
TEMED	14 μl	7,5 μl
APS 10%	65 μl	30 μl
Total Volume	**10 ml**	**3 ml**

Table 7. *Volumes necessary to prepare the resolving and the stacking gels.*

3.4.3.3.5. Electrotransfer and blocking

Electrotransfer and blocking was carried out as previously described for the detection of caspases and cytochrome *c* (section ***3.3.3.2.6.***). For detection of MAPKs, membranes were blocked in TBS-T with 5% (w/v) BSA 1 hour at room temperature.

3.4.3.3.6. Antibodies incubation and reprobing

Blocked membranes were incubated with an appropriate dilution of the corresponding primary antibody in TBS-T and BSA and/or dry milk. Incubation dilution, time and temperature were as follows:

o   Polyclonal anti-phospho ERK1/2 [Cell Signalling, Beverly, MA (USA)] was used in TBS-T with 2% dry milk at 1:1000 dilution and incubated for 1-2 hours.

o   Monoclonal anti-ERK2 [Upstate, Dundee, Scotland (UK)] was used in TBS-T with 2% dry milk at 1:1000 dilution and incubated for 1 hour at room temperature.

o   Polyclonals anti-phospho-p38 and anti-p38 [Cell Signalling] were used in TBS-T with 5% (w/v) BSA at 1:1000 dilutions and incubated overnight at 4ºC.

o   Monoclonal anti-phospho-JNK and polyclonal anti-JNK [Cell signalling] were used in TBS-T with 1% (w/v) BSA and 0.5% dry milk from 1:500 to 1:1000 dilutions and incubated overnight at 4ºC.

After incubation with the primary antibody, washing, incubation with the corresponding secondary antibody, analysis of equal protein load with anti-α-actin and

protein detection were performed in the same way described for caspases and cytochrome *c* detection (sections ***3.3.3.2.7.*** and ***3.3.3.2.8.***) except for detection of total ERK2, which was performed by incubating with a secondary antibody anti-mouse AP conjugated [Promega] in TBS-T with 2% (w/v) dry milk at 1:2000 dilution for 1 hour at room temperature. Once the incubation was completed, blots were washed tree times and visualized by detection of alkaline peroxidase incubating in CDS (100 mM Tris-HCl, 100 mM Na Cl pH 9.4 and 5 mM Mg Cl$_2$) with 0.3% of BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and 0.6% of NBT (nitro blue tetrazolium) [Promega] till colour visualization.

3.4.3.4. Evaluation of MAPKs activation and polyphenolic fractions protection

Quantification of active MAPKs (phospho-ERK1/2, -p38 and −JNK1/2) was carried out in order to estimate possible protection of the tested polyphenolic fractions. Since membranes used to analyse active MAPKs were different from the ones used for total MAPKs analysis, α-actin was used as the internal load control and density of active MAPKs bands was referred to density of α-actin bands. Thus, the relative density of the bands was quantified by the automated digitising system UN-SCAN-IT [Silk Scientific, (USA)] and referred to α-actin. Density of UVB or UVA radiated non-treated cell bands was considered as 100% of activation and results were expressed as a percentage of them.

3.4.4. DETECTION OF INTRACELLULAR H$_2$O$_2$ AFTER UV RADIATION OF HUMAN QUIESCENT KERATINOCYTES HaCaT

Intracellular levels of hydrogen peroxide were analysed by flow cytometry after UVB or UVA radiations of keratinocytes HaCaT pre-treated or not with the total fraction **OW** or the derived fractions **IV, V** and **XI**. To do so, dihydrorhodamine 123 (DHR) was used as a specific fluorescent dye probe, since the intracellular release of H$_2$O$_2$ irreversibly oxidizes DHR and it is converted to red fluorescent compound rhodamine 123 [Royall et al., 1993].

3.4.4.1. Culture and pre-treatment of human keratinocytes HaCaT

For this assay, 83,000 keratinocytes HaCaT were seeded in 12-well microtitre plates in standard culture medium. The number of cells to culture in these plates was calculated considering the results obtained for the cell screening of quiescent cells (explained in

section **3.4.1.1.**), and obeyed the relationship of areas * number of cells of screening ((A$_{12 \text{ wells}}$/A$_{96 \text{ wells}}$) * 7,0000 cells). After 24 hours of seeding, medium was changed for another one without serum and incubated 48 hours prior to the treatment with the polyphenolic fractions for 6 hours at the same concentrations indicated for the analysis of MAPKs activation (section **3.4.3.1.**)

3.4.4.2. Incubation with DHR, irradiation and post-incubation

Once the treatment with polyphenolic fractions was completed, the medium was removed and fresh medium with DHR at a final concentration of 5 µM (from a stock of 10 mM in DMSO) was added to all the wells. After 30 minutes of incubation, the medium was removed and the cells were washed with PBS prior to UV radiation, followed by 30 minutes of post-incubation as explained for the analysis of MAPKs activation (section **3.4.3.2**).

3.4.4.3. Detection of intracellular H$_2$O$_2$ and evaluation of possible polyphenolic fractions protection

After post-incubation time, the cells were washed with PBS, trypsinized and collected by centrifugation at 500 x *g*. Then, medium was removed and pellets were washed with PBS previous to fix cells with 400 µl of 0.5% formaldehyde in PBS. Finally, cells were placed on ice and analysed by measuring the fluorescence intensity of 10,000 cells at 488 nm in a Epics XL flow cytometer. The results were expressed as a percentage of mean fluorescence intensity of non-irradiated DHR stained cells, considering them as 100%.

4. RESULTS

4.1. ANTITUMORAL EFFECT OF POLYPHENOLIC FRACTIONS OW, I AND II AT CELLULAR AND METABOLIC LEVELS

4.1.1. CELL VIABILITY

4.1.1.1. Screening of adherent cells

To calculate the optimum number of adherent cells necessary to perform reliable cell viability studies, a cell screening from 500 to 20,000 cells was carried out at 72 hours in human melanomas A-375 and SK-Mel-28, and mouse hepatoma Hepa-1c1c7 by following the two different MTT assays described in section *3.3.1.1.1.* of Materials and Methods. An estimation of cell number range in which absorbance obeyed Beer-Lambert's law was made by plotting the number of seeded cells versus absorbance determined at 550 nm (Figure 8).

MTT 0.2 mg/ml (4h)   **MTT 0.5 mg/ml (1h)**

A

B

MTT 0.2 mg/ml (4h)　　　　　　　**MTT 0.5 mg/ml (1h)**

C
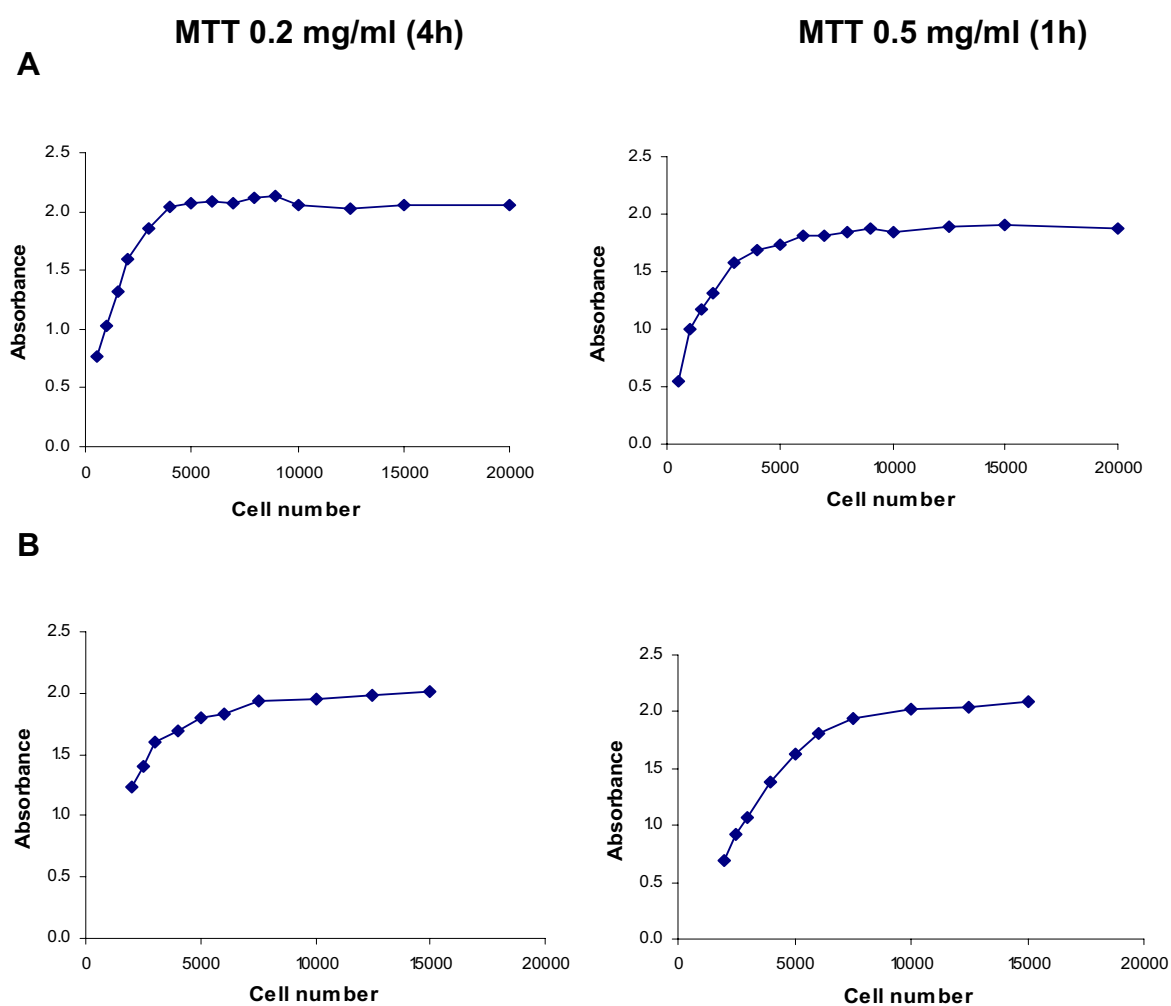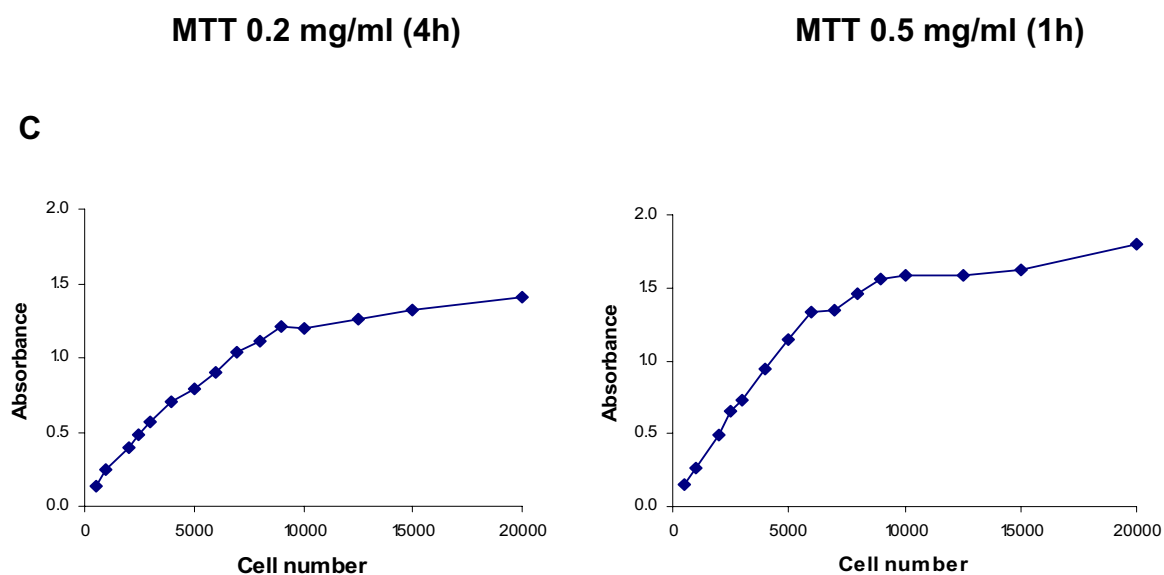
Figure 8. *Cell number versus absorbance after incubation of human melanomas A-375 (**A**) and SK-Mel-28 (**B**), and mouse hepatoma Hepa-1c1c7 (**C**) with MTT 0.2 mg/ml for 4 hours or MTT 0.5 mg/ml for 1 hour for a cell number range between 500 and 20,000.*

These graphs showed that the cell number was directly proportional to the absorbance in the range between 500 and 5,000 cells for human melanoma A-375, and 500 and 6,000 cells for human melanoma SK-Mel-28 and mouse hepatoma Hepa-1c1c7. Since the two MTT treatments (MTT 0.2 mg/ml and MTT 0.5 mg/ml with incubation periods of 4 and 1 hours respectively) appeared to be valid, MTT 0.5 mg/ml was selected for the rest of viability assays because it was more time efficient. In order to limit the optimum cell number, with an absorbance value between 1 and 1.5, another screening was performed in a shorter cell number range (between 500 and 2,000 for A-375, and 2,000 and 6,000 for SK-Mel-28 and Hepa-1c1c7) but only treating with MTT 0.5 mg/ml (Figure 9).

A　　　　　　　　　　　　　　　　**B**

C
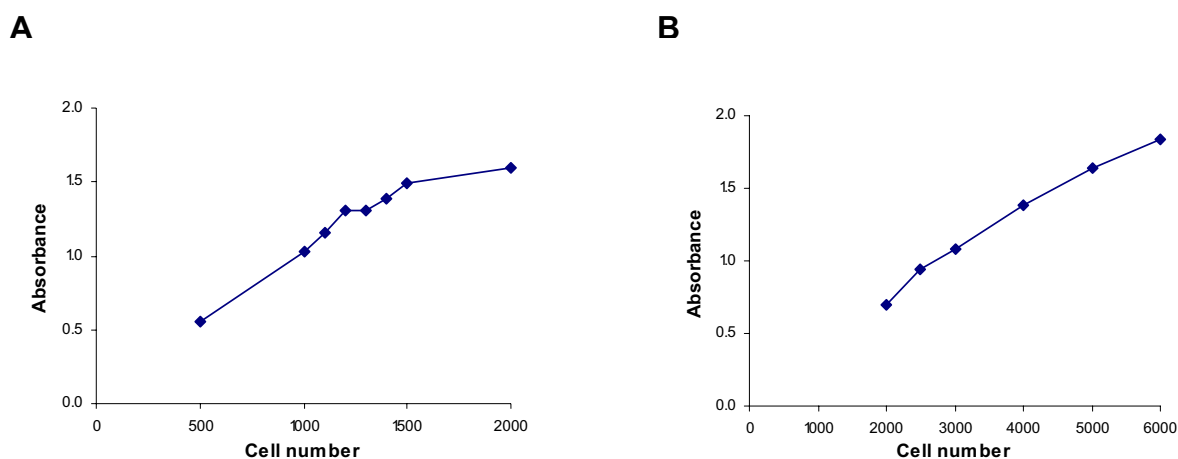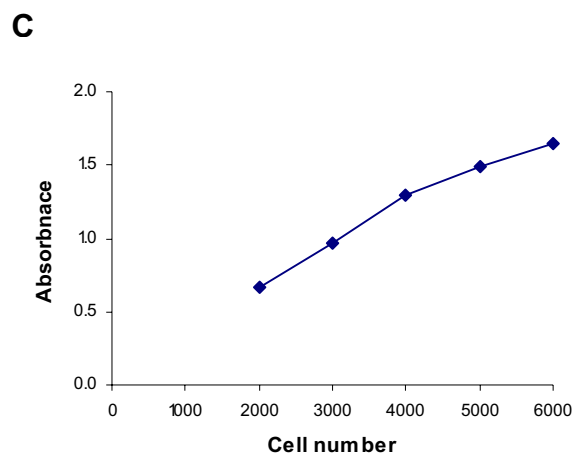
Figure 9*. Cell number versus absorbance after 1 hour of incubation with MTT 0.5 mg/ml for for a cell number range between 500 and 2,000 for A-375 (**A**) and 2,000 to 6,000 for SK-Mel-28 (**B**) and Hepa-1c1c7 (**C**).*

According to the results obtained in these screenings (Figure 9), the optimum cell number to carry out viability assays was estimated, considering an absorbance around 1.3. Thus, it was decided that 1,200 cells would be used for the human melanoma A-375 and 4,000 cells for the human melanoma SK-Mel-28 and the mouse hepatoma Hepa-1c1c7 to carry out future assays.

4.1.1.2. Cell viability

The effect of polyphenolic fractions on cell viability of human melanomas A-375 and SK-Mel-28, mouse hepatoma Hepa-1c1c7 and leukemia Jurkat cells, as well as primary peripheral blood lymphocytes (PBLs) was studied after 72 hours of treatment with the total polyphenolic fraction **OW** or the derived fractions **I** and **II** as indicated in section **3.3.1.2.** of Material and Methods.

4.1.1.2.1. Tumoral cells

Cell viability assays for adherent cells were performed by culturing the number of cells calculated in the previous screenings referred above (1,200 cells for human melanoma A-375 and 4,000 cells for human melanoma SK-Mel-28 and mouse hepatoma Hepa-1c1c7). In the case of leukemia Jurkat cells, 20,000 cells were seeded.

The respective IC$_{50}$ values for each polyphenolic fraction (concentration of product that causes 50% inhibition of cell growth) were determined by testing the effect on cell viability of different fraction dilutions on a scale of 0.1 µg/ml to 200 µg/ml after 72 hours

of treatment. The results obtained by absorbance determination of formazan dye uptake at 550 nm were represented considering control as 100% of cell viability. Representation of these results (Figure 10) allowed us to calculate the IC_{50} values for the fractions **OW**, **I** and **II** in each type of tumoral cells, using the data analysis and graphics program Grafit 3.0 (Table 8).

A

■ **Fraction OW (53 ± 4 µg/ml)**

● **Fraction I (48 ± 4 µg/ml)**

▲ **Fraction II (70 ± 1 µg/ml)**

B

■ **Fraction OW (70 ± 4 µg/ml)**

● **Fraction I (127 ± 6 µg/ml)**

▲ **Fraction II (137 ± 5 µg/ml)**

C

■ **Fraction OW (43 ± 2 µg/ml)**

● **Fraction I (61 ± 2 µg/ml)**

▲ **Fraction II (81 ± 1 µg/ml)**
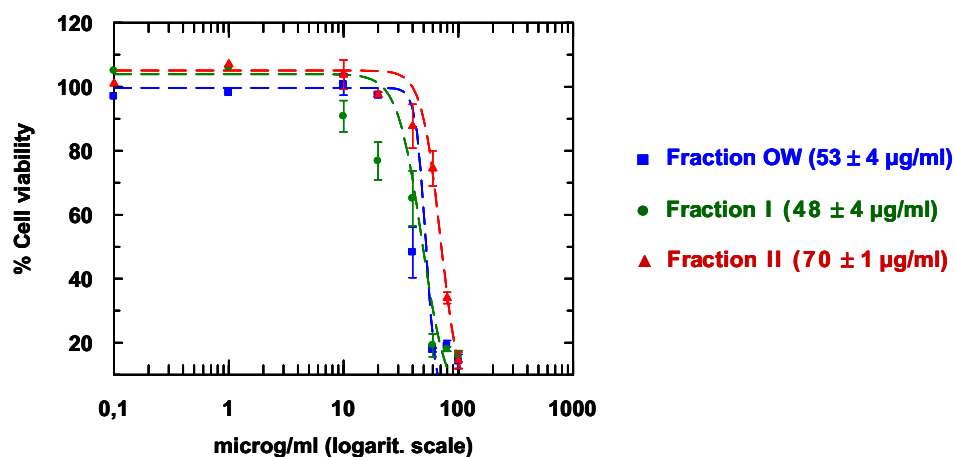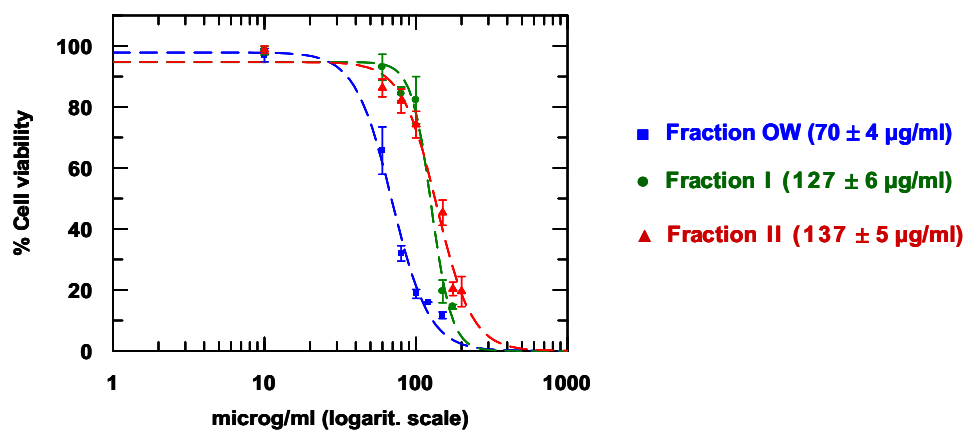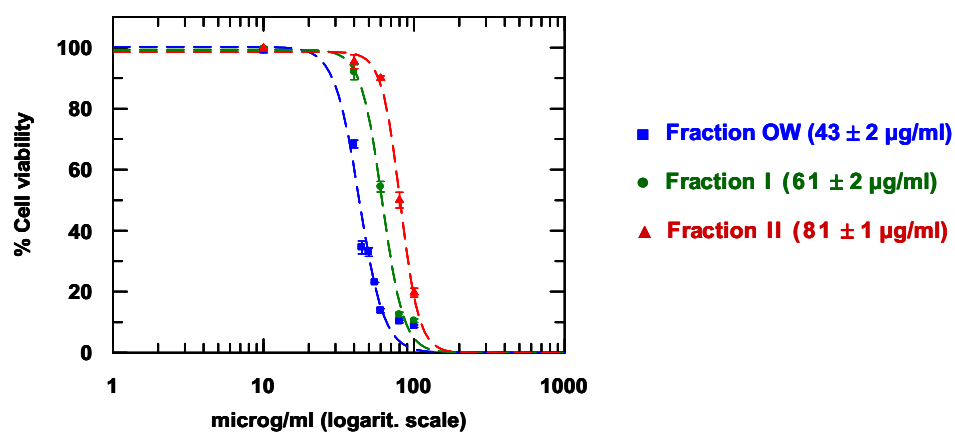
D
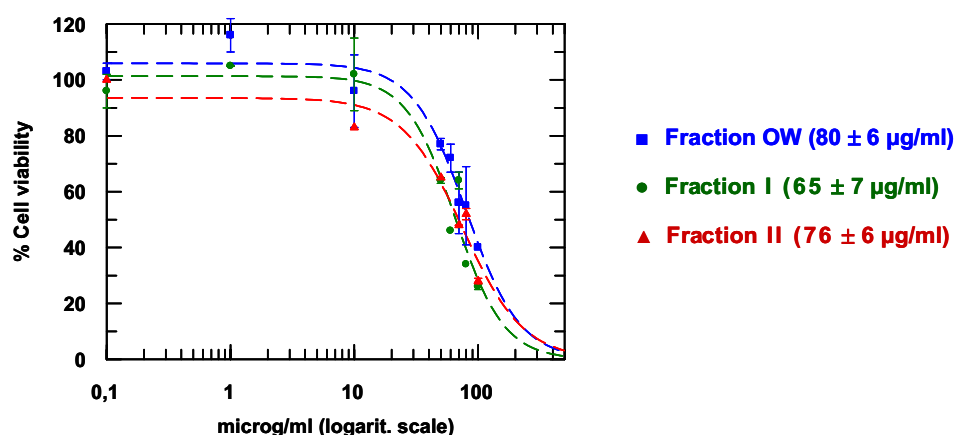
Figure 10. *Effect on cell viability of human melanomas A-375 (**A**) and SK-Mel-28 (**B**), mouse hepatoma Hepa-1c1c7 (**C**) and leukemia Jurkat cells (**D**) after72 hours of treatment with the polyphenolic fractions **OW**, **I** or **II**. The IC$_{50}$ values for each fraction are expressed as mean ± SD (n=3).*

Cell line	IC50 ± SD (μg/ml)		
	Fraction OW	**Fraction I**	**Fraction II**
A-375	53 ± 4	48 ± 4	70 ± 1
SK-Mel-28	70 ± 4	127 ± 6	137 ± 5
Hepa-1c1c7	43 ± 2	61 ± 2	81 ± 1
Jurkat	80 ± 6	65 ± 7	76 ± 6

Table 8. *IC$_{50}$ values obtained for the tumoral cell lines treated with the polyphenolic fractions **OW**, **I** and **II**. Results are expressed as mean ± SD (n=3).*

As observed in Table 8, the concentration of polyphenolic fractions that caused 50% inhibition in cell viability of tumoral cells ranged from 40 μg/ml to 140 μg/ml, showing that the fraction **II** (fraction with higher percentage of gallate groups) was about 30-50% less effective in all of them, except for leukemia Jurkat cells, where the three fractions seemed to have the same efficiency. Furthermore, the derived fractions **I** and **II**, had a similar effect on the viability of fast growth human melanoma A-375, mouse hepatoma Hepa-1c1c7 and leukemia Jurkat cells, being more evident for the derived fraction **II**. The derived fractions **I** and **II** showed a less marked effect on cell viability of slow growth human melanoma SK-Mel-28 (IC$_{50}$ value about 50% higher than the corresponding values in the other tumoral cells).

4.1.1.2.2. Primary peripheral blood lymphocytes

Cytotoxicity determination of the total polyphenolic fraction **OW** and the derived fractions **I** and **II** was carried out in Peripheral Blood Lymphocytes (PBLs). In this case the respective IC_{50} values for each polyphenolic fraction were determined by testing the effect on cell viability of different fraction dilutions on a scale of 5 µg/ml to 1,000 µg/ml and the results obtained were represented and analyzed as explained above for the tumoral cells (Figure 11).
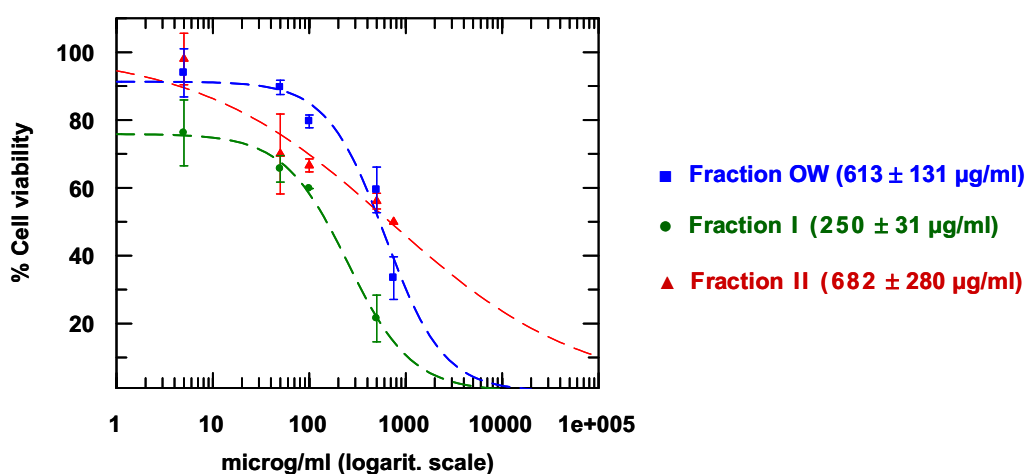
Figure 11. *Effect on cell viability of PBLs after72 hours of treatment with the polyphenolic fractions **OW**, **I** and **II**. The IC_{50} values for each fraction are expressed as mean ± SD (n=3).*

In general the polyphenolic fraction concentrations that caused 50% cell death in primary PBLs were much higher than the concentrations that provoked 50% inhibition growth in the tumoral cell lines (Table 8). Thus, IC_{50} values for the fractions **OW** and **II** in PBLs were approximately 8-fold higher than the respective values in its tumoral analogous Jurkat cells. However, the derived fraction **I** (polyphenolic fraction with the lowest content in gallate) caused 50% of cytotoxicity to PBLs only at a concentration 4-fold higher than its IC_{50} in Jurkat cells.

4.1.2. CELL CYCLE ANALYSIS

The effect of the total polyphenolic fraction **OW** and the derived fractions **I** and **II** on the cell cycle of tumoral cell lines tested in this study (human melanomas A-375 and SK-Mel-28, mouse hepatoma Hepa-1c1c7 and leukemia Jurkat cells) was studied after 72 hours of treatment. The tested fraction concentrations were their respective IC_{50} values (Table 8) and a higher dose that caused 80% of cell growth inhibition (IC_{80}) (Table 9), which was estimated by extrapolation in cell viability curve (Figure 10). The effect of these fractions on the cell cycle was analyzed in a flow cytometer by detecting fluorescence of PI when it binds to DNA, and the percentage of cells in each of the different phases of the cell cycle was calculated using Multicycle program.

	IC80 (µg/ml)		
Cell line	**Fraction OW**	**Fraction I**	**Fraction II**
A-375	60	60	100
SK-Mel-28	125	175	200
Hepa-1c1c7	60	80	100
Jurkat	150	150	150

Table 9. *IC_{80} values for the polyphenolic fractions **OW**, **I** and **II** in the tumoral cell lines.*

In Figure 12 is displayed an illustrative example of the cell cycle profile obtained for the different tumoral cell lines after treatment with the polyphenolic fractions at their respective IC_{50} and IC_{80} values.

IC$_{50}$ IC$_{80}$

A

B

C
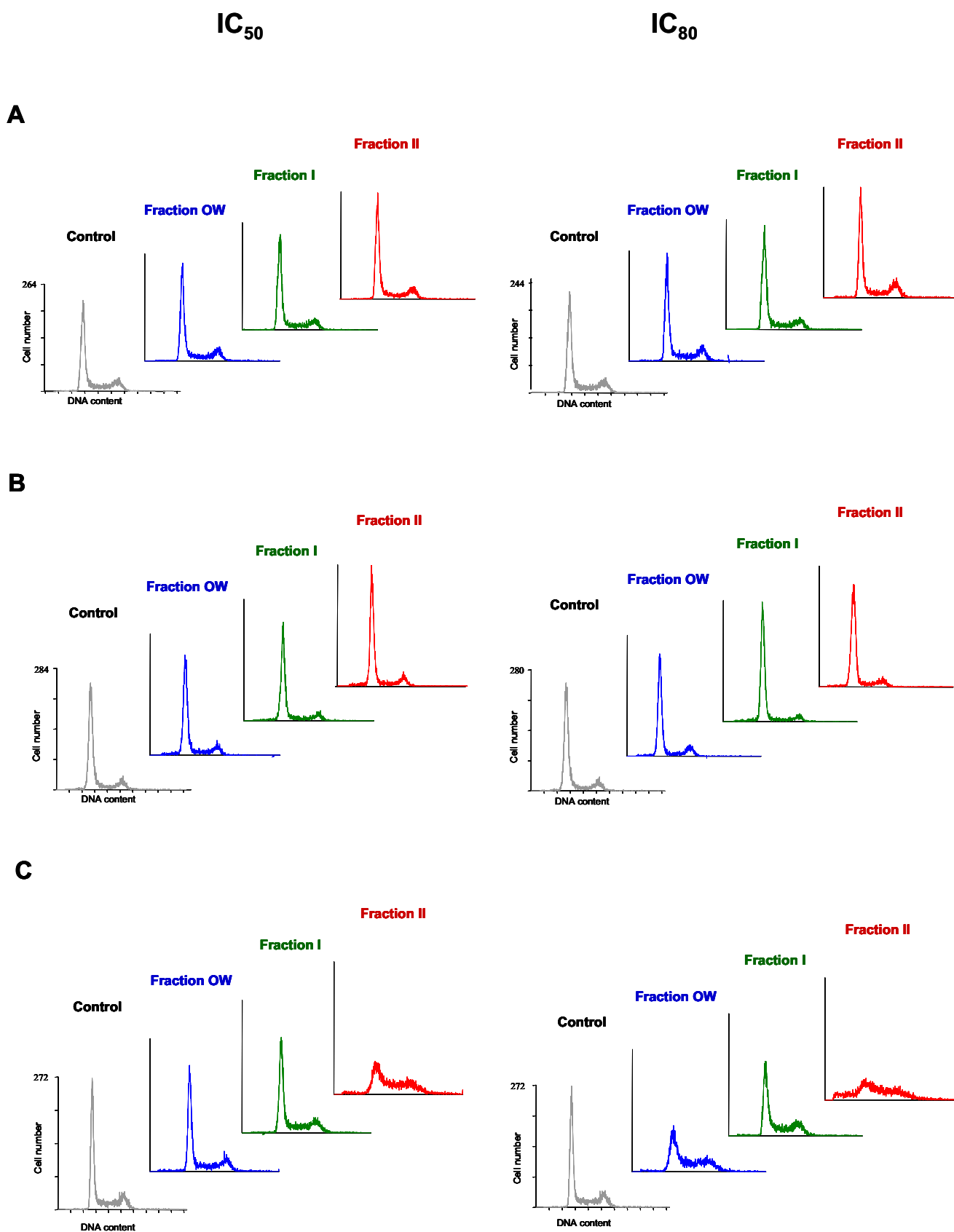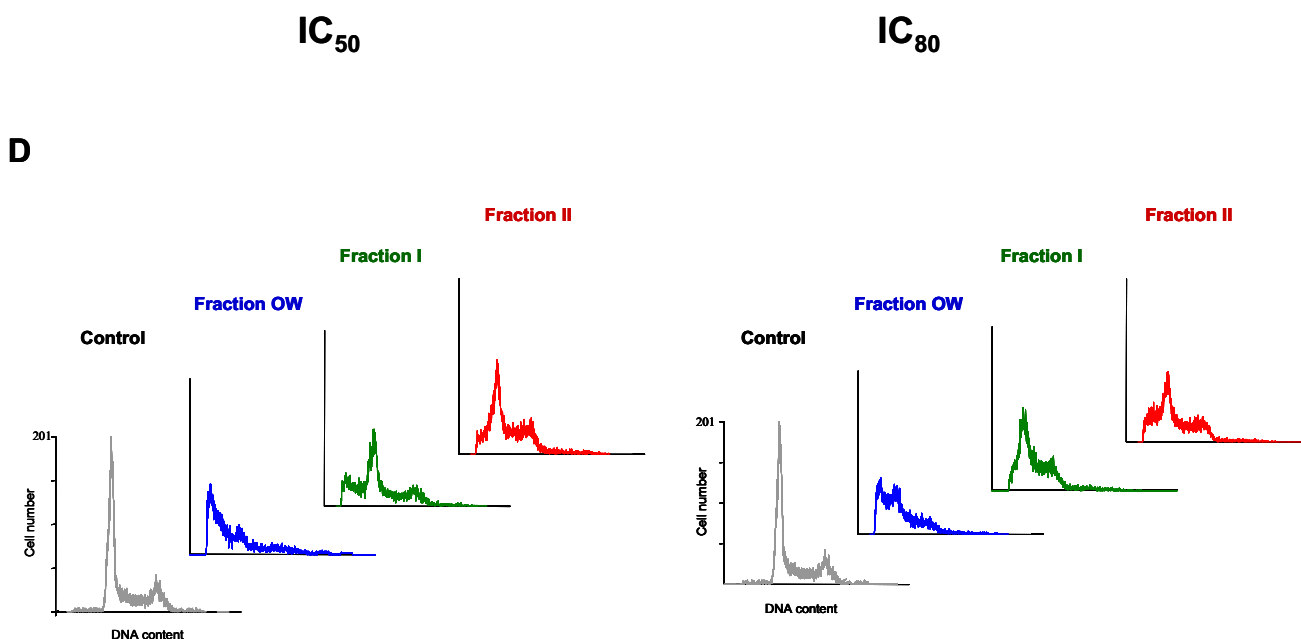
Figure 12. *Cell cycle profile of A-375 (**A**), SK-Mel-28 (**B**), Hepa-1c1c7 (**C**) and Jurkat (**D**) control cells or after 72h of treatment with the polyphenolic fractions **OW**, **I** or **II** at concentrations equal to their respective IC$_{50}$ and IC$_{80}$ values.*

Figure 12 shows that none of the polyphenolic fractions seemed to alter cell cycle of the human melanomas A-375 or SK-Mel-28 at concentrations equal to their respective IC$_{50}$ and IC$_{80}$ values (Figure 12A, 12B). However, the total fraction **OW** at concentration equivalent to its IC$_{80}$ value and the derived fraction **II** at both, its IC$_{50}$ and IC$_{80}$ values modified cell cycle profile of mouse hepatoma Hepa-1c1c7, being the fraction **II** effect so strong that resulted in an aberrant profile (Figure 12C). Additionally, the total polyphenolic fraction **OW**, as well as the derived fractions **I** and **II**, exerted such a strong effect on the cell cycle of leukemia Jurkat cells (Figure 12D) that produced apparent aberrant cell cycle profiles, like the fraction **II** in Hepa-1c1c7 cells (Figure 12C).

The percentage of cell cycle distribution calculated for the human melanomas A-375 and SK-Mel-28 and hepatoma Hepa-1c1c7 allowed us to estimate differences between control cells and cells treated with the polyphenolic fractions (**OW**, **I** or **II** for A-375 and SK-Mel-28 cells and **OW** or **I** for Hepa-1c1c7 cells) at their respective IC$_{50}$ and IC$_{80}$ concentrations (Table 10). Cell cycle distribution percentage of Hepa-1c1c7 cells treated with the derived fraction **II**, as well as treated Jurkat cells was not calculated since the aberrant cell cycle profile did not allowed to properly evaluate them.

IC50

	% G0/G1 (Mean ± sem)			%S (Mean ± sem)			%G2/M (Mean ± sem)		
	A-375	SK-Mel-28	Hepa-1c1c7	A-375	SK-Mel-28	Hepa-1c1c7	A-375	SK-Mel-28	Hepa-1c1c7
Control	59.4 ± 3.0	73.5 ± 0.9	57.7 ± 1.5	25.7 ± 1.8	16 ± 0.6	26.7 ± 0.8	14.9 ± 1.2	10.5 ± 0.7	15.6 ± 0.8
Fraction OW	60.8 ± 0.8	72.8 ± 1.3	58.7 ± 2.4	24.7 ± 0.5	16.3 ± 1.6	27.1 ± 0.8	14.5 ± 0.8	10.8 ± 0.6	14.2 ± 1.6
Fraction I	63 ± 1.8	74.4 ± 1.4 *	54.6 ± 3.4	23.8 ± 0.6	15.6 ± 1.2	29.1 ± 2.3	13.1 ± 1.2	10.0 ± 0.5	16.3 ± 1.3
Fraction II	64.4 ± 0.6	76.9 ± 0.3 *		21.3 ± 0.8	13.1 ± 0.4		14.4 ± 0.6	10.0 ± 0.5	

IC80

	% G0/G1 (Mean ± sem)			%S (Mean ± sem)			%G2/M (Mean ± sem)		
	A-375	SK-Mel-28	Hepa-1c1c7	A-375	SK-Mel-28	Hepa-1c1c7	A-375	SK-Mel-28	Hepa-1c1c7
Control	59.4 ± 3.0	73.5 ± 0.9	57.7 ± 1.5	25.7 ± 1.8	16 ± 0.6	26.7 ± 0.8	14.9 ± 1.2	10.5 ± 0.7	15.6 ± 0.8
Fraction OW	59.9 ± 1.0	76.4 ± 1.6	39.5 ± 3.0 *	24.8 ± 0.5	12.9 ± 1.0	40.3 ± 2.9 *	15.3 ± 1.5	10.7 ± 1.0	20.2 ± 0.4 *
Fraction I	64.3 ± 1.1	79.8 ± 1.4 *	48.6 ± 3.9	23.1 ± 0.4	10.6 ± 1.0 *	29.8 ± 2.4	12.5 ± 1.1	9.5 ± 0.5	21.6 ± 2.8
Fraction II	59.7 ± 2.2	79.3 ± 0.9 *		22.6 ± 1.5	8.0 ± 1.0		17.7 ± 0.9	12.7 ± 0.3 *	

Table 10. Cell cycle distribution of SK-Mel-28, A-375 and Hepa-1c1c7 control cells or after 72 hours of treatment with the polyphenolic fractions *OW*, *I* or *II* at concentrations equal to their respective IC50 or IC80 values. Percentage of cells in each phase of the cell cycle (G0/G1, S and G2/M) are expressed as Mean ± sem (n=3).

Cell cycle distribution percentages illustrated in Table 10 show a slight increase of cells in G0/G1 phase of human melanomas A-375 and SK-Mel-28 treated with the derived fractions **I** or **II** at their respective IC_{50} and IC_{80} doses, although it was only significant for SK-Mel-28 cells. Moreover, similar G0/G1 cell cycle arrest was observed in SK-Mel-28 cells treated with the total fraction **OW** at concentration equal to its IC_{80} value, but it was not significant. In the case of Hepa-1c1c7 cells, the derived fraction **I** apparently decreased number of cells in G0/G1 phase in a dose dependent manner, although it was not statistically significant. The total fraction **OW** caused that effect in a significant way at IC_{80} dose, accompanied by an increase of cells in S and G2/M phases.

4.1.3. APOPTOSIS

The capacity of the polyphenolic fractions to induce apoptosis was studied at the same conditions explained in section **4.1.2.** for cell cycle analysis. Thus, the effect of the total polyphenolic fraction **OW** and the derived fractions **I** and **II** was tested in all the tumoral cell lines (human melanomas A-375 and SK-Mel-28, mouse hepatoma Hepa-1c1c7 and leukemia Jurkat cells) after 72 hours of treatment. Furthermore, a study of the possible apoptotic mechanism caused by these fractions was performed in leukemia Jurkat cells, the tumoral cell line in which the polyphenolic fractions induced more apoptosis.

4.1.3.1.   Apoptosis detection

Detection of apoptosis was performed after 72 hours of treatment with the total polyphenolic fraction **OW** or the derived fractions **I** and **II** at concentrations equal to their respective IC_{50} and IC_{80} values (Tables 8 and 9). Results obtained for all the tumoral cell lines are summarized in Figure 13 expressed as percentage of cells in early apoptosis (annexin V positive and PI negative) and late apoptosis/necrosis (annexin V positive and PI positive).
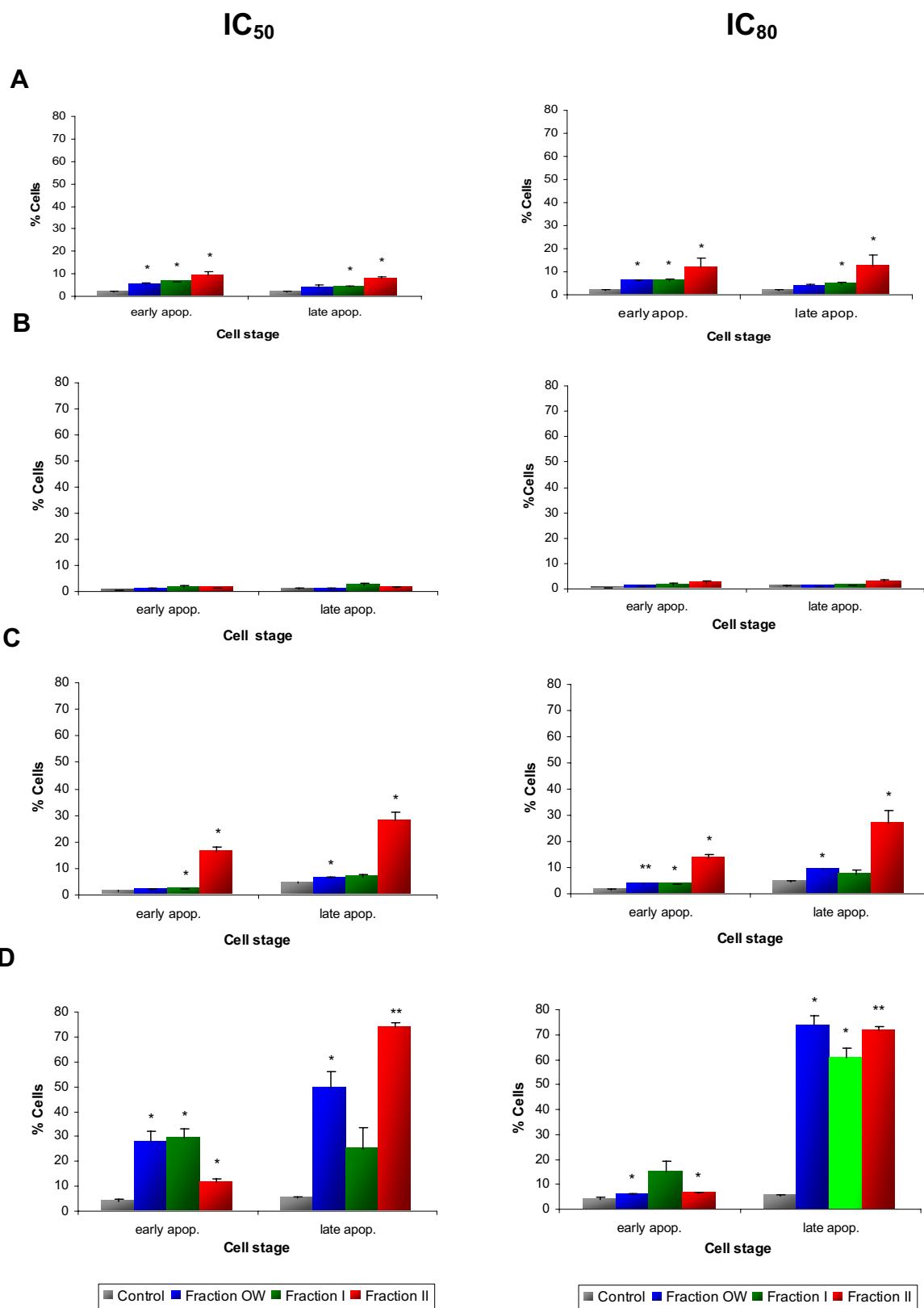
Figure 13. *Percentage of early (Annexin V^+ PI^-) and late (Annexin V^+ PI^+) apoptotic A-375 (**A**), SK-Mel-28 (**B**), Hepa-1c1c7 (**C**) and Jurkat (**D**) cells after 72 hours of treatment with the polyphenolic fractions **OW**, **I** or **II** at concentrations equal to their respective IC_{50} or IC_{80}. Results are expressed as mean ± sem (n=3). * p < 0.05 ** p < 0.001.*

Results showed that none of the three fractions induced apoptosis in the human melanoma SK-Mel-28 (Fig. 13B). In human melanoma A-375, the fraction **II** (fraction with high percentage of gallate groups) was the one that caused more significant apoptosis (9% and 12% early apoptosis plus 8% and 12% late apoptosis/necrosis at concentrations equal to their respective IC_{50} and IC_{80} values) (Fig. 13A). Although the fractions **OW** and **I** slightly induced significant apoptosis in mouse hepatoma Hepa-1c1c7, the fraction **II** was again the one that generated more significant apoptosis in this cell line (15% and 13% early apoptosis plus 23% and 22% late apoptosis/necrosis at concentrations equal to their respective IC_{50} and IC_{80} values) (Fig. 13C). Interestingly, at their respective IC_{50} and IC_{80} doses, the polyphenolic fractions **OW** and **II** induced a great degree of apoptosis in leukemia Jurkat cells (about 80% early apoptosis plus significant late apoptosis compared to the total 50% apoptosis induced by the fraction **I** at its IC_{50}). It is worth notice that the percentage of early apoptosis was higher in the cells treated with concentrations equal to their respective IC_{50} and that of late apoptosis higher in the cells treated with concentrations equal to their respective IC_{80} (Fig. 13D).

4.1.3.2. Analysis of caspases activation and release of cytochrome *c* in leukemia Jurkat cells.

Non-adherent leukemia Jurkat cells (the tumoral cell line with the highest percentage of apoptosis triggered by the polyphenolic fractions) was used to determine if the apoptosis induced by the total polyphenolic fraction **OW** and the derived fractions **I** and **II** was caspase-dependent and if cytochrome *c* was released. Thus, cell extracts were analyzed after 6 hours of treatment at concentrations equal to their IC_{50} values (Table 8), considering Jurkat cells treated with staurosporine for 3 hours as the internal positive control.

The results represented in Figure 14 showed that 6 hours of treatment with the polyphenolic fractions was sufficient for activation of both, initiator caspases (caspase-9 and -8) and effecter caspase-3. Thus, procaspase-9 (47 KDa) was proteolytically cleaved into smaller active fragments of 37 and 35 KDa; procaspase-8 (55/50 KDa) into 40 and 36 KDa subunits and procaspase-3 (35 KDa, not shown in figure 7 because anti-caspase-3 used in this study did not detect the proenzyme form) into 19 and 17 KDa fragments. Although all the polyphenolic fractions induced activation of caspases, the effect was more evident for the total fraction **OW** and the derived fraction **II** (fractions with high content in gallate groups) (Fig. 14A). Furthermore, the presence of cytochrome *c* (14 KDa) in the cytosolic fraction of cell lysate was observed after treatment with any of the

three fractions, being it more accentuated for the fraction **II** (Fig. 14B). For all the caspases and cytochrome *c*, α-actin expression demonstrated equal amount of loaded protein. It is worth notice that less amount of protein was loaded for the positive control as indicated in section ***3.3.3.2.4.*** of Materials and Methods.
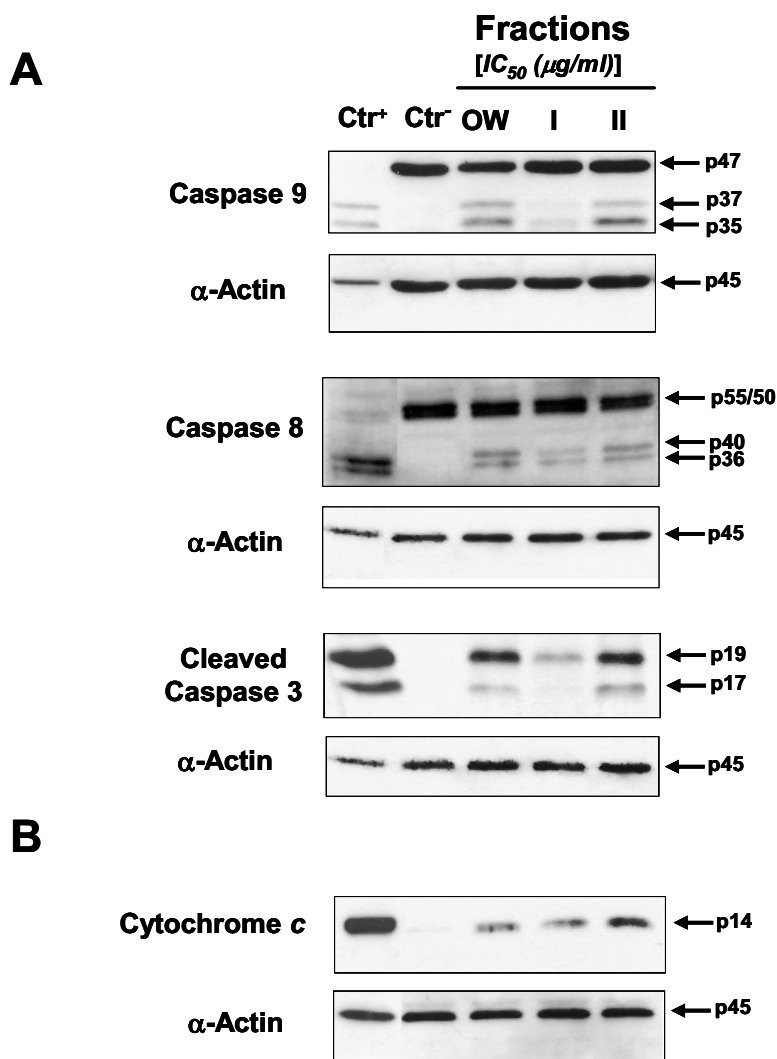
Figure 14. *Effect of the polyphenolic fractions **OW**, **I**, or **II** on processing of caspase proteins (**A**) and release of cytochrome c (**B**) of leukemia Jurkat cells after 6 hours of treatment at concentrations equal to their respective IC$_{50}$ values. α-Actin expression was used as loading control. Blots are representative of three independent experiments.*

4.1.4. METABOLIC PROFILE OF LEUKEMIA JURKAT CELLS

Like for the study of the apoptosis mechanism, non-adherent leukemia Jurkat cells were chosen as the model cell line to determine the effect of the total polyphenolic fraction **OW** and the derived fractions **I** and **II** on specific metabolic fluxes such as the pentose cycle and glycolysis, as well as nucleic acid synthesis. Therefore, metabolic profile was studied after 24 and 48 hours of treatment at concentration equal to 40 µg/ml as related in section **3.3.4.** of Materials and Methods.

4.1.4.1. Stable isotope incorporation into glucose

The analysis of ^{13}C label distribution in glucose from original Jurkat cells culture medium demonstrated that the initial enrichment in the tracer was about 50%, since the mass isotopomer m2 ($[1,2\text{-}^{13}C_2]$ D-glucose) was 47%.

4.1.4.2. Stable isotope incorporation into RNA Ribose

To estimate the nucleic acid precursor synthesis in leukemia Jurkat cells treated with the polyphenolic fractions **OW**, **I** or **II**, measurements of the molar enrichment of RNA ribose with ^{13}C from $[1,2\text{-}^{13}C_2]$ D-glucose were carried out by calculating the SIGmn value (m1*1 + m2*2 + m3*3 + m4*4). As shown in Figure 15, since SIGmn value decreased after treatment with any of the polyphenolic fractions in a statistically significant manner, an inhibition of *de novo* nucleic acid synthesis occurred for all the treated cells with respect to the control. Moreover, that effect was higher for the derived fraction **II** (fraction with high percentage of gallate groups) whose percentage of inhibition was about 20%, while it was approximately 10% for the fractions **OW** and **I**. Furthermore, those percentages were the same for both 24 and 48 hours treatments, showing a non-time dependent inhibition of *de novo* nucleic acid synthesis.
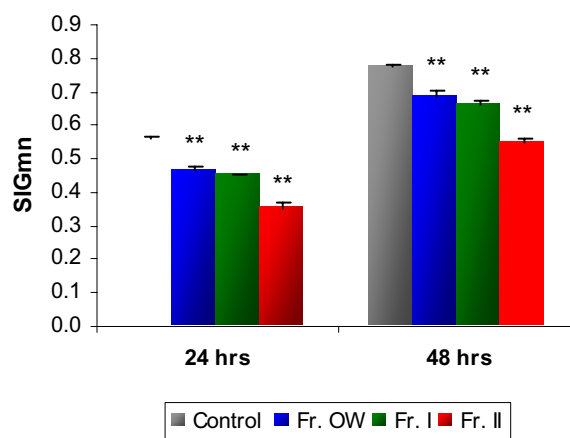
Figure 15. *Molar enrichment (SIGmn) of ^{13}C in ribose, synthesized from $[1,2-^{13}C_2]$-glucose, of leukemia Jurkat cells either untreated, or treated 24 or 48 hours with the polyphenolic fractions **OW**, **I** or **II** at a concentration equal to 40 µg/ml. Values are expressed as mean ± sem (n=4). * p < 0.05 ** p < 0.001.*

4.1.4.3. Stable isotope incorporation in lactate

The isotopomer distribution and the total label incorporation in lactate were measured in order to estimate the pentose cycle activity and the anaerobic glycolysis as indicated in section ***3.3.4.3.2.*** of Materials and Methods.

We observed a significant decrease in the SIGmn value (m1*1 + m2*2 + m3*3) of Jurkat cells treated with the fractions **OW**, **I** or **II** with respect to the control. After 24 hours of treatment all the fractions exerted similar effect, but in incubations for 48 hours the derived fraction **II** triggered a higher decrease. For the isotopomers m1 (with one ^{13}C label), resulting from the oxidative pentose phosphate pathway $[1,2-^{13}C_2]$ D-glucose catabolism; and m2 (with two^{13}C labels), resulting from direct $[1,2-^{13}C_2]$ D-glucose catabolism through anaerobic glycolysis, only the reduction observed in m2 was significant for all the fractions, being higher after 48h of treatment with the derived fraction **II**, which was indicative of a decrease in anaerobic glycolysis (Table 11).

	Control			
	m0	**m1**	**m2**	**SIGmn**
24 hrs	0,8656 ± 0,0014	0,0089 ± 0,0005	0,1243 ± 0,0009	0,2610 ± 0,0026
48 hrs	0,8158 ± 0,0010	0,0135 ± 0,0004	0,1691± 0,0010	0,3562 ± 0,0018

	Fraction OW			
	m0	**m1**	**m2**	**SIGmn**
24 hrs	0,8785 ± 0,0004**	0,0094 ± 0,0000	0,1113 ± 0,0003**	0,2345 ± 0,0007**
48 hrs	0,8325 ± 0,0006**	0,0131 ± 0,0005	0,1526 ± 0,0003**	0,3236± 0,0009**

	Fraction I			
	m0	**m1**	**m2**	**SIGmn**
24 hrs	0,8817 ± 0,0012**	0,0092 ± 0,0002	0,1083 ± 0,0011**	0,2283 ± 0,0023**
48 hrs	0,8372 ± 0,0008**	0,0140 ± 0,0008	0,1470 ± 0,0005**	0,3134 ± 0,0010**

	Fraction II			
	m0	**m1**	**m2**	**SIGmn**
24 hrs	0,8741 ± 0,0023*	0,0161 ± 0,0021*	0,1083 ± 0,0004**	0,2373 ± 0,0023**
48 hrs	0,8516 ± 0,0012**	0,0135 ± 0,0002	0,1329 ± 0,0013**	0,2850 ± 0,0016**

Table 11. *Lactate isotopomers derived from $[1,2\text{-}^{13}C_2]$D-glucose in control cells and after treatment with the polyphenolic fractions **OW**, **I** or **II** at a concentration equal to 40 µg/ml. m2 isotopomer results from $[1,2\text{-}^{13}C_2]$D-glucose catabolism through anaerobic glycolysis and m1 isotopomer from oxidative pentose phosphate pathway. The m0-m2 and SIGmn values are expressed as molar enrichment (mean ± sem) (n=4). * p<0.05, ** p<0.001.*

4.2. <u>PROTECTIVE EFFECT OF THE POLYPHENOLIC FRACTIONS OW, IV, V AND XI AGAINST UV DAMAGE</u>

4.2.1. DNA FRAGMENTATION IN HUMAN KERATYNOCYTES HaCaT IRRADIATED WITH UVB LIGHT

4.2.1.1. Screening of human keratinocytes HaCaT

The correct number of HaCaT cells to complete later experiments of DNA fragmentation was determined as indicated in section **3.4.1.1.** of Materials and Methods by carrying out a screening from 1,000 to 20,000 cells. Results showed cell number was directly proportional to absorbance in all the cell number range at the tested incubation time (24 plus 24 hours) (Figure 16):
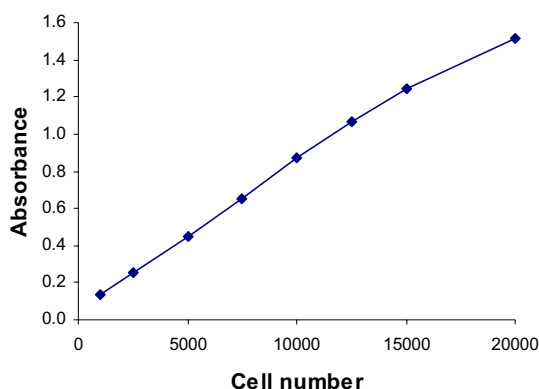
Figure 16. *Cell number versus absorbance of keratinocytes HaCaT after 24 hours plus 24 hours of seeding for a cell number range between 1,000 and 20,000.*

Since there was a lineal proportion along all the cell number range, about 10,000 cells were selected as the optimum number necessary to achieve an 80-100% confluence after 24h of seeding (necessary culture time previous to UV radiation performed in later studies), as estimated by microscope observation.

4.2.1.2. Screening of the total fraction OW

Once determined the optimum culture cell number as 10,000, a screening of the total fraction **OW** was made from 1 to 10 µg/ml in order to discard possible concentrations responsible for altering cell viability of keratinocytes HaCaT.

Results obtained let us confirm that the total fraction **OW** did not decrease cell viability of HaCaT cells up to concentration of 10 µg/ml after 24 hours of incubation considering control as 100% of cell viability (Figure 17):
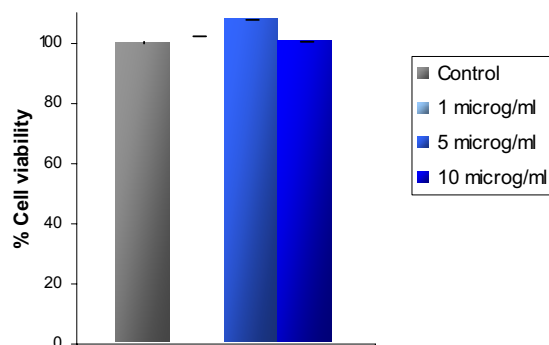
Figure 17. *Cell viability percentage of keratinocytes HaCaT treated with 1, 5 or 10 µg/ml of the total fraction* ***OW*** *for 24 h.*

4.2.1.3. Analysis of DNA fragmentation and possible protective effect of the total fraction OW

Detection of DNA fragmentation provoked by UVB radiation in human keratinocytes HaCaT and potential protection by the total polyphenolic fraction **OW** was performed as largely described in section **3.4.1.3.** of Materials and Methods. The extensive conditions tested for processes of pre-treatment, irradiation and post-incubation let us achieve the following conclusions:

1. Treatment of 24 hours with the total fraction **OW** did not induce DNA fragmentation up to 10 µg/ml (Figure 18).
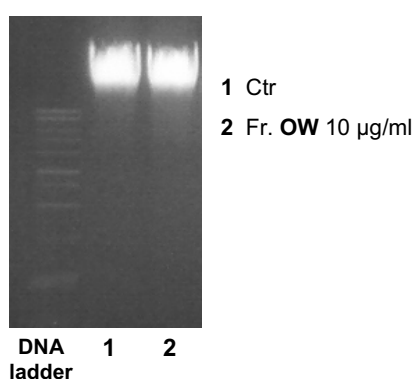
Figure 18. *Absence of DNA fragmentation in HaCaT cells treated 24 hours with the total fraction* ***OW*** *at 10 µg/ml*

2. Irradiating in PBS or culture medium did not alter the production of DNA fragmentation (Figure 19).

1 Ctr
2 UVB 0.3 J/cm^2 (PBS)
3 UVB 0.3 J/cm^2 (medium)

DNA ladder **1** **2** **3**
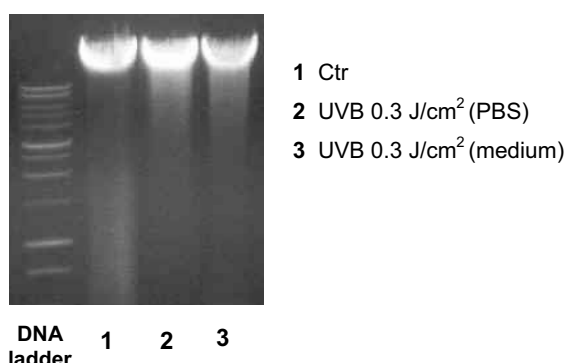
Figure 19. *DNA fragmentation induced by UVB radiation in HaCaT cells irradiated in PBS or culture medium.*

3. Optimum post-incubation time was 24 hours, since fragmentation at 12 and 18 hours was not so clear (Figure 20).

A   **B**   **C**

1 Ctr
2 UVB 0.3 J/cm^2

DNA ladder **1** **2**
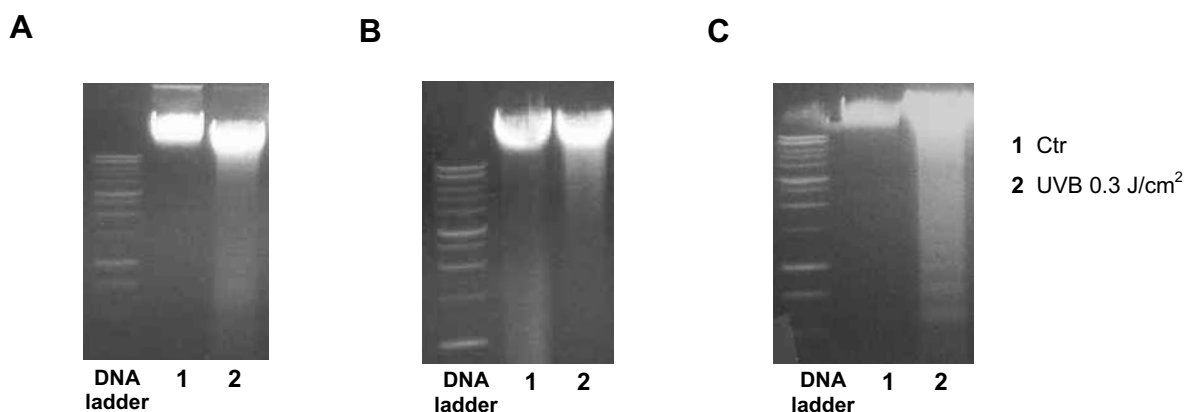
Figure 20. *DNA fragmentation induced by UVB radiation in HaCaT cells after 12 hours (**A**), 18 hours (**B**) or 24 hours (**C**) of post-incubation.*

4. DNA fragmentation was observed for both types of post-incubations conditions: culture medium with 5% or 10% FCS (Figure 21).

1 Ctr
2 UVB 0.3 J/cm^2- 5% FCS
3 UVB 0.3 J/cm^2- 10% FCS

DNA ladder **1** **2** **3**
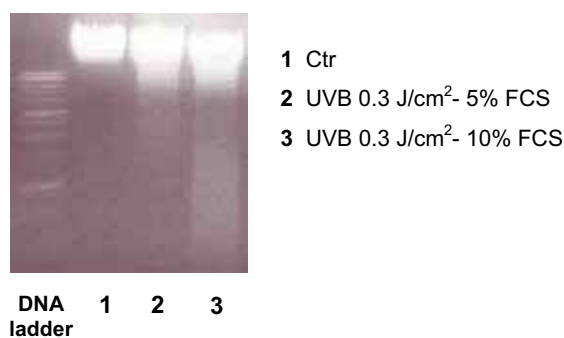
Figure 21. *DNA fragmentation induced by UVB radiation in HaCaT cells after post-incubation in culture medium with 5 or 10% FCS.*

5. No protective effect of the total fraction **OW** at 5 µg/ml was observed for any of the conditions tested: only pre-treated; pre-treated and irradiated; or pre-treated, irradiated and post-incubated with the fraction (Figure 22).

1 Ctr
2 UVB 0.3 J/cm^2
3 Fr. **OW** 5 µg/ml + UVB 0.3 J/cm^2
4 Fr. **OW** 5 µg/ml + UVB 0.3 J/cm^2 with Fr. **OW**
5 Fr. **OW** 5 µg/ml + UVB 0.3 J/cm^2 with Fr. **OW** + Fr. **OW** 5 µg/ml
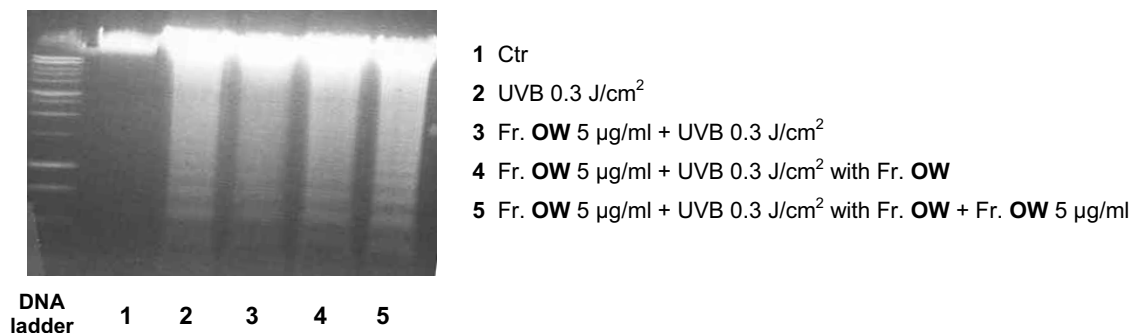
Figure 22. DNA fragmentation induced by UVB radiation in HaCaT cells pre-treated (24h); pre-treated (24h) and irradiated; or pre-treated (24h), irradiated and post-incubated (24h) with 5 µg/ml of the total fraction OW.

6. Only slight protective effect of the total fraction **OW** was observed when the concentration was increased to 10 µg/ml for both conditions: only pre-treated or pre-treated and post-incubated with the fraction (Figure 23).

1 Ctr
2 UVB 0.3 J/cm^2
3 Fr. **OW** 10 µg/ml + UVB + Fr. **OW** 10 µg/ml
4 Fr. **OW** 10 µg/ml + UVB
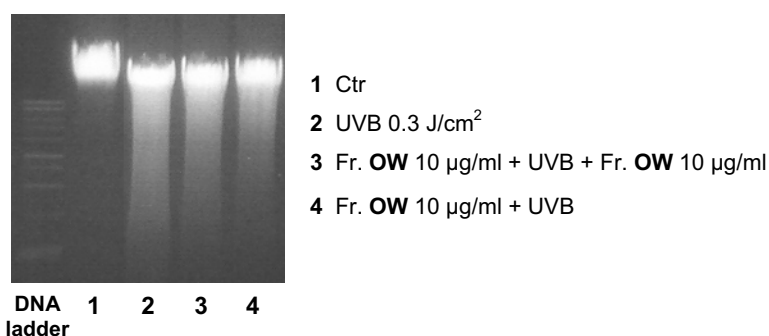
Figure 23. DNA fragmentation induced by UVB radiation in HaCaT cells pre-treated (24h); or pre-treated (24h), and post-incubated (24h) with 10 µg/ml of the total fraction.

As it is shown, UVB-induced DNA fragmentation, a later process in UV-induced apoptosis [Collins et al., 1997], was caused by a considerably elevated radiation dose (0.3 J/cm^2). Moreover, protective effect of the total fraction **OW** against that damage was not much accentuated and it occurred with concentrations equal to 10 µg/ml. Because of it, we though it was interesting to study the possible molecular mechanisms underlying this apoptotic cell death, which are activated by shorter UV radiation doses, and the effect of the polyphenolic fractions in these early events.

4.2.2. UV CYTOTOXICITY IN HUMAN QUIESCENT KERATINOCYTES HaCaT

4.2.2.1. Screening of human quiescent keratinocytes HaCaT

Due to the fact that the UV-induced cell death and MAPKs (mitogen-activated protein kinases) activation had to be analyzed on quiescent HaCaT adherent cells, a new screening was performed. The optimum number of HaCaT cells was determined by culturing from 7,000 to 12,500 cells as indicated in section **3.4.2.1.** of Materials and Methods. Although the absorbance seemed to increase proportionally to the cell number for both incubation times (6 hours for posterior MAPKs analysis and 6 plus 24 hours for later UV cytotoxicity assays) (Figure 24), 7,000 was chosen as the most adequate number of cells since it resulted in 80-100% cell confluence after 24 hours of seeding plus 48 hours of serum deprivation (necessary culture time previous to pre-treatment and/or UV radiation performed in later studies).
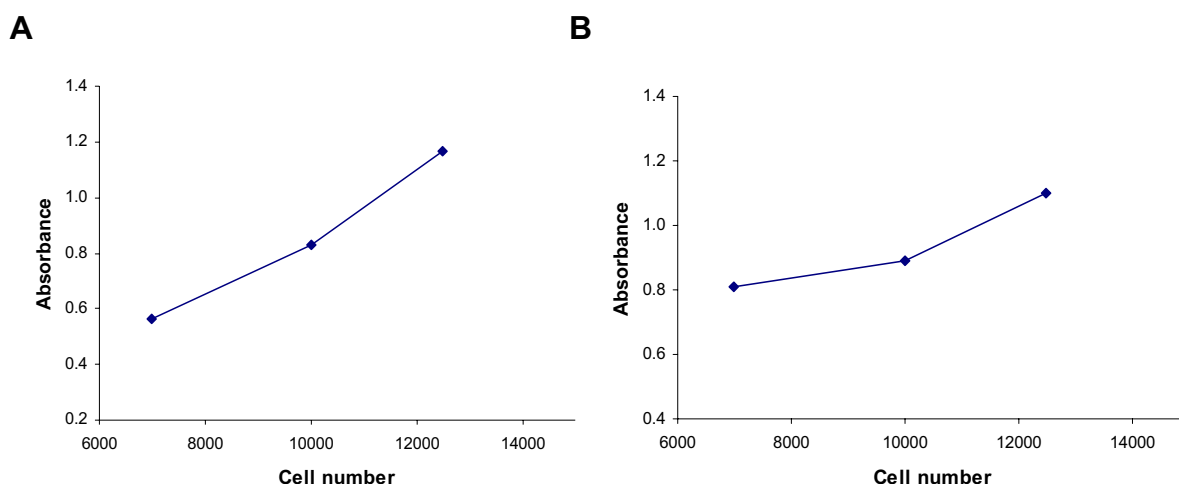
Figure 24. *Cell number versus absorbance of keratinocytes HaCaT after 24 h of seeding plus 48 h of serum deprivation followed by 6h (**A**) or 6 plus 24h (**B**) for a cell number range between 7,000 and 12,500 cells.*

4.2.2.2. Screening of polyphenolic fractions

Once the optimum cell number was estimated to be 7,000, and to check if 6 hours pre-treatment with the polyphenolic fractions (**OW**, **V, IV** and **XI**) altered viability of HaCaT cells, a concentration screening from 5 to 20 µg/ml was carried out as explained in section **3.4.2.2.** of Materials and Methods. As performed in other screenings, absorbance of control was considered as 100% of cell viability. Results showed that none of the tested concentrations for the total fraction **OW** and the derived fraction **V** altered

cell viability respect to the control. However, concentrations higher than 5 μg/ml for the derived fractions **IV** and **XI** reduced cell viability of quiescent keratinocytes HaCaT in a significant manner (20% and 50% for concentrations equal to 10 and 20 μg/ml respectively) (Figure 25).
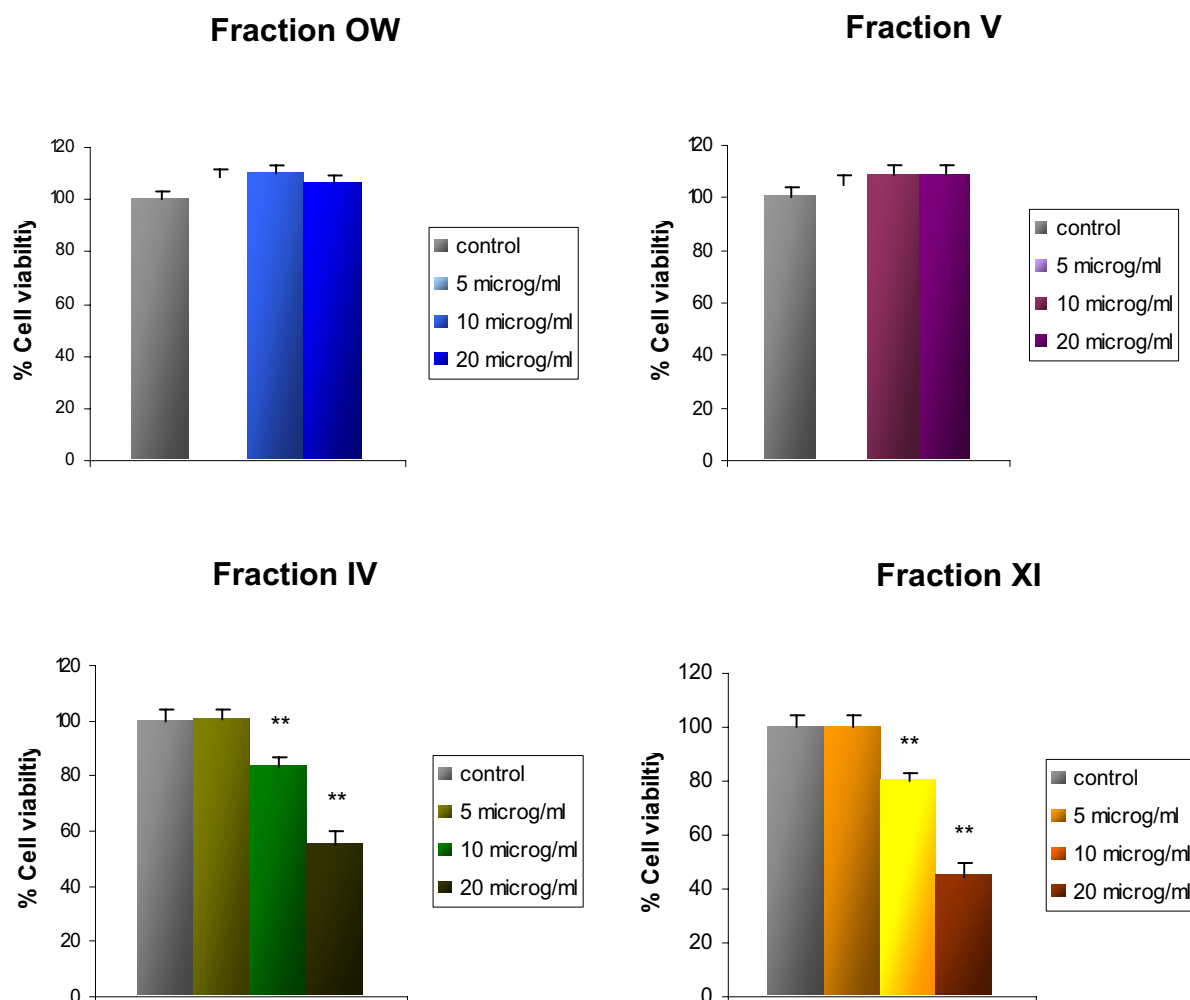
Figure 25. *Cell viability percentage of quiescent keratinocytes HaCaT treated with 5, 10 and 20 μg/ml of the total fraction OW or the derived fractions V, IV or XI for 6 h and post-incubated 24h. Results are expressed as mean ± sem (n=3). * p < 0.05 ** p < 0.001.*

4.2.2.3. Determination of UV cytotoxicity and possible protective effect of polyphenolic fractions

Cytotoxicity caused by UVB and UVA radiations on human quiescent keratinocytes HaCaT was studied as described in section **3.4.2.3.** of Material and Methods. Additionally, to investigate the potential protective capacity of polyphenolic fractions against UV-induced cell death, 6 hours pre-treatments with fraction concentrations that

not affect cell viability (5, 10 and 20 µg/ml for the total polyphenolic fraction **OW** or the derived fraction **V**, and 5 µg/ml for the derived fractions **IV** or **XI)**, were tested.

It was proven that both UVB and UVA radiation induced significant cell death of quiescent keratinocytes HaCaT at all the irradiation intensities tested (0.03 and 0.05 J/cm^2 for UVB, or 10, 20 and 30 J/cm^2 for UVA) (Figure 26).

UVB   **UVA**
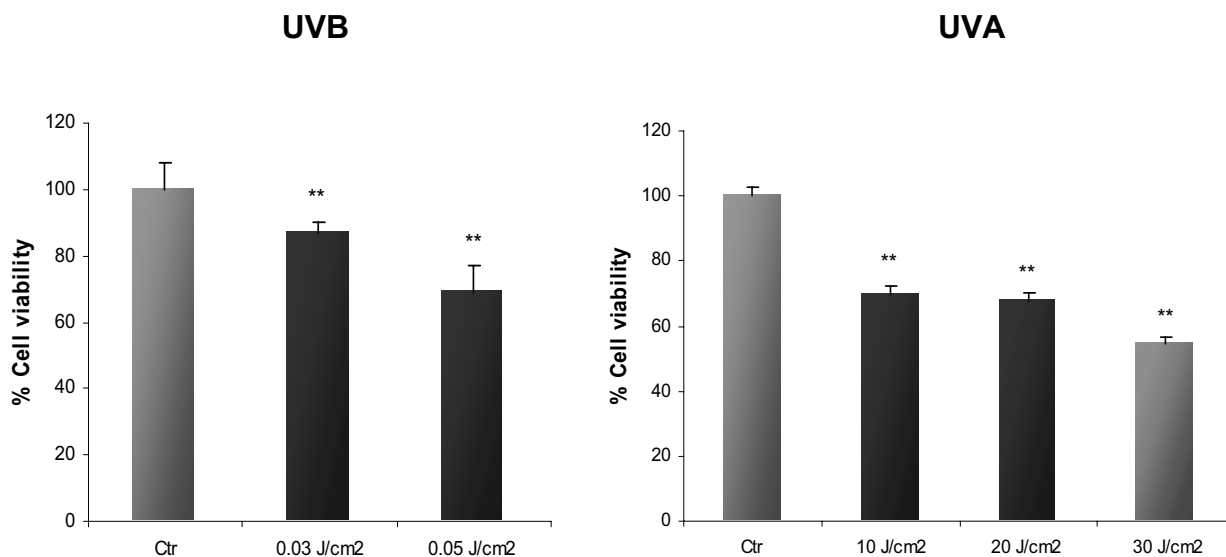
Figure 26. *Cell viability percentage of quiescent keratinocytes HaCaT after irradiation with UVB (0.03 or 0.05 J/cm^2) or UVA (10, 20 or 30 J/cm^2) and 24h of post-incubation. Results are expressed as mean ± sem (n=3). * p < 0.05 ** p < 0.001*

Finally, an irradiation intensity that caused about 30% of cell death in both UVB and UVA radiations was selected for posterior experiments where the potential protective capacity of polyphenolic fractions was tested after pre-treatments of 6 hours. Thus, pre-treated cells at the concentrations indicated above were irradiated at 0.05 J/cm^2 for UVB or 20 J/cm^2 for UVA and post-incubated without fraction for 24 hours before determination of cell viability. Assays of cells pre-treated with the polyphenolic fractions showed that none of the concentrations tested (5, 10 or 20 µg/ml for the total polyphenolic fraction **OW** or the derived fraction **V** and 5 µg/ml for the derived fractions **IV** or **XI**) seemed to avoid the cytotoxicity caused by either UVB or UVA radiations (Figure 27).
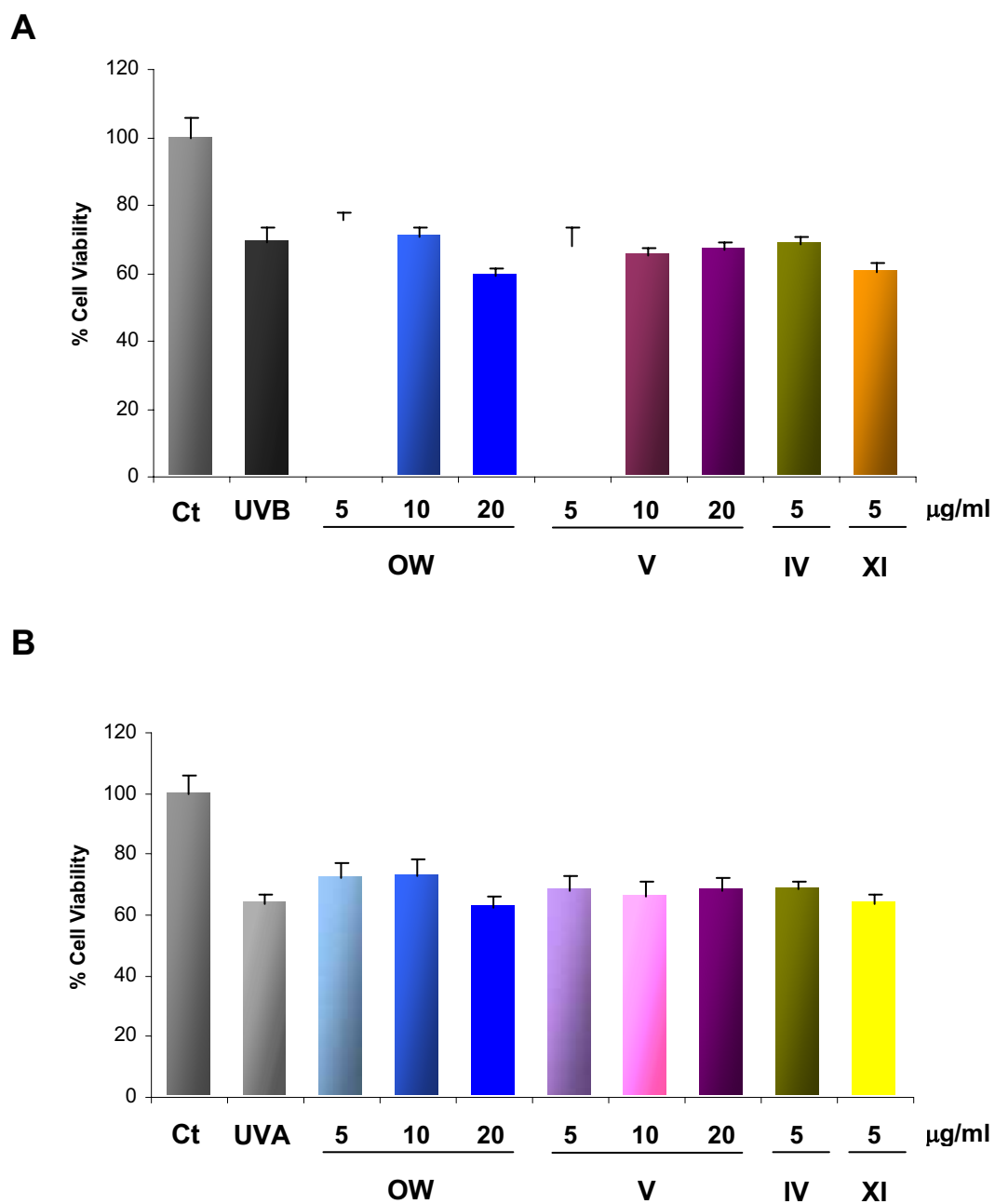
Figure 27. *Cell viability percentage of quiescent keratinocytes HaCaT pre-treated 6 hours with the polyphenolic fractions **OW**, **V**, **IV** or **XI**, irradiated with UVB (**A**) or UVA (**B**) lights and post-incubated for 24 hours. Results are expressed as mean ± sem (n=3-4). * $p < 0.05$ ** $p < 0.001$.*

4.2.3. UV-INDUCED MAPKs ACTIVATION IN HUMAN QUIESCENT KERATINOCYTES HaCaT

4.2.3.1. Detection of active and total MAPKs and possible protective effect of polyphenolic fractions

Activation of mitogen-activated protein kinases ERK1/2, p38 and JNK1/2 was studied after UV radiation of HaCaT cells pre-treated with the polyphenolic fractions at the same concentrations tested before in the study of UV- induced cell death (5, 10 or 20 µg/ml for the total polyphenolic fraction **OW** or the derived fraction **V** and 5 µg/ml for the derived fractions **IV** or **XI**).

4.2.3.1.1. Inhibition of ERK1/2 activation by the total polyphenolic fraction OW

Initial studies of MAPKs activation in quiescent HaCaT cells pre-treated with the total fraction **OW** at 20 µg/ml for 24 hours and post-incubated 3 hours showed, interestingly, that this fraction was able to reduce the baseline active ERK1/2 level (phospho-ERK1/2) of control non-irradiated cells, without changing the total amount of protein (ERK2). On the contrary, absence of inhibition in slight baseline phospho-p38 and -JNK1/2 levels of control non-irradiated cells seemed to be found for the same assay (Figure 28).
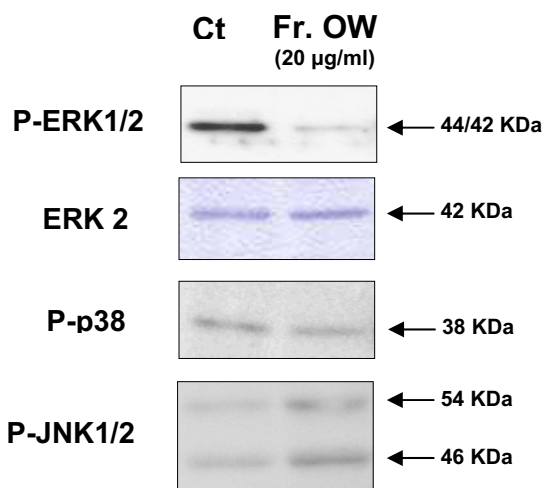
Figure 28. *Expression of active MAPKs (phospho-ERK1/2, -p38 and –JNK1/2) and total ERK2 in non-irradiated keratinocytes HaCaT treated or not with 20 µg/ml of the total fraction **OW** for 24 hours*

In view of the fact that 24 hours incubation with the total polyphenolic fraction **OW** seemed to inhibit ERK1/2 activation, we decided to test it more accurately by reducing

the incubation time. In short, quiescent HaCaT cells were pre-treated with the fraction **OW** at 10 or 20 µg/ml for 6 hours and post-incubated 30 minutes with standard culture medium supplemented with growth factors (10% FCS) to activate ERK1/2. Results indicated a dose dependent inhibition of phospo-ERK1/2 by the total fraction **OW** after 6 hours of treatment (Figure 29).
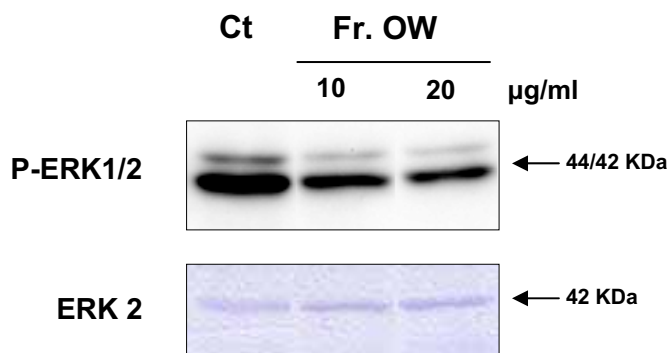
Figure 29. *Expression of phospho-ERK1/2 and ERK2 in non-irradiated keratinocytes HaCaT treated with 10 or 20 µg/ml of the total fraction OW for 6 hours.*

Thus, confirmation of baseline phospho-ERK1/2 level inhibition by the fraction **OW** let us discard these MAPK for the study of possible protective effect of polyphenolic fractions against UV damage, since the results would be difficult to evaluate. Besides, these extracellular receptor kinases (ERK1/2) are poorly activated in stress conditions as oxidative stress [Assefa et al.,1997], which we are interested in.

4.2.3.1.2. Timing of p-38 and JNK1/2 activation after UVB or UVA radiations.

To properly settle an optimum post-incubation time for p38 and JNK1/2 activation after both UVB (0.05 J/cm^2) and UVA (20 J/cm^2) lights, timing from 30 minutes to 2 hours was set up with quiescent HaCaT cells. The results showed that 30 minutes were sufficient for the activation of both p38 and JNK1/2 after the two types of exposure. Moreover, that activation seemed to be more accentuated after UVA radiation. The phospho levels of the two MAPKs (phospho-p38 and phospho-JNK1/2) appeared to decrease with longer post-incubation times, being that reduction more accentuated after UVA radiation (Figure 30A).

The amount of total proteins seemed identical for non-irradiated and irradiated cells upon both UVB and UVA radiations. The apparent over expression in the total p38 protein

of UVA respect to UVB was due to a protein load error as demonstrated by the analysis of α-actin (Figure 30B).
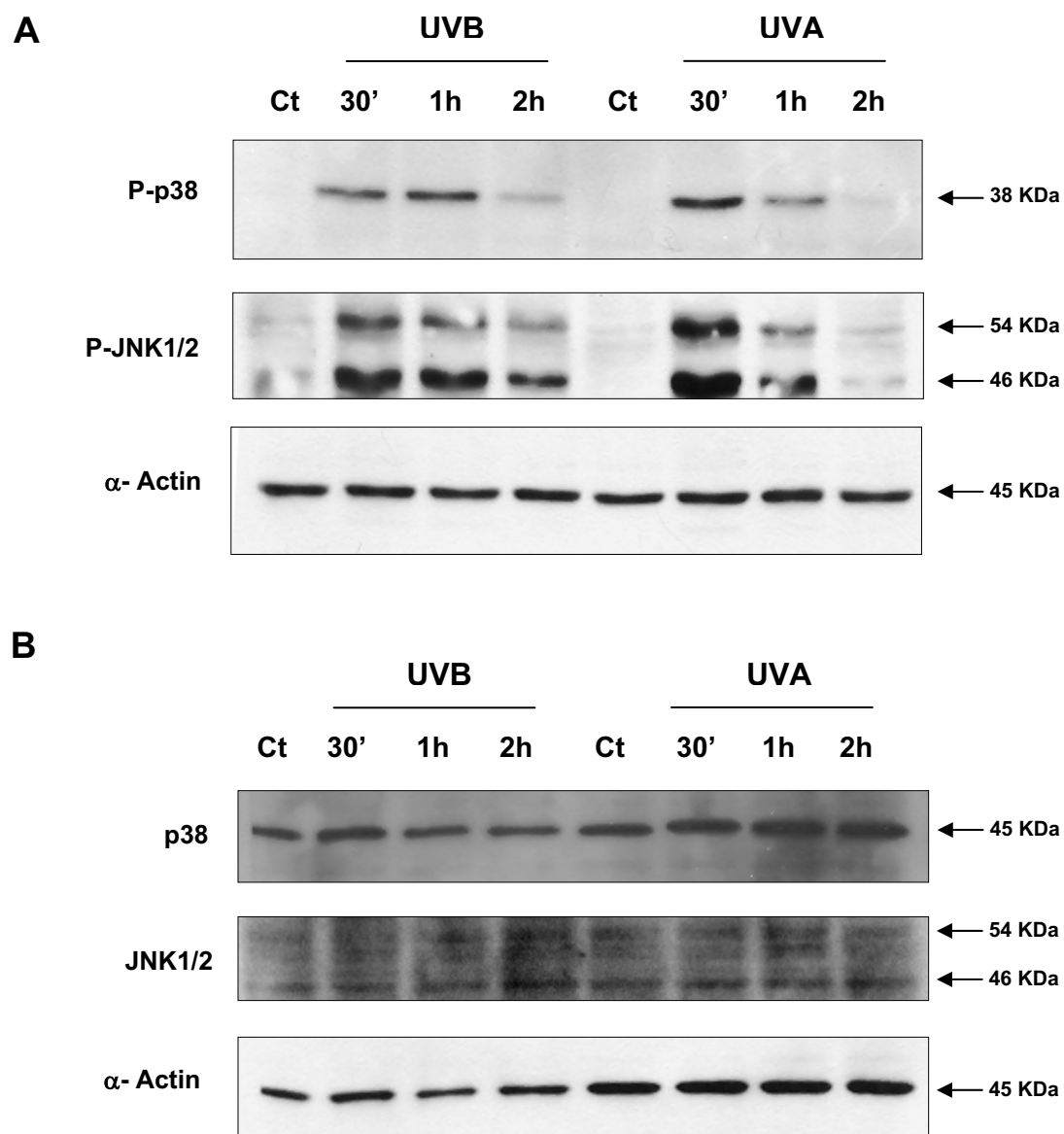
Figure 30. *Expression of active (phospho-p38 and -JNK1/2) (**A**) and total (p38 and JNK1/2) (**B**) MAPKs in UVB or UVA radiated keratinocytes HaCaT after 30 min., 1 and 2 hours of post-incubation. α-actin was used as loading control.*

4.2.3.1.3. Evaluation of p38 and JNK1/2 activation after UVB or UVA radiations and possible protective effect of polyphenolic fractions

Once the optimum post-incubation time had been determined as 30 minutes, activation of MAPKs that respond to various stress events such as oxidation processes (p38 and JNK1/2) [Johnson et al., 2002] was studied, as well as the possible capacity of the total polyphenolic fraction **OW** and the derived fractions **V**, **IV** and **XI** to avoid such activation. In this case, the concentrations tested 5, 10 and 20 µg/ml for the total polyphenolic fraction **OW** and only 5 µg/ml for all the derived fractions (**V**, **IV** and **XI**).

As shown in Figure 31A, 6 hours pre-treatment with any of the polyphenolic fractions seemed to protect against p38 and JNK1/2 activation. When cells were irradiated with UVA this effect was clearer for the highest concentration of the total fraction **OW** (20 µg/ml) and 5 µg/ml for the derived fractions **V, IV** and **XI**. Similar results were obtained for UVB radiation except for the derived fraction **V** at 5 µg/ml, which apparently did not inhibit p38 activation.

The amount of total proteins seemed identical for non-irradiated and irradiated cells at both UVB and UVA radiations (Figure 31B).

A

B
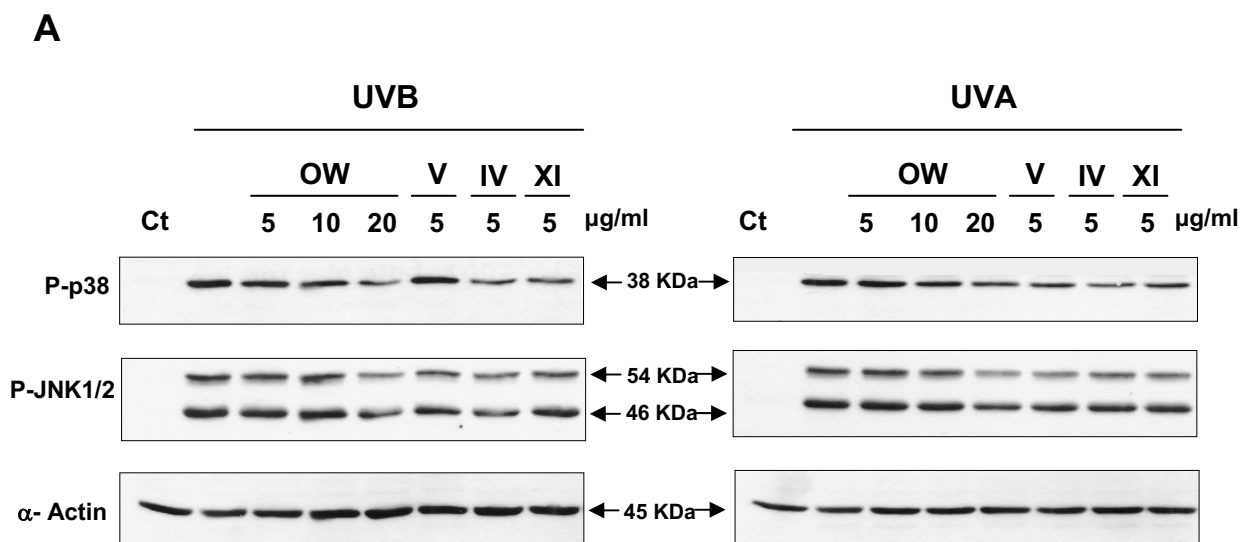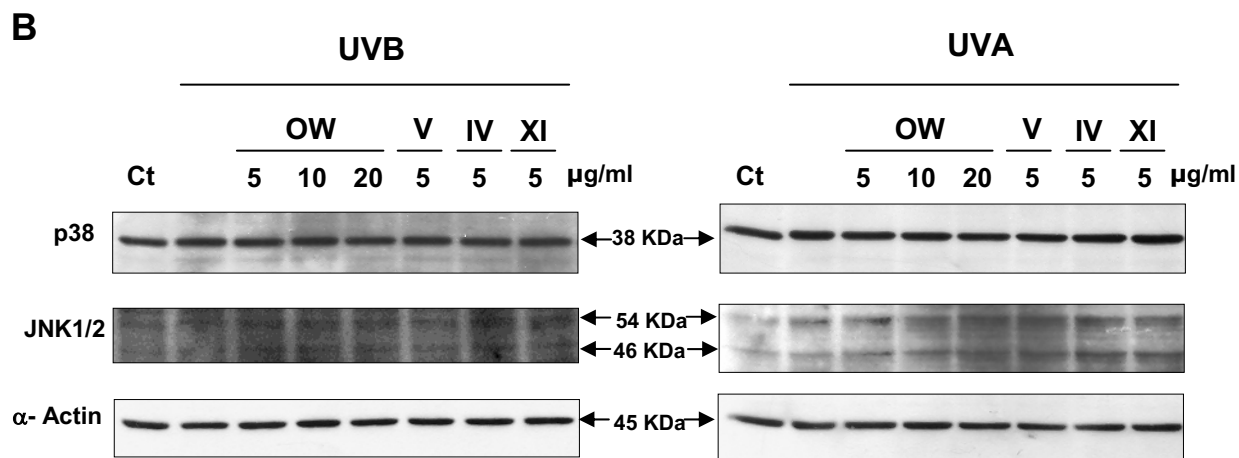
Figure 31. *Expression of active (phospho-p38 and -JNK1/2) (**A**) and total (p38 and JNK1/2) (**B**) MAPKs in keratinocytes HaCaT pre-treated with the polyphenolic fractions, UVB or UVA radiated and post-incubated 30 min. α-Actin expression was used as loading control. Blots are representative of three independent experiments.*

Quantification of active forms of tested MAPKs (phospho-p38 and -JNK1/2) (Figure 31A) was carried out referring density of phospho-p38 or –JNK1/2 to density of the corresponding α-actin bands and considering density of UV-radiated non-treated cell bands as 100% activation.

The results demonstrated the dose dependent protective effect against p38 activation for the total fraction **OW** and this effect seemed to be higher in UVB-radiated cells (Figure 32A), where all fractions decreased activation in a significant manner. Moreover, protection of the derived fractions **V**, **IV** and **XI** was quite similar for both UVB and UVA-radiated cells and it was higher for the fractions **IV** and **XI**, which presented about 50-60% reduction, while the fraction **V** resulted in 25% decrease. Additionally, 50-60% reduction by derived the fractions **IV** and **XI** was only achieved at 10-20 µg/ml of the total fraction **OW** in UVB-radiated cells. When cells were UVA radiated, the highest concentration of the total fraction **OW** (20 µg/ml) was not even able to reduce p38 activation at the same extend that the derived fractions **IV** and **XI**. As occurred for p38, protection against UV-induced JNK1/2 activation (Figure 32B) was generally higher on UVB-radiated cells and dose dependent, as observed for the fraction **OW**. However, the protective effect of the total fraction **OW** (5 µg/ml) was similar to the effect produced by the derived fractions **V**, **IV** and **XI** at the same concentration (about 30-40% inhibition); and the inhibitory effect achieved by the highest concentration of the total fraction **OW** (20 µg/ml) was around 60-70%.

A
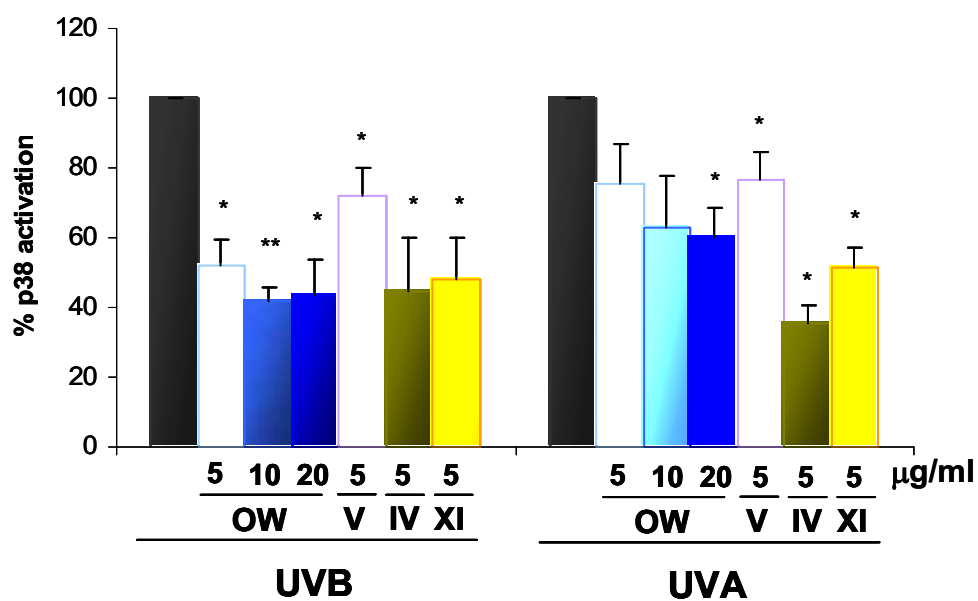
B
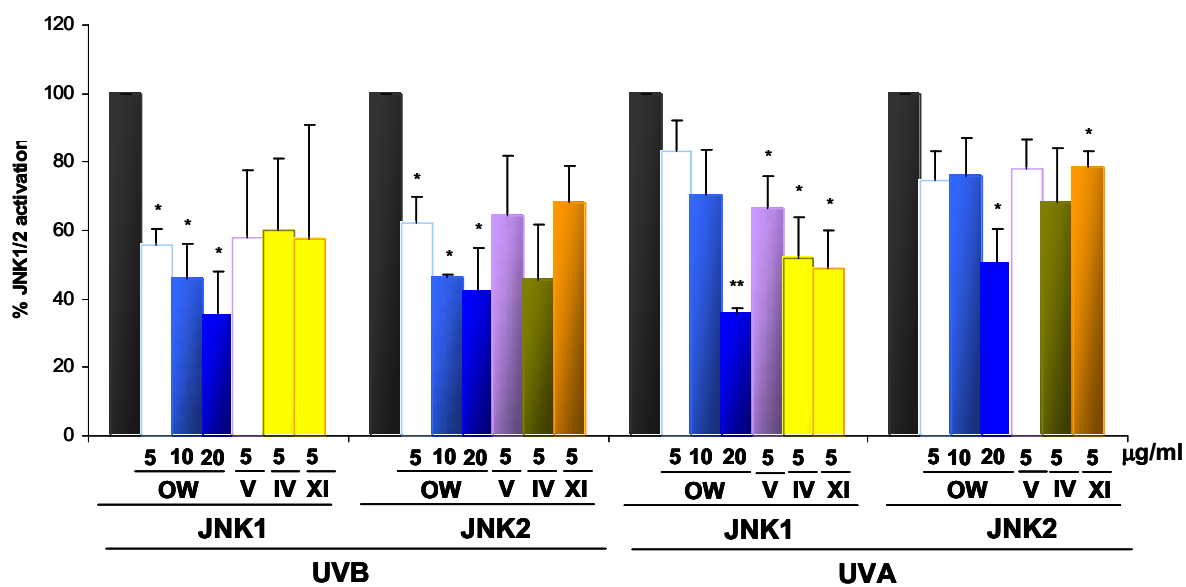
Figure 32. *Percentage of p38 (**A**) and JNK1/2 (**B**) activation of keratinocytes HaCaT pre-treated with the polyphenolic fractions **OW**, **V**, **IV** or **XI**, and UVB or UVA radiated. Expression of P-p38 and P-JNK1/2 is referred to α-Actin.. Results are expressed as mean ± sem (n=2-3). * p < 0.05 ** p < 0.001*

4.2.4. INTRACELLULAR H$_2$O$_2$ AFTER UV RADIATION OF HUMAN QUIESCENT KERATINOCYTES HaCaT

4.2.4.1. Detection of intracellular H$_2$O$_2$ after UV exposure and possible protective effect of polyphenolic fractions

Ultraviolet radiation is a potent inductor of reactive oxygen species (ROS) such as superoxide radical ($\cdot$O$^-_2$), hydroxyl radical ($\cdot$OH$^-$) and hydrogen peroxide (H$_2$O$_2$) [Peus et al., 1998]. To study the potential protective effect of the total polyphenolic fraction **OW** and the derived fractions **V**, **IV** and **XI** against this induction, the increase in intracellular levels of H$_2$O$_2$ was studied in quiescent keratinocytes HaCaT pre-treated or not with those fractions, UV radiated and post-incubated 30 minutes as explained in section **3.4.4.** of Materials and Methods.

4.2.4.1.1. Reduction of baseline H$_2$O$_2$ levels by polyphenolic fractions

ROS, including hydrogen peroxide are not only generated under cellular stress but are also constitutively produced in organisms during normal cellular function as a consequence of aerobic metabolism [Halliwell, 1995]. Therefore, it was interesting to note that the studied polyphenolic fractions (total fraction **OW** and derived fractions **V**, **IV** and **XI**) decreased baseline H$_2$O$_2$ levels in a significant manner. Additionally, that reduction was dose dependent as observed for the total fraction **OW** and was lower for the derived fraction **V** at 5 µg/ml respect to the other derived fractions **IV** and **XI** at the same concentration (about 20% instead of 40-50% reduction of the fractions **IV** and **XI**). Moreover, the decrease in H$_2$O$_2$ levels observed for 5 µg/ml of the derived fractions **IV** and **XI** was only achieved by 10-20 µg/ml of the total fraction **OW** (Figure 33).
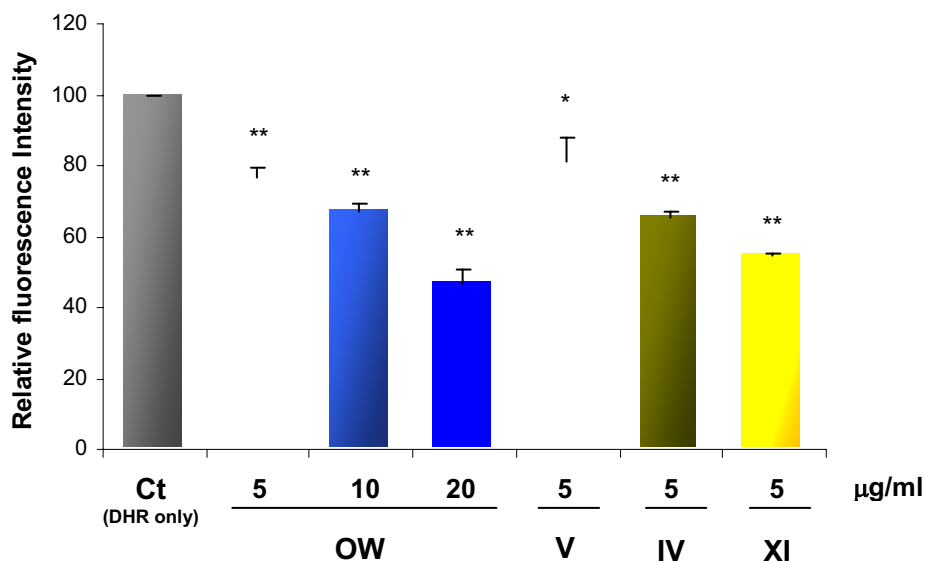
Figure 33. *Relative fluorescence intensity of quiescent keratinocytes HaCaT pre-treated 6 hours with the polyphenolic fractions **OW**, **V, IV** or **XI**, stained with DHR and post-incubated 30 minutes. Results are expressed as mean ± sem (n=3). * p < 0.05 ** p < 0.001*

4.2.4.1.2. Increase of intracellular H_2O_2 after UV radiation

As previously described [Peus et al., 1998], oxidative stress caused by UV radiation increases baseline of ROS such as intracellular H_2O_2. Our studies in UV radiated quiescent keratinocytes HaCaT confirmed that augment, being significant for both UVB and UVA radiation, although it was about 6-fold higher after UVA exposure and less than 2-fold higher after UVB radiation (Figure 34).
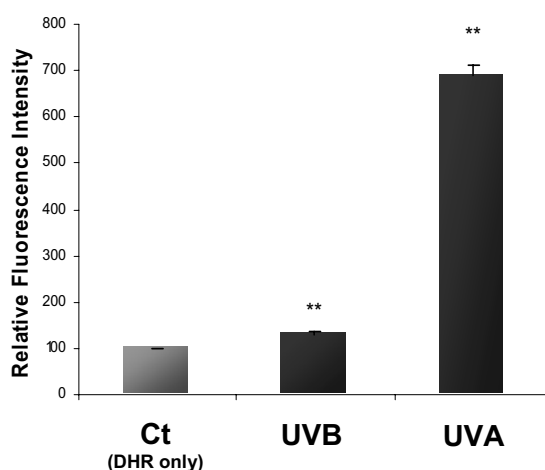
Figure 34. *Relative fluorescence intensity of quiescent keratinocytes HaCaT stained with DHR, irradiated with UVB or UVA lights and post-incubated 30 minutes. Results are expressed as mean ± sem (n=3). * p < 0.05 ** p < 0.001*

4.2.4.1.3. *Possible protective effect of polyphenolic fractions against UV-induced intracellular H_2O_2 increase*

As explained above, the potential capacity of the polyphenolic fractions to protect against intracellular H_2O_2 generation induced by UV radiation was studied in quiescent keratinocytes HaCaT pre-treated with the fractions at the same concentrations used for MAPKs detection assays (5, 10 and 20 µg/ml for the total polyphenolic fraction **OW** and 5 µg/ml for the derived fractions **V**, **IV** and **XI**).

The results showed all fractions reduced hydrogen peroxide levels at the tested concentrations in a significant manner after both UVB and UVA radiations, except for the fraction **V**, which failed to protect at 5 µg/ml (the concentration studied for the derived fractions). Moreover, the decrease produced by the total fraction **OW** was dose dependent and 10-20 µg/ml of that fraction was necessary to achieve the same reduction (50-60%) than that generated by the derived fractions **IV** and **XI** after UVB or UVA exposures (Figure 35). Additionally, these polyphenolic fractions were able to reduce generation H_2O_2 to lower values than the control non-irradiated cells when were UVB radiated (Figure 35A), which was probably due to their ability of decreasing baseline H_2O_2 levels in non-irradiated cells. Since increase in H_2O_2 production was much higher after UVA radiation, the polyphenolic fractions **OW**, **IV** and **XI** were not able to reduce it to such a extend as the found for UVB radiation (Figure 35B).

A
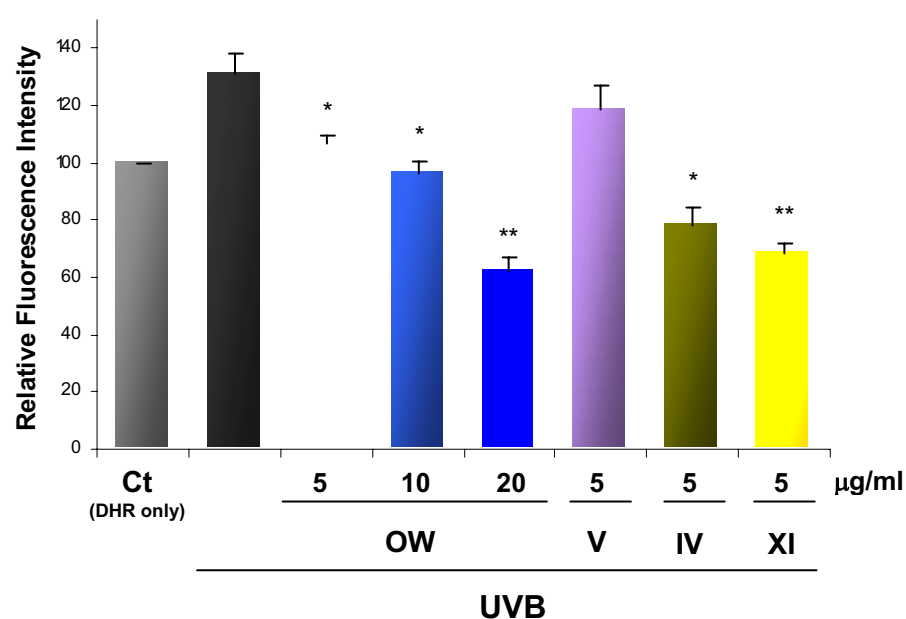
B
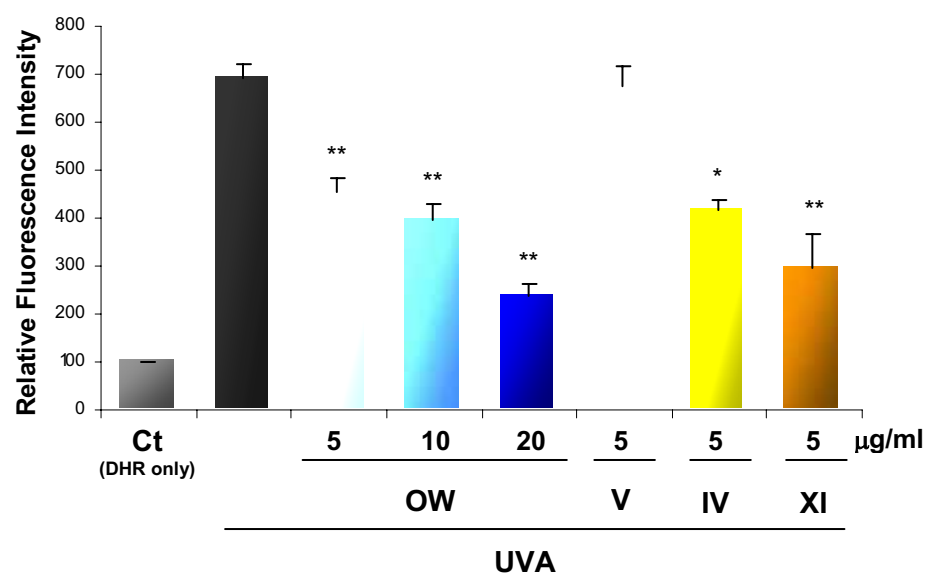
Figure 35. *Relative fluorescence intensity of quiescent keratinocytes HaCaT pre-treated with the polyphenolic fractions **OW**, **V**, **IV** or **XI**, stained with DHR, irradiated with UVB (**A**) or UVA (**B**) light, and post-incubated 30 minutes. Results are expressed as mean ± sem (n=3). * p < 0.05 ** p < 0.001*

5. DISCUSSION

5.1. ANTITUMORAL EFFECT OF THE POLYPHENOLIC FRACTIONS OW, I AND II AT CELLULAR AND METABOLIC LEVELS

Impressive progress is being made in defining the role that bioactive compounds have in reducing the risk of major chronic diseases such as cancer and the underlying biological mechanisms that account for these effects. Antioxidants such as polyphenols, which are constituents of various classes of foods and beverages, have been thoroughly tested during recent years [Yang et al., 2001]. Flavonoids belong to a vast group of phenolic compounds that are widely distributed in all foods of plant origin. Compelling data from *in vitro* and *in vivo* laboratory studies, epidemiological investigations, and human clinical trials indicate that flavonoids have important effects on cancer chemoprevention and therapy [Galati et al., 2004].

The beneficial anticarcinogenesis effects of antioxidants as flavonoids have been attributed to their ability to scavenge endogenous ROS. However, their pro-oxidant action rather than their antioxidant function may be an important mechanism for their anticancer and apoptosis-inducing properties, as reactive oxygen species and flavonoid radicals generated can damage DNA, lipids and other biological molecules [Decker et al., 1997; Hadi et al., 2000; Li et al., 1994]. Additionally, recent studies have speculated that flavonoids may not act as conventional hydrogen-donating antioxidants but may affect cellular function through actions at protein kinase and lipid kinase signalling pathways, [Williams et al., 2004].

5.1.1. Composition of studied polyphenolic fractions and their importance

The polyphenolic fractions used in this study (total fraction **OW**, and the derived fractions **I** and **II**), were obtained from a grape by-product consisting of grape skins, seeds and stems and were mainly composed by flavanol monomers with or without gallate groups, glycosylated flavonols and mostly procyanidin oligomers. In contrast, the predominant flavanols in green tea are monomeric catechins, such as (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin 3-*O*-gallate (ECG) and (−)-epigallocatechin 3-*O*-gallate (EGCG), the last one being the major flavanol component [Ho, 1992]. Although the antioxidant and antiproliferative properties of these monomeric flavanols have been demonstrated in numerous *in vitro* assays [Ahmad et al., 1997; Smith et al., 2001; Kuo et al., 2003], it has also been shown that the green tea major component (EGCG), which contains a pyrogallol group in the B ring as shown in Figure

1A in section **1.3.1.** of Introduction, can be pro-oxidant through the formation of hydrogen peroxide under different experimental conditions. This pro-oxidant effect, detected at the higher concentrations of tea polyphenols in cell culture systems, has hardly been noticed or not detected at all for EC and ECG [Long et al., 2000], which are major components of grape procyanidins [Prieur et al., 1994; Souquet et al., 2000]. Moreover, recent studies carried out in our laboratory showed the flavanol derivative 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (Cys-ECG) obtained from grape procyanidins and lacked of the pyrogallol group in the B ring, has a similar antioxidant capacity than EGCG but with the advantage that Cys-ECG enhanced the scavenging capacity by hydrogen atom donation rather than by electron transfer, which is related to the pro-oxidants effects of catechins with a pyrogallol group in the B ring such as EGC and EGCG as shown Figure 1A in section **1.3.1.** of Introduction [Lozano et al., 2005]. Hence, the polyphenolic fractions used in this study, obtained from a grape by-product consisting of grape skins, seeds and stems, may have a great safety advantage compared to green tea extracts because the polyphenolic fractions analyzed contain vary low quantities of trihydroxylated EGC and EGCG.

Recent studies carried out by Raza et al have demonstrated low concentrations of EGCG up to 10 µM did not affect level of peroxides in PC12 cells, while a marked increase was observed at higher concentrations from 50 to 800 µM. [Raza et al., 2005]. Those results have suggested an induction of antioxidant defence metabolism by EGCG at low concentrations, whereas the antioxidant effects of EGCG are masked by the increased oxidative stress at high concentrations, which advises that dietary flavonoids could be potentially more of an oxidative risk than a benefit when taking large amounts in the form of a concentrated supplement [Decker et al., 1997].

5.1.2. Antiproliferative effect of polyphenolic fractions and absence of cytotoxicity to PBLs

The analysis performed in the human melanomas SK-Mel-28 and A-375, the mouse hepatoma Hepa-1c1c7 and human leukemia Jurkat cells showed that the polyphenolic fractions studied (total fraction **OW**, and the derived fractions **I** and **II**), inhibited the growth of those tumour cell lines. These results are in overall agreement with numerous studies that earlier demonstrated the antiproliferative effects of flavonoids [Kampa et al., 2000; Briviba et al., 2002; Rodriguez et al., 2002]**.** In the case of the specific polyphenolic fractions tested in this study, the concentration that altered cell viability was

within the micromolar range, being the slow growth human melanoma SK-Mel-28 the tumoral cell line with the highest IC_{50} values.

It has been described that the antioxidant activity depends on polymerization and increases with galloylation [Plumb et al., 1998]. Although the derived fraction **II** is particularly rich in EGC (31% galloylation) and oligomeric flavanols, it was not the most efficient fraction in inhibiting cell growth, since their IC_{50} values were approximately 30-50% higher than the corresponding values for the fractions **OW** and **I**, except for leukemia Jurkat cells, where it seemed to have the same efficiency to the fractions **OW** and **I**. This is probably due to the fact that this derived fraction **II** also contains glycosylated flavonols which may decrease its antioxidant properties and its subsequent antiproliferative activity [Plumb et al., 1999]. Potential synergistic effect on the polyphenolic compounds of the two derived fractions **I** and **II** was observed for SK-Mel-28 and Hepa-1c1c7 cells, where IC_{50} values for the total fraction **OW** were lower than their respective IC_{50} values for the derived fractions **I** or **II**, but not for A-375 or Jurkat cells.

Since we were interested in studying the metabolic changes induced by the polyphenolic fractions at initial stages, when cell death was not as much as fifty per cent, the study of substrate carbon flow using mass isotopomer distribution analysis (MIDA) was carried out on leukemia Jurkat cells treated with the polyphenolic fractions (**OW**, **I** or **II**) for shorter times (24 and 48 hours) at lower concentrations (40 µg/ml) than the treatments performed on analysis of cell cycle and apoptosis (72 hours; IC_{50} values Table 8 in Results section). That study showed a reduction in *de novo* synthesis of the nucleic acid precursor ribose, in leukemia Jurkat cells treated with any of the polyphenolic fractions studied (fractions **OW**, **I** or **II**), although that effect was more accentuated after treatment with the fraction **II**. These data led us to consider that the polyphenolic compounds of those fractions may control tumour cell proliferation at least in part through the regulation of glucose carbon redistribution.

The analysis of cell viability carried out with normal human peripheral blood lymphocytes (PBLs) confirm these polyphenolic fractions as very specific antiproliferative agents with very low cytotoxicity to non-proliferative normal cells. This combination of high efficacy and safety was more pronounced for the total fraction **OW** and the derived fraction **II**, each of which resulted cytotoxic to PBLs only at concentrations about 8-fold higher than their respective IC_{50} values in Jurkat cells. However, the derived fraction **I**

was more cytotoxic to PBLs than the fractions **OW** and **II**, beginning its effect only at concentration 4-fold higher than its IC_{50} value in Jurkat cells.

5.1.3. Effects on cell cycle and induction of intrinsic mitochondrial apoptosis pathway

The slight cell cycle arrest in G0/G1 phase observed in human melanomas SK-Mel-28 and A-375 treated with the derived fractions **I** or **II** concord with the effect of EGCG in several tumour cell lines, including breast, epidermoid, prostate, and head and neck squamous cell cancers [Masuda et al., 2001; Lin et al., 1999; Ahmad et al., 2000; Liberto et al., 2000; Gupta et al., 2003]. Moreover, the results obtained in the present study are in agreement with the induction of apoptosis *in vivo* by polyphenols described in colon tumours [Caderni et al., 2000] and show that the degree of galloylation of polyphenolic fractions correlates with the capacity to alter cell cycle and induce apoptosis. Accordingly, the derived fraction with the highest degree of polymerization and galloylation (fraction **II**, 31% galloylation) induced apoptosis in all the tumoral cell lines analyzed, except for the slow growth human melanoma SK-Mel-28, where none of the polyphenolic fractions triggered apoptosis. Interestingly, leukemia Jurkat cells were the tumoral cells more affected after treatment with the polyphenolic fractions, exhibiting a significantly affected cell cycle and a strong induction of apoptosis. Additionally, those effects were more accused for the total fraction **OW** and the derived fraction **II**, (fractions with 15 and 31% galloylation respectively, against 7% for the derived fraction **I**).

Apoptosis can be induced by many different pathways from which two major pathways have been identified according to the initiator caspase: the extrinsic death receptor pathway, involving caspase-8 and the intrinsic mitochondrial pathway, in which various signals can trigger the release of harmful proteins to mitochondria into the cytoplasm, leading to activation of caspase-9. Both pathways seem to join at the level of caspase-3 which located downstream in the caspase cascade [Hengartner et al., 2000]. The analysis of caspases activation and release of cytochrome *c* carried out in leukemia Jurkat cells demonstrated treatment with the polyphenolic fractions resulted in release of cytochrome *c*, as well as caspases-9, -8 and -3 cleavages. Moreover, the study of substrate carbon flux using mass isotopomer distribution analysis (MIDA) showed a significant decrease in anaerobic glycolysis in treated leukemia Jurkat cells. That glycolysis depletion after treatment with the polyphenolic fractions could be responsible for an intrinsic mitochondrial apoptotic pathway as shown by previous studies [Danial et

al., 2003; Xu et al., 2005] where inhibition of glycolysis caused dephosphorylation of Bad, a pro-apoptotic Bcl-2 family member, which provoked translocation of Bax to the mitochondria followed by loss of mitochondrial membrane integrity, release of cytochrome *c* and later activation of caspases-9 and -3. The finding that the polyphenolic fractions also provoked caspase-8 cleavage could be explained by the fact that in type II apoptotic cells, like leukemia Jurkat cells, this activation has been shown to occur downstream of mitochondria [Ozoren et al., 2002; Slee et al., 1999]. These results are in agreement with recent studies made by Zhao et al in which EGCG triggered apoptosis in nasopharyngeal carcinoma NPC via the mitochondrial signal transduction mechanism [Zhao et al., 2004] and with others performed by Nakazato et al where the increase in ROS production generated by the same flavonoid in human malignant B cells was correlated to loss of mitochondrial transmembrane potential and the release of mitochondrial cytochrome *c* and Smac/DIABLO, followed by capase-9 and -3 activation [Nakazato et al., 2005].

Although intrinsic mitochondrial pathway seems to be the best candidate for apoptosis induced for the polyphenolic fractions analyzed, there is the possibility that the extrinsic mechanism may be operative at the same time and caspase-8 was activated by binding of components of these fractions to death receptors, as shown before for EGCG [Hayakawa et al., 2001; Kawai et al., 2005]. Therefore, complementary studies in involvement of death receptors and Bcl-2 family members such as the pro-apoptotic member Bid will be necessary for discarding the existence of the extrinsic pathway, since truncated Bid, cleavaged by caspase-8, could be triggering cytochrome *c* release and the breakdown of mitochondrial membrane potential in an extrinsic Fas pathway [Li et al., 1998].

5.1.4. Efficacy of polyphenolic fractions as antitumoral agents

An overview of the results obtained in this study allows us to corroborate the efficacy of oligomeric polyphenolic compounds rich in gallate esters (fractions **OW** and **II**) as antiproliferative agents *in vitro* systems independent of their capacity as radical scavengers, since the antiradical power is similar for the three polyphenolic fractions tested. The findings of these analyses show the total fraction **OW** and its derived fraction **II** to be the most attractive products due to their low cytotoxic effects in primary cells and their capacity to selectively alter the cell cycle and induce intrinsic mitochondrial apoptosis in tumour cells. Moreover, treatment with these fractions results in intracellular metabolic changes, restricting the ability of leukemia cells to proliferate, being higher for

the fraction **II** which, as previously indicated, is particularly rich in ECG containing oligomeric flavanols. Thus, the restriction of transformed cells' access to glucose for use in rapid, high-quantity *de novo* nucleic acid synthesis required for proliferation is likely to be an effective approach for developing new cancer therapies [Cascante et al., 2002; Boros et al., 2002], where bioactive compounds such as polyphenols may play a role as anticarcinogens with no known toxic effects.

5.2. PROTECTIVE EFFECT OF THE POLYPHENOLIC FRACTIONS OW, IV, V AND XI AGAINST UV DAMAGE

UV radiation is a major source of adverse reactions in the skin and is the most efficient environmental carcinogen known [Matsui et al., 1991]. Many harmful effects of UV radiation, including carcinogenesis, solar erythema and premature skin aging [Fisher et al., 1997], are associated with the generation of reactive oxygen species (ROS) such as singlet oxygen, superoxide radicals, hydroxyl radical and H_2O_2 [Darr et al., 1994]. An integrated defence system comprising non-enzymatic and enzymatic antioxidants, including catalase, glutathione, and superoxide dismutase, is thus crucial in protecting the skin from oxidative stress. Severe depletion of endogenous skin antioxidants during oxidative stress caused by prolonged exposure to UV radiation results in insufficient protection and hence cellular damage [Shindo et al., 1994; Fuchs et al., 1989]. Consequently, administration and/or topical application of antioxidants are a promising strategy to counteract UV radiation-induced oxidative damage and have been under study [Stewart et al., 1996; Darr et al., 1996].

Among the various agents shown to possess chemopreventive activity, there is growing emphasis on the use of naturally occurring substances. Many studies have suggested a role for EGCG and other flavonoids in preventing UV-induced skin damage in both, *in vitro* systems [Tobi et al., 2002; Basu-Modak et al., 2003; Katiyar et al., 2001] and *in vivo* systems [Katiyar et al., 1995; Elmets et al., 2001]. More recently, a cream based formulation of green tea polyphenols, or EGCG alone, has been shown to attenuate solar UVB-induced oxidative stress-mediated ERK1/2, JNK, and p38 activation in SKH-1 hairless mouse skin [Vayalil et al., 2003].

5.2.1. Composition of studied polyphenolic fractions

Once we had seen that polymerization and percentage of galloylation are important elements to increase the antitumoral efficacy of the polyphenolic compounds, at both cellular and metabolic levels, we decided to analyze the possible protective effect of fractions differing in both parameters. Thus, the fractions **IV** and **XI** (derived from the fraction **OW**) containing oligomeric flavanols with 25% and 31% galloylation respectively, differing in the mean molecular weight and the mean degree of polymerization, were evaluated. Moreover, to confirm the efficacy of gallate groups and degree of polymerization, the fraction **V** (derived from the fraction **I**) composed by monomeric flavanols with less than 1% galloylation was studied.

5.2.2. Slight protective effect against UVB-induced DNA fragmentation in human keratinocytes HaCaT

DNA fragmentation, a process that results from the activation of endonucleases, is one of the later steps during the apoptotic cell death [Collins et al., 1997]. Since some studies have reported the ability of antioxidants as protectors against apoptosis caused by UVB radiation [Ahn et al., 2002; Maalouf et al., 2002; Takahashi et al., 2004], we decided to investigated the effect of the total polyphenolic **OW** on UVB-induced DNA fragmentation of human keratinocytes HaCaT.

There are quite substantial/extensive differences in procedures described in literature, referred to the radiation, the pre-treatment and post-incubation processes for human keratinocytes, which is perhaps due to differences in the UV sources as well as to varying cell-type sensitivities to UV-induced damage. For example, Takahashi et al observed DNA fragmentation in SV40-transformed human keratinocytes 12 hours after irradiation with an UVB dose of 0.03 J/cm^2 [Takahashi et al., 2004], but Shimizu et al detected DNA laddering in HaCaT cells exposed to UVB radiation at a dose of 0.25 J/cm^2 after 8 hours of post-incubation with medium containing 5% FCS instead of 10% of standard culture medium [Shimizu et al., 1999]. Because of this, we decided to test different conditions as large described in section **3.4.1.3.** of Materials and Methods referred to polyphenolic treatment, radiation and post-incubation states. Thus, contrary to Shimuzu et al. study we needed 24 hours of post-incubation to observe clear DNA fragmentation in HaCaT after a similar UVB dose (0.3 J/cm^2), independently on irradiating in PBS or medium and post-incubating with standard culture medium or medium deprived of 5% serum.

When keratinocytes HaCaT were pre-treated 24 hours with the total fraction **OW** at concentrations of 5 µg/ml any protection against UVB-induced DNA fragmentation was observed. Moreover, no improvement was detected when irradiated and/or post-incubated with the polyphenolic fraction. When we increased polyphenolic fraction concentration to 10 µg/ml, we only observed slight protective effect at both conditions (only pre-treated, or pre-treated and post-incubated with the fraction). These preliminary results allowed us to consider polyphenolic fractions could be promising protective agents against UVB-induced damage. However, as DNA fragmentation is a quite later process in UV-induced apoptosis and the radiation dose we needed to trigger this effect was too high, we decided to use lower UV radiation dose and study the potential early molecular events responsible for this cell death.

5.2.3. Protective effect against UV-induced oxidative damage in human quiescent keratinocytes HaCaT

Mitogen-activated protein kinases (MAPKs) are important cellular signalling components that transducer various extracellular signals through serial phosphorylation cascades [Marshall et al., 1994]. In mammalian cells, three distinct MAPK cascades have been identified: ERK, p38 and JNK. The ERK pathway is predominantly activated by growth factors. In contrast, p38 and JNK pathways are mainly stress induced [Johhnson et al., 2002]. All pathways seem to be activated in keratinocytes in response to both UVB [Assefa et al., 1997; Peus et al., 1999] and UVA exposures [Klotz et al., 1997; Huang et al., 2005].

Firstly, we considered the study of the three MAPK cascades but preliminary experiments with the total fraction **OW** allowed us to discard analysis of ERK pathway since that polyphenolic fraction was able to inhibit baseline activation of ERK1/2, which could be related to the growth inhibition capacity of this fraction observed in the previous study performed with tumoral cells, discussed in section **5.1.2.** However, this baseline inhibition did not happen for p38 or JNK1/2. Furthermore, as we were interested in the potential capacity of polyphenolic fractions as protectors against oxidative damage induced by UV radiation, we decided to analyze activation of p38 and JNK mitogen-activated protein kinases which are extensively described as activated under stress conditions [Cano et al., 1995; Johhnson et al., 2002]. These MAPKs can be phosphorylated by exposure to radical oxygen species as H_2O_2 and this phenomenon has been described as inhibited by pre-treatment with classical antioxidants [Guyton et al., 1997; Lo et al., 1996]. The higher H_2O_2 production we observed after UVA radiation

(600% respect to 50% H_2O_2 increase in UVB radiated cells) was in agreement with the higher activation of p38 and JNK1/2 after UVA radiation compared to UVB exposure detected in the timing performed on quiescent keratinocytes HaCaT.

Our results showed a general protective effect of the total polyphenolic fraction **OW** and the derived fractions **V, IV** and **XI** against UV-induced activation of p38 and JNK1/2, concomitant to a reduction in UV-induced H_2O_2 production by the fractions **OW**, **IV** and **XI** in a dose dependent manner, as seen for the fraction **OW**. The finding showing that inhibition of UV-induced MAPKs phosphorylation was relatively better for UVB than UVA radiation could be related to the lower increase in H_2O_2 production after UVB radiation, which was practically reverted by the fractions **OW**, **IV** and **XI**. In more detail, we observed high concentration of the total fraction **OW** (10-20 µg/ml) was necessary to achieve 50% inhibition in phosphorylation of p38, like the derived fractions **IV** and **XI** at concentrations 2 or 4-fold lower (5 µg/ml). However, the derived fraction **V** at the same concentration (5 µg/ml) reduced activation only 25%. A similar behaviour was found when decrease in UV-induced H_2O_2 production was analysed. On the contrary, inhibitory effect of all the derived fractions (**V**, **IV** and **XI**) on UV-induced JNK1/2 phosphorylation was similar to the total fraction **OW**, since the same percentage of inhibition (about 30%) was achieved for all the fractions at the same concentration (5 µg/ml). Thus, polymerization and galloylation (the derived fractions **IV** and **XI** are composed of procyanidin oligomers and riched in gallate groups, contrary to the fraction **V**, which is composed of monomeric flavanols without gallate groups) seem to be important in inhibition of UV-induced activation of p38 but not of JNK1/2. Although both MAPKs can be activated by ROS generated under oxidative stress conditions, this different effect may be related to distinct activation stimuli or regulation mechanisms for JNK1/2 and p38 pathways, which could be also responsible for the lower protective effect of these polyphenolic fractions against JNK1/2 activation compared to p38. Thus, Katiyar and colleagues observed distinct levels and kinetics of phosphorylation and inhibition of ERK1/2, JNK1/2 and p38 by EGCG treatment in keratinocytes which strongly implicate independent mechanisms of regulation of each pathway [Katiyar et al., 2001]. Although the exact mechanisms of inhibition of UV-induced phosphorylation of p38 and JNK1/2 proteins by these polyphenolic fractions may not be clearly explained by the data obtained in this study, it appears that not only their antioxidant properties contributed to that inhibition (antiradical power of the polyphenolic fractions tested is quite similar) but also other structural characteristics such as polymerization and presence of gallate groups.

Cell death induced by UV radiation seemed not to be avoided by polyphenolic fraction treatment, maybe due to other mechanisms responsible for this death apart from implication of ROS, which could also help to elucidate why UV-induced activation of JNK1/2 is less affected by these fractions since JNK has been described as important in controlling programmed cell death or apoptosis [Tournier et al., 2000].

5.2.4. Efficacy of polyphenolic fractions as protective agents against UV damage

Like for the study of antitumoral effect at cellular and metabolic levels, a general idea of the results derived from this analysis allow us to confirm the importance of characteristics such as polymerization and galloylation in the protective capacity of these polyphenolic fractions against UV-induced damage. Hence, the derived fraction composed by monomeric flavanols and >1% galloylation (fraction **V**) is less active when compared to the total fraction **OW** and its derived fractions **IV** and **XI** (fractions composed of oligomeric flavanols and high percentage of galloylation as indicated in Scheme 1 and Table 1 of Materials and Methods). Thus, in an era in which the incidence of the adverse effects of UV radiation is rising at an alarming rate, the introduction of natural chemopreventive agents such as polyphenols may prove to be a new alternative for protection against damage caused by exposure to UV radiation.

6. CONCLUSIONS

1. **Polyhenolic fractions rich in procyanidin oligomers and gallate esters (total OW, 15% galloylation and derived fraction II, 31% galloylation) are the most efficient as antitumoral agents, active at both cellular and metabolic levels with low cytotoxicity:**

   o They inhibit tumoral cell growth at concentrations not cytotoxic to primary pheripheral blood lymphocytes (PBLs).

   o They greatly affect the cell cycle of mouse hepatoma Hepa-1c1c7 and leukemia Jurkat cells, generating an aberrant cell cycle profile, most clearly triggered by the fraction **II**.

   o The derived fraction **II** induces apoptosis in all the tumoral cell lines studied except for the slow growth human melanoma SK-Mel-28.

   o They trigger cytochrome *c* release and cleavage of caspases-9, -3 and -8 in leukemia Jurkat cells, suggesting that the polyphenols activate the intrinsic mitochondrial apoptosis pathway.

   o They reduce *de novo* synthesis of the nucleic acid precursor ribose in leukemia Jurkat cells, being the derived fraction **II** the most effective.

   o They inhibit glycolisis of leukemia Jurkat cells.

2. **Polymerization and percentage of galloylation are also important in the efficacy of the polyphenolic fractions (OW and derived fractions V, IV and XI) as protectors against damage induced by UV radiation:**

   o The total fraction **OW** slightly protects against DNA fragmentation caused by UVB radiation in human keratinocytes HaCaT.

   o None of the polyphenolic fractions avoids UV-induced cell death.

   o The total fraction **OW** inhibits baseline activation of ERK1/2 mitogen-activated protein kinases in human quiescent keratinocytes HaCaT.

   o All the polyphenolic fractions tested inhibit activation of p38 and JNK1/2 mitogen-activated protein kinases in human quiescent keratinocytes HaCaT, being higher for p38 and UVB-radiation.

   o The derived fractions **IV** (25% galloylation) and **XI** (31% galloylation) are the most efficient fractions in inhibiting UV-induced activation of p38.

o   All the polyphenolic fractions tested show the same efficacy to inhibit UV-induced JNK1/2 activation.

o   Baseline levels of H_2O_2, as well as its UV-induced production in human quiescent keratinocytes HaCaT were reduced by the polyphenolic fractions **OW**, **IV** and **XI**.

Briefly, the degree of polymerization and particularly the gallate content are important factors influencing the beneficial efficiency of procyanidins as selective inhibitors of tumoral cells proliferation and protective agents against UV-induced damage.

7. RESUMEN

7.1. INTRODUCCIÓN

El cáncer constituye la segunda causa de mortalidad en los países desarrollados, en los que una de cada cinco personas fallece debido a esta enfermedad. Como la historia de la medicina ha demostrado para otras enfermedades, la falta de tratamientos se debe básicamente al insuficiente conocimiento de los mecanismos biológicos que causan la aparición y progresión del cáncer. A pesar de que en las últimas décadas se ha avanzado enormemente en el desciframiento de las bases moleculares y celulares del cáncer y en menor medida en su tratamiento clínico, no parece inminente el control de esta enfermedad hasta el punto deseable. Precisamente, el descubrimiento de la elevada complejidad del cáncer ha conducido a que hoy sea considerado como un grupo relativamente heterogéneo de enfermedades más que una única etiopatogenia [Alberts et al., 1994; Muñoz, 1997].

7.1.1. Oxidantes intracelulares: especies reactivas de oxígeno (ROS)

Pequeñas cantidades de especies reactivas de oxígeno (ROS) tales como anión superóxido ($O_2^{\cdot-}$), peróxido de hidrógeno (H_2O_2), singlete de oxígeno ($^1O_2$), radical hidroxilo ($^{\cdot}OH$), y peroxil radical ($RO_2^{\cdot}$) se generan en el organismo como consecuencia del metabolismo aeróbico [Chandra et al., 2000; Matés, 2000; Halliwell, 1995]. A pesar de que la fosforilación oxidativa mitocondrial es una de las principales fuentes de estas especies en células eucariotas, también pueden producirse por exposición a radiación ionizante o UV, a agentes oxidantes químicos o pueden ser liberadas por células fagocíticas que aparecen en respuesta a invasión bacteriana o a un tumor [Slater et al., 1995; Kong et al., 2000]. Cuando estas especies se acumulan en el interior de la célula, se produce una rápida respuesta para intentar contrarrestar está pérdida del balance redox de la célula, incluyendo arresto de células en alguna fase de su ciclo, aumento de transcripción genética y puesta en marcha de mecanismos de transducción de señal y reparación del DNA dañado. Así, esta respuesta determinará si una célula morirá por necrosis apoptosis o sobrevivirá y proliferará [Chandra et al, 2000; Matés et al., 2000; Kong et al., 2000].

7.1.2. Antioxidantes y polifenoles.

Numerosos estudios epidemiológicos y experimentales han puesto de manifiesto el hecho de que los micronutrientes con propiedades antioxidantes, presentes en los

alimentos, tienen la capacidad de inhibir la carcinogenesis mediante su efecto en los mecanismos moleculares responsables del inicio, promoción o progresión del tumor. [Wattenberg, 1992]. Un grupo de estos micronutrientes ampliamente estudiado en los últimos años es el de los polifenoles, los cuales son componentes importantes de los tejidos vegetales [Kampa et al., 2000; Li et al., 2000]. Las uvas y el vino contienen altas concentraciones de compuestos polifenólicos (12.6-22.4 mmol/l) que juegan un papel importante en protección cardiovascular, efecto anticancerígeno y protección de la membrana celular [Miller et al, 1995; Brenna et al., 2001; Kampa et al., 2000]. Los flavonoides son los compuestos polifenólicos más abundantes, conociéndose hasta 4000 estructuras diferentes [Middleton et al, 2000]. Se clasifican en tres grandes grupos:

o   Flavanoles:

Los flavanoles más importantes son las catequinas, las cuales son muy abundantes en el té, el vino tinto y el chocolate. Las uvas y el chocolate contienen principalmente (+) catequina y (-) epicatequina, mientras que las principales catequinas del té son galocatequinas y sus esteres galato. Tanto las pepitas como las pieles de las uvas contienen monómeros, oligómeros y polímericas proantocianidinas, siendo el grado de polimerización más alto para los flavanoles de las pieles [Yang et al., 2001; Souquet et al., 1996; Prieur et al., 1994].
(Figura 1 de *Introduction*, página 4)

o   Flavonoles:

El principal flavonol que se consume en la dieta es la quercetina, el cual está presente en gran cantidad de frutas, vegetales y bebidas como el té o el vino, además de ser abundante en los racimos y pieles de las uvas, junto con otras clases de flavonoles. La quercetina normalmente se encuentra glucosilada, siendo la D-glucosa el azúcar más frecuente [Yang et al., 2001; Soleas et al.; 1997; Souquet Et al., 2000].
(Figura 2 de *Introduction*, página 5)

o   Isoflavonas:

La única fuente característica de isoflavonas son las semillas de soja. Las isoflavonas primarias de la soja son la genesteína y didzeina, las cuales son generalmente consideradas como fitoestrógenos [Yang et al., 2001].
(Figura 3 de *Introduction*, página 5)

Polifenoles como los flavonoides han demostrado ser potentes antioxidantes, interfiriendo con el potencial oxidativo/antioxidativo de la célula o actuando como

captadores de radicales libres, aunque también se les ha asignado actividad como agentes pro-oxidantes [Rice-Evans et al., 1996; Hanasaki et al., 1994; Ohshima et al., 1998]. El balance entre sus potenciales propiedades antioxidantes/pro-oxidantes ha sido atribuido a sus características estructurales y a las concentraciones a las que se utilizan, sugiriendo una inducción de su papel antioxidante a bajas concentraciones y producción de ROS a elevadas [Raza et al., 2005].

Además de la importancia de las características estructurales y físico-químicas de los flavonoides ingeridos en la dieta, su absorción, farmacocinética, biotransformación y la actividad de sus metabolitos son críticos en sus efectos biológicos sobre los organismos vivos [Williams et al., 2004].

A pesar de que numerosos estudios han demostrado que la ingesta de estos antioxidantes puede tener un considerable efecto protector frente el estrés oxidativo producido en el organismo por la pérdida del balance entre ROS y su mecanismo endógeno de defensa [Wiseman et al., 1996; Prior et al., 1999; Wattenberg et al., 1992], publicaciones más recientes han alertado de los posibles riesgos de un gran consumo de antioxidantes como los polifenoles [Vitaglione et al., 2004; Scalbert et al., 2005], los cuales ponen de manifiesto la necesidad de llevar a cabo más estudios epidemiológicos para asegurarse de su potencial efecto protector y para poder determinar sus óptimos niveles de ingesta.

7.1.2.1. Polifenoles empleados en este estudio

Los polifenoles empleados en este estudio incluyen determinadas fracciones obtenidas en el Instituto de Investigaciones Químicas y Medioambientales (IIQAB-CSIC), mediante extracción y fraccionamiento de un residuo procedente del prensado de uvas blancas de la variedad Parellada (*Vitis vinifera*). La extracción y composición de las distintas fracciones polifenólicas se resume en el Esquema 1 de *Materials and Methods* (página 25). En dicho centro también se determinó el poder antiradicalario de las mismas así como su grado medio de polimerización (mDP), peso molecular medio (mMW) y porcentaje de galoización (resumidos en Tabla 1 de *Materials and Methods,* página 26).

Las fracciones polifenólicas escogidas para llevar a cabo el análisis de sus posibles capacidades antitumorales y/o protectoras frente al daño causado por la radiación UV fueron las siguientes:

- o Extracto o fracción total **OW**: compuesta de ácido gálico (GA); monómeros de flavanol como catequina (Cat), (-)-epicatequina (EC); flavonoles glucosilados y olígomeros de procianidinas.

- o Fracción **I**: formada por monómeros y pequeños olígomeros de procianidinas con un bajo contenido en galato.

- o Fracción **II**: compuesta de flavonoles y olígomeros de procianidinas de elevado peso molecular y porcentaje de galoización.

- o Fracción **IV**: compuesta de olígomeros de procianidinas con un porcentaje considerable de galoización.

- o Fracción **V**: formada por monómeros de flavanoles sin grupos galato.

- o Fracción **XI**: composición similar a la de la fracción **IV**, pero con oligómeros de procianidinas de mayor peso molecular y contenido en galato.

Las procianidinas que componen dichas fracciones están mayoritariamente formadas por monómeros de (-)-epicatequina (EC) y (−)-epicatequina 3-*O*-galato (ECG), contrario a lo que sucede en el té, donde el mayor componente es (−)-epigalocatequina 3-*O*-galato (EGCG) [Ho, 1992]. Determinados estudios han atribuido propiedades pro-oxidantes al monómero EGCG pero no al EC o ECG [Long et al., 2000] lo cual confiere una ventaja a las fracciones polifenólicas estudiadas en relación a los polifenoles del té.

7.1.3. Cultivos celulares en la investigación del cáncer

A pesar de que el crecimiento celular incontrolado y el cáncer dependen en último término del comportamiento de las células dentro de un organismo vivo, la mayoría de los análisis para evaluar las potenciales características antitumorales de gran cantidad de sustancias se realizan previamente en células en cultivo. Las investigaciones llevadas a cabo con cultivos celulares permiten poder elegir el tipo de célula diana que se quiere analizar, manipular las condiciones en las que se encuentran dichas células y seguir los cambios que se producen en las mismas, así como controlar los efectos o procesos que generan las sustancias a analizar en dichas células. Además, se pueden manipular genéticamente y controlar su estado de crecimiento haciéndolas quiescentes si es necesario. Todas estas características hacen que las investigaciones del crecimiento normal o tumoral dependen altamente del uso de células en cultivo [Lodish et al., 1995].

7.1.3.1. Cultivos celulares utilizados en este estudio

o En los análisis dirigidos a estudiar el posible efecto antitumoral de las fracciones polifenólicas se emplearon una batería de líneas tumorales, así como un cultivo primario para estimar la posible citotoxicidad de estas fracciones:

- Hepa-1c1c7: línea de hepatoma de ratón.

- SK-Mel-28 y A-375: líneas de diferentes melanomas humanos.

- Jurkat: leucemia de linfocitos T

- PBLs: cultivo primario de linfocitos de sangre periférica.

o En los estudios encaminados a determinar el posible efecto protector de las fracciones polifenólicas frente al daño causado por la radiación UV se utilizó una línea celular no tumoral de queratinocitos humanos (HaCaT). Dicha línea se empleo tanto en sus condiciones normales de crecimiento como en estado quiescente.

7.1.4. Ciclo celular

El ciclo celular se puede definir como la serie de sucesos por los que una célula da lugar a dos células hijas y comprende el periodo entre dos divisiones mitóticas. La duración del mismo puede variar ampliamente de un tipo celular a otro, pero se puede considerar que el ciclo completo de una típica célula de mamífero conlleva unas 24 horas. Consta de cuatro fases (Figura 4 de Introduction, página 8):

o Fase G1 o fase de crecimiento que dura entre 6 y 12 horas y en la que las células pueden entrar en un estado de reposo (G0) en el que pueden permanecer incluso años.

o Fase S o fase de síntesis corresponde al tiempo (6-8 horas) durante el cual la célula replica su DNA.

o Fase G2 en la que la célula se prepara para la división celular y tiene una duración de unas 6 horas.

o Fase M o mitosis en la que se produce la división celular y comprende de 30 a 60 minutos.
[Alberts et al., 1994; Muñoz, 1997]

Normalmente este proceso se desarrolla de una manera totalmente normal. Además existen puntos de control que sirven de freno durante el ciclo, asegurándose que una fase no se inicia antes de que la anterior haya finalizado. Sin embargo, cuando se producen mutaciones en genes claves en la regulación, el control de la división se altera y en algunas ocasiones se produce una división celular incontrolada que puede derivar en cáncer [Hutchison et al, 1995].

7.1.5. Apoptosis

La apoptosis o muerte celular programada es un mecanismo clave que utiliza el organismo para eliminar células superfluas en un lugar determinado, porque estén en exceso, dañadas o sean potencialmente peligrosas; o en el timo para eliminar los linfocitos autorreactivos que reconocen antígenos propios. Hoy día se sabe que la apoptosis también está relacionada con un gran número de enfermedades como el cáncer [Muñoz, 1997; Johnstone et al., 2002]

La apoptosis se diferencia de la necrosis por distintas características morfológicas y bioquímicas, como la condensación de la cromatina, la fragmentación del DNA formando una escalera (DNA ladder) y la rotura de las células en una serie de trozos de citoplasma rodeados de membrana, denominados cuerpos apoptóticos, que son englobados y digeridos por otras células, tipo macrófagos o similares, sin que se produzca liberación de material celular al medio y, por tanto, sin reacción antiflamatoria. Dichos eventos están asociados con la activación de específicas cistein proteasas conocidas como caspasas y la pérdida de asimetría de la membrana plasmática, provocando una externalización de la fosfatidilserina, la cual es reconocida específicamente por la anexina V [Matés et al., 2000; Slater et al, 1995; Muñoz, 1997; Fadok et al., 1992].

Hoy en día existen dos mecanismos de apoptosis asociados a la activación de caspasas que han sido muy bien caracterizados (Figura 5 de Introduction, página 12):

o Mecanismo extrínseco o mediado por receptores de muerte, en el que la unión de un ligando a su receptor de muerte provoca la activación de caspasa-8, la cual procesa la caspasa-3 e inicia la cascada proteolítica que desencadena la apoptosis.

o Mecanismo intrínseco o mitocondrial, el cual se inicia con la permeabilización de las membranas mitocondriales y la liberación al citosol de ciertas proteínas

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apoptogénicas como Smac/DIABLO o citocromo *c*, requeridos para la activación de caspasa-9, la cual termina desencadenando la respuesta apoptótica.
[Hengartner, 2000; Chinnaiyan et al., 1995; Muzio et al., 1998]

Otros reguladores apoptóticos que parecen tener un papel importante en el control de ambos mecanismos de apoptosis son los miembros de la familia de proteínas Bcl-2 presentes en la membrana mitocondrial. Así, algunos son antiapoptóticos, (ej. Bcl-2 y Bcl-X$_L$), los cuales pueden desestabilizarse durante condiciones de estrés celular debido a la inducción de los proapoptóticos (ej. Bax, Bad y Bak), generándose poros en la membrana y provocando la liberación de las correspondientes proteínas mitocondriales apoptogénicas que activan caspasas e inducen apoptosis. Además, la caspasa-8 también puede activar a Bid (otra proteína proapotótica de la familia Bcl-2) y desencadenar la respuesta apoptótica [Adams et al., 1998; Reed et al., 1998].

Existen numerosas evidencias que demuestran que las especies reactivas de oxígeno (ROS) juegan un papel importante en la apoptosis, aunque también existen datos que indican que no son esenciales para la misma [Kehrer, 2000].

7.1.6. Metabolismo de la glucosa

La oxidación de la glucosa se conoce como respiración celular y comprende los siguientes procesos: glucólisis, formación de acetil coenzima A, Ciclo de Krebs y la cadena de transporte electrónico. La glucólisis tiene lugar en la mayoría de las células y consiste en la oxidación de glucosa a piruvato, proporcionando a la célula ATP y poder reductor. Debido a que este proceso no requiere oxígeno, se conoce como respiración anaeróbica. La formación de acetil CoA a partir de piruvato es un paso de transición entre la glucólisis y el Ciclo de Krebs, que permite preparar al piruvato para su entrada en dicho ciclo. Tanto el Ciclo de Krebs como la cadena de transporte electrónico necesitan oxígeno para producir ATP por lo que se conocen como respiración aeróbica. Así, bajo condiciones aeróbicas, la mayoría de las células convierten el piruvato a acetil CoA, el cual une la glucólisis con el Ciclo de Krebs. Sin embargo, en un ambiente anaeróbico, las células reducen el piruvato a lactato. El objetivo de esta glucólisis anaeróbica no es la producción de lactato sino la reoxidación del NADH para poder continuar con la producción de ATP vía glucólisis. Además de la glucólisis existe otro mecanismo de oxidación de glucosa conocido como la ruta de las pentosas fosfato, la cual proporciona ribosa-5-fosfato necesaria para la síntesis de nucleótidos y ácidos nucleicos además de poder reductor en forma de NADPH. Las enzimas que catalizan dichas reacciones son

muy abundantes en tejidos con grandes demandas de NADPH y en células de crecimiento rápido que necesitan ribosa-5-fosfato para síntesis de DNA. La ruta de las pentosas fosfato se divide en dos fases:

o Fase oxidativa: la glucosa-6-fosfato pasa a ribosa-5-fosfato y CO_2, generándose NADPH.

o Fase no oxidativa: se producen varios pasos de interconversión entre cetosas y aldosas de 3, 4 y 7 carbonos, regenerándose glucosa-6-fosfato, fructosa-6-fosfato y gliceraldehido-3-fosfato, todos ellos metabolitos de la glucólisis.

Estas fases tienen diferentes funciones y pueden ocurrir de forma independiente, aunque se ha aceptado que se suceden formando un ciclo donde la oxidativa genera ribosa-5-fosfato y NADPH y la no-oxidativa recicla el exceso de ribosa-5-fosfato a glucólisis (Figura 6 de Introduction, página 15) [Elliot et al., 1997].

Las células tumorales han reprogramado su metabolismo para poder sustentar su elevada tasa de proliferación. Así, se ha observado un aumento en la síntesis *de novo* del precursor ribosa-5-fosfato de ácidos nucleicos y determinados estudios han demostrado que la fase no-oxidativa de la ruta de las pentosas fosfato tiene un papel importante en la síntesis de ácidos nucleicos de las células tumorales [Hatse et al., 1999; Boros et al., 1997; Cascante et al., 2000]. Asimismo, también se ha comprobado que existe un considerable aumento de la glucólisis en las células tumorales [Warburg, 1956; Gatenby, 1995; Mathupala et al., 2001].

7.1.7. Radiación ultravioleta

La radiación ultravioleta (UV) emitida por el sol cubre una pequeña parte del espectro electromagnético (400-100 nm), pero incluso en esta porción del espectro los efectos biológicos que provoca varían mucho con la longitud de onda, por lo que se ha dividido en tres regiones:

o UVA: 400- 315 nm
o UVB: 315-280 nm
o UVC: 280-100 nm

Tanto la radiación UVB como la UVA alcanzan la superficie terrestre y causan daño en las células de la piel. El efecto más generalizado que producen es el eritema solar o

enrojecimiento. El término *Dosis Mínima de Eritema* (MED) se usa convencionalmente para evaluar la respuesta de la piel a la radiación UV. Así, MED es la dosis mínima que se requiere para provocar un eritema en individuos con piel blanca no expuesta previamente al sol. Aunque este término se utiliza ampliamente, Diffey y colaboradores han propuesto el término *Dosis Estándar de Eritema* (SED), de forma que un SED es equivalente a la exposición a una radiación de 100 J/cm^2. Así por ejemplo, 30-40 SED serían equivalentes a la radiación de un día soleado en Europa y 4 SED provocarían eritema moderado en un individuo de piel blanca, pero apenas inducirían eritema en una piel previamente expuesta al sol [CIE Standard, 1998; Diffey et al., 1997; De Grujil, 2000; Diffey, 2002].

La radiación UVB es absorbida solo por la epidermis y la parte superior de la dermis y su intensidad en la luz solar es 100 veces menor que la radiación UVA, la cual penetra hasta la dermis. Sin embargo, la radiación UVB es mucho más efectiva en la inducción de efectos biológicos que la UVA. Mientras la radiación UVA provoca daño a través de intermediarios como ROS, la luz UVB es directamente absorbida por el DNA de las células. Así, las lesiones más comunes son la formación de 8-hidroxidesoxiguanosina (8-OHdG) y dímeros de pirimidina y ciclobutano después de exposiciones a radiaciones UVA y UVB respectivamente. Este daño provocado al DNA genera una rápida respuesta de la célula, la cual se para en alguna de las fases de su ciclo para reparar el DNA dañado o muere. Sin embargo, en los últimos años se ha visto que la radiación UV también ejerce otra serie de efectos biológicos específicos a través de la activación de proteinquinasas, factores de transcripción y receptores de membrana [Griffiths et al., 1998; Assefa et al.,2005].

7.1.8. Proteinquinasas activadas por mitógenos (MAPKs) y radiación UV

Las proteinquinasas activadas por mitógenos (MAPKs) son una gran familia de serin/treonin quinasas que son activadas a través de una doble fosforilación en una secuencia específica de tres aminoácidos Thr-X-Tyr (donde X depende del tipo de quinasa). Estas MAPKs sirven como sustratos de fosforilación de las MAPK quinasas (MAPKKs), las cuales son a su vez fosforiladas por las MAPKK quinasas (MAP3Ks), de las cuales existen muchos tipos que permiten activar vías específicas de MAPKS en respuesta a diferentes estímulos.

En organismos pluricelulares se han caracterizado tres importantes subfamilias de MAPKs:

- o Quinasas reguladas por señales extracelulares (ERK1 and ERK2)

- o Quinasas N-terminal de c-Jun (JNK1, JNK2 y JNK3)

- o Quinasas p38 (p38α, p38β, p38γ, y p38δ, de las cuales la p38α es la mejor caracterizada y la que se expresa en la mayoría de los tipos celulares)

El nivel de expresión de la subfamilia ERK de MAPKs es bastante elevado y estas quinasas intervienen en la regulación de la meiosis, mitosis y en funciones post-mitóticas en las células diferenciadas. Son activadas fundamentalmente por factores de crecimiento y diferenciación en vías mediadas por receptores de tirosinquinasas, receptores acoplados a proteínas G heterotriméricas o receptores de citoquinas. Sin embargo, JNK y p38 MAPKs se activan principalmente en respuesta a gran variedad de señales de estrés o de inflamación como citoquinas, radiación ionizante o UV, quimioterapia, choque térmico y estrés hiperosmótico (Figura 7 de Introduction, página 19). Las consecuencias transcripcionales de la activación de una de las cascadas de MAPK pueden variar dependiendo del tipo de célula y del contexto biológico en el que se provoca el estímulo. Incluso en el mismo tipo de célula la respuesta fisiológica a dicha activación puede variar, indicando la existencia de otros mecanismos adicionales importantes en la transducción de dichas señales [Johnson et al., 2002; Lewis et al., 1998; Kyriakis et al., 2001].

La activación de MAPKs inducida por la radiación UV es una de las primeras respuestas de la célula y depende del tiempo, dosis y longitud de onda de la irradiación, así como del tipo de célula y de las condiciones experimentales [Kabuyama et al., 2001; Shimizu et al., 1999; Englaro et al., 1998; Tada et al., 2002]. Numerosos estudios han confirmado que las especies reactivas de oxígeno (generadas en el citosol como resultado de la absorción de radiación UV en presencia de oxígeno o procedentes de orgánulos como la mitocondria) contribuyen significativamente a señales de transducción como la activación de cascadas de MAPKs, inducidas tanto por radiaciones UVB como UVA) [Foote et al., 1968; Zamzami et al., 1995].

7.1.9. Protección del daño inducido por la radiación UV

En los últimos años existe una creciente alarma sobre los efectos dañinos de la radiación UV sobre la piel. Así por ejemplo, entre 1960 y 1986 se produjo un aumento del 400% en la aparición de melanomas en ciertas áreas de USA [Glass et al., 1989]. El aumento en el agujero de ozono y el uso de bronceadores como las lámparas ultravioleta

ha contribuido en gran medida a ello. Así, se ha intentado promover campañas de información para educar a la población sobre como protegerse de los efectos nocivos del sol, además de potenciar el estudio de posibles agentes quimiopreventivos con capacidad para proteger de dichos efectos. Entre estos agentes se encuentran los antioxidantes, cuya aplicabilidad se ha estudiado tanto para aplicación por vía oral como tópica. Así, varios estudios han demostrado los efectos protectores de antioxidantes como los polifenoles en el desarrollo del cáncer de piel inducido por la radiación UV [Lu et al., 2000; Elmets et al., 2001; Katiyar et al., 1995].

7.2. CONCLUSIONES

Los resultados obtenidos en el estudio de las posibles propiedades antitumorales y/o fotoprotectoras de las fracciones polifénolicas indicadas en la sección ***7.1.2.1.*** han permitido obtener las siguientes conclusiones:

1. **Las fracciones polifenólicas ricas en oligómeros de procianidinas y ésteres galato (fracción total OW, 15% de galoización y fracción II, 31% galoización) son las más eficientes como agentes antitumorales, activos tanto a nivel celular como metabólico, y además poseen baja citotoxicidad:**

   o Inhiben el crecimiento tumoral a concentraciones no citotóxicas para un cultivo primario de linfocitos humanos de sangre periférica (PBLs). Así, sus valores de IC$_{50}$ (concentración de producto que causa 50% inhibición del crecimiento tumoral) para las células tumorales está comprendido entre 40 y 140 µg/ml (Tabla 8 de *Results*, página 59). Sin embargo, se necesita una concentración aproximadamente 8 veces superior para producir el mismo efecto en el cultivo primario de PBLs (Figura 11 de *Results*, página 60).

   o Afectan en gran medida al ciclo celular del hepatoma de ratón Hepa-1c1c7 y de las células leucémicas Jurkat, generando un perfil aberrante del mismo, lo cual es más claro tras el tratamiento con la fracción **II** (Figura 12 de *Results*, páginas 62 y 63).

   o La fracción **II** induce apoptosis en todas las líneas tumorales estudiadas, excepto en el melanoma humano de crecimiento lento SK-Mel-28. (Figura 13 de *Results*, página 66)

   o Inducen la liberación de citocromo *c* al citosol y provocan la activación de las caspasas-9, -3 y -8 en células leucémicas Jurkat (Figura 14 de *Results*, página 68), lo cual sugiere que los polifenoles componentes de estas fracciones podrían estar activando la vía intrínseca mitocondrial de apoptosis.

   o Reducen la síntesis *de novo* de ácidos nucleicos en células leucémicas Jurkat, ya que inhiben la producción de su precursor ribosa, y este efecto es mayor después del tratamiento con la fracción **II** (Figura 15 de *Results*, página 70).

o Inhiben la glucólisis de las células leucémicas Jurkat (Tabla 11 de *Results*, página 71).

2. La polimerización y el porcentaje de galoización también son importantes en la eficacia de las fracciones polifenólicas estudiadas (OW, V, IV y XI) como protectores frente al daño inducido por la radiación ultravioleta:

o La fracción total **OW** ejerce un ligero efecto protector sobre la fragmentación de DNA causada por una alta dosis de radiación UVB (0.3 J/cm^2) en queratinocitos humanos HaCaT, tanto si solo se pre-trata como si se pre-trata y post-incuba con dicha fracción (Figura 23 de *Results*, página 75).

o Ninguna de las fracciones polifenólicas estudiadas evita la muerte celular causada por la radiación UV en queratinocitos humanos quiescentes HaCaT (Figura 27 de *Results*, página 79).

o La fracción total **OW** inhibe los niveles basales de activación de las ERK1/2 (MAPKs activadas principalmente por factores de crecimiento) en queratinocitos humanos quiescentes HaCaT, y lo hace de una forma dosis dependiente. Sin embargo, esto no sucede para las MAPKs activadas principalmente bajo situaciones de estrés, como son la p38 y las JNK1/2 (Figuras 28 y 29 de *Results*, páginas 80 y 81).

o Todas las fracciones polifenólicas analizadas inhiben la activación de las MAPKs afectadas por señales de estrés (p38 y JNK1/2) en queratinocitos humanos quiescentes HaCaT irradiados con UVB (0.05 J/cm^2) o UVA (20 J/cm^2). Además esta inhibición es algo mayor sobre p38 y/o para células que han sido irradiadas con UVB (Figuras 31 y 32 de *Results*, páginas 83-85).

o Las fracciones **IV** (25% galoización) y **XI** (31% galoización), ricas en oligómeros de procianidinas, son las más eficientes a la hora de inhibir la activación de p38 provocada por la radiación UV en queratinocitos humanos quiescentes HaCaT (Figura 32A de *Results*, página 85).

- o Todas las fracciones polifenólicas estudiadas muestran la misma eficacia respecto a la inhibición en la activación de JNK1/2 en queratinocitos humanos quiescentes HaCaT (Figura 32B de *Results*, página 85).

- o Todas las fracciones polifenólicas disminuyen los niveles basales de H_2O_2 de queratinocitos humanos quiescentes HaCaT, aunque este efecto es mucho más pronunciado para las fracciones **OW**, **IV** y **XI** (Figura 33 de *Results*, página 87).

- o Las fracciones polifenólicas ricas en oligómeros de procianidinas y grupos galato (**OW**, **IV** y **XI**) inhiben el aumento en la producción de H_2O_2 generado por la radiación UV en queratinocitos humanos quiescentes HaCaT. Sin embargo, la fracción compuesta de monómeros sin grupos galato (**V**) no es capaza de inhibir dicha producción (Figura 35 de *Results*, páginas 88 y 89).

En resumen podemos decir que los resultados de este estudio apuntan a que el grado de polimerización, y en particular el de galoización, son factores importantes en el efecto beneficioso de las procianidinas como inhibidores selectivos de la proliferación tumoral y como agentes protectores del daño causado por la radiación UV.

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9. ANNEXES

9.1. PUBLICATIONS

Matito, C., Mastorakou, F., Centelles, J. J., Torres, J. L. & Cascante, M. (2003). Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur J Nutr*, **42**(1), 43-9.

Torres, J. L., Varela, B., Garcia, M. T., Carilla, J., Matito, C., Centelles, J. J., Cascante, M., Sort, X. & Bobet, R. (2002). Valorization of grape (Vitis vinifera) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J Agric Food Chem*, **50**(26), 7548-55.

ORIGINAL CONTRIBUTION

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Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7

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■ **Summary** *Background* Grapes and wine contain high concentrations of polyphenolic compounds. Although their cancer protective effect has been well documented, their activity as anticarcinogens should be cautiously considered since the molecular bases of action and their applicability to human cancer prevention are still unclear. *Aim of the study* We studied the antioxidant/antiradical activity and the antiproliferative effect *in vitro* of different polyphenolic mixtures, extracted from grapes and fractionated through RP-HPLC. *Methods* The polyphenolic fractions were chemically characterized and their antioxidant/antiradical activity was determined by the DPPH assay. Mouse hepatoma Hepa-1c1c7 cells were used to study the cell growth inhibition capacity of these fractions by MTT assay. Their capacity of altering cell cycle and possible induction of apoptosis was examined using FACS analysis. *Results* The original polyphenolic fraction OW, which contained gallic acid (GA), (+)-catechin (Cat), (–)-epicatechin (Ec), glycosylated flavonols (F) and procyanidin oligomers was fractionated into fraction I, composed of monomers and small oligomers, and fraction II that included flavonols and procyanidin oligomers of higher molecular weight. The three polyphenolic fractions tested showed quite similar antiradical activity, although fraction I was the most potent antiradical agent (lowest ED_{50} value: 9 μg). Fraction II was the least potent cell growth inhibitor (highest IC_{50} value: 100 μg/ml) but showed the strongest effect on the cell cycle of Hepa-1c1c7, inducing apoptosis in those cells. The original fraction OW was demonstrated to have the most potent cell growth inhibition effect (lowest IC_{50} value: 43 μg/ml). However, it only appeared to alter cell cycle of Hepa-1c1c7 at concentrations higher than its IC_{50} and did not induce apoptosis in those cells. A similar effect on cell cycle and apoptosis was encountered for fraction I. *Conclusions* The polyphenolic fractions tested in this study were potent antiradical agents and exerted an antiproliferative effect in mouse hepatoma Hepa-1c1c7 cells; the fraction with the highest degree of polimerization and galloylation (fraction II) had the most influence on the cell cycle and induction of apoptosis on Hepa-1c1c7.

■ **Key words** polyphenols – antioxidants – RP-HPLC – Hepa-1c1c7 – cell growth inhibition – cell cycle – apoptosis

Introduction

Cancer is one of the leading causes of death in the prosperous countries of the world, where one person in five die because of this disease [1]. Numerous experimental and epidemiological studies have shown that antioxidant-micronutrients present in food can inhibit carcinogenesis by affecting the molecular events in the initiation, promotion or progression states [2–5]. Among these micronutrients, polyphenols, constituents of plant tissues, were intensively studied in recent years [6–8].

Grapes and wine contain high concentrations of polyphenolic compounds (12.6–22.4 mmol/l) [9], which are absorbed from the upper gastrointestinal tract [10] and distributed in the body, showing an increased affinity for the heart, liver and kidney. Chronic ingestion of products rich in polyphenols is necessary to obtain biologically effective concentrations [11, 12]. Flavonoids are the largest class of polyphenolic compounds. Thus, over 4000 structurally unique compounds have been identified in plant sources [13]. Flavanols and flavonols are important flavonoids present in grapes and wine. Flavanols such as catechins, together with oligomeric and polymeric proanthocyanidins, are constituents of both grape skins and seeds, the mean degree of polymerization being higher for skin flavanols; and flavonols as quercetin are particularly abundant in grape skins and stems [14–17]. Polyphenols have been shown to be potent antioxidants, interfering with the oxidative/antioxidative potential of the cell or acting as free radical scavengers [18–20]. The cancer protective effect of dietary polyphenols has been well documented in numerous studies [7, 21, 22]. However their activity as anticarcinogens should be cautiously considered since the molecular bases of action and their applicability to human cancer prevention are still unclear [14].

Apart from the putative anticarcinogenic effect as antioxidants, polyphenols have been described to possess antiproliferative activity [23–25]. Catechins from grape and tea influence the cell cycle by inhibiting certain kinases [26–29]. They have also been shown to trigger apoptosis [30–32].

In the present work, we have extracted, fractionated and characterized different polyphenolic mixtures obtained from grape. Their antioxidant/antiradical activity, together with their antiproliferative effect, capacity to alter cell cycle and induction of apoptosis were examined on mouse hepatoma Hepa-1c1c7.

Materials and methods

■ Materials

The starting material, provided by Bodegas Miguel Torres, S. A. (Vilafranca del Penedés, Spain) was the by-product from pressing destemmed Parellada grapes (*Vitis vinifera*) and consisted of skins, seeds and stems. The by-product was cooled immediately after pressing and then frozen (–20 °C). Deionized water and preparative grade acetonitrile (CH₃CN, Scharlau, Barcelona, Spain) were used for preparative HPLC. Analytical grade methanol was obtained from Panreac (Montcada i Reixac, Spain), HPLC grade CH₃CN was obtained from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) from Fluorochem (Derbyshire, UK). Cysteamine hydrochloride, acetic acid and hydrochloric acid 37 %

(v/v) were provided by Merck. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95 %) was from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97 %) from Aldrich (Milwaukee, USA) and (–)-epicatechin from Sigma Chemical Co, (Saint Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), antibiotic (10,000 U/ml penicillin, 10,000 µg/ml streptomycin) and Dulbecco's phosphate buffer saline (PBS) were provided by Gibco-BRL (Eggenstein, Germany). Fetal calf serum (FCS) was purchased from Invitrogen (Carlsbad, CA, USA). Trypsin EDTA solution C (0.05 % trypsin –0.02 % EDTA) was obtained from Biological Industries (Kibbutz Beit Haemet, Israel). Trypan blue solution 0.2 %, 3-[4,5-dimethylthiazol-2yl]–2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI) and Igepal CA-630 were from Sigma Chemical Co (St. Louis MO, USA). α,α,α-Tris (hydroxymethyl)aminomethane was purchased from Aldrich-Chemie (Steinheim, Germany), RNase free of DNase from Roche Diagnostics (Mannheim, Germany) and rh Annexin V/FITC Kit from Bender MedSystems.

■ Methods

Preparative chromatography

Preparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters (Milford, USA) Prep LC 4000 pumping system with a Waters PrepPack 1000 module fitted with a PrepPack Waters cartridge (300 x 47 mm i. d.) filled with VYDAC (The Separations Group, Hesperia, USA) C₁₈, 300 Å pore size, 15–20 µm particle size stationary phase, at a flow rate of 100 ml/min. The column was equilibrated with filtered (0.45 µm) deionized water and the fractions were eluted with mixtures of CH₃CN/water as stated in the Results section.

Analytical chromatography

Analysis of the polyphenolic fractions was carried out by RP-HPLC on either a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC™ C₁₈, 300 Å pore size, 5 µm particle size, 250 x 4.6 mm i. d. column, or a Smart® System (Amersham-Pharmacia Biotech) equipped with a µPeak Monitor (Amersham-Pharmacia Biotech) and fitted with a µRPC C2/C18 SC 2.1/10 (100 x 2.1 mm i. d.) column (Amersham-Pharmacia Biotech). Eluents: [A] 0.10 % (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water/CH₃CN [1:4]. Gradient elution 0 to 50 % [B] over 38 min. Flow rate 1.5 ml/min (VYDAC™ C₁₈) or 0.2 ml/min (µRPC) with detection at 214 nm (VYDAC™ C₁₈) or 214, 280, 320 and 365 nm (µRPC).

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Chemical characterization of oligomeric procyanidins

The mean degree of polymerization (mDP), mean molecular weight (mMW) and galloylation (molar percentage of gallyl ester containing species) of the procyanidin oligomers within the fractions were estimated by thiolysis and HPLC essentially as described [33–34] except that cysteamine was used instead of toluene-α-thiol [35]. Briefly, the terminal flavan-3-ols units were released as such by acid cleavage, whereas the extension moieties were released as the cysteamine derivatives on the fourth position of the flavonoid system. The resulting mixtures were submitted to analytical RP-HPLC (μRPC column, gradient 8–18 % [B] over 30 min) and the molar amount (nmol) of all the released moieties calculated from the peak areas and calibration curves obtained with pure samples. mDP = total nmoles/nmoles terminal units, mMW = total mass/nmoles terminal units, galloylation = [nmoles (–)-epicatechin-gallate (ECG) + nmoles cysteamine-ECG]/total nmoles.

Antioxidant/antiradical activity

The free radical scavenging activity was evaluated by the DPPH method [36, 37]. The samples (0.1 ml) were added to aliquots (3.9 ml) of a solution made up with 4.8 mg DPPH in 200 ml of MeOH (initial concentration of DPPH ca. 60 μM) and the mixture incubated for 1 h at room temperature. The results were plotted as the degree of absorbance disappearance at 517 nm ((1-A/A_0)x100) against μg of sample. Each point was acquired in triplicate. A dose-response curve was obtained for every product. The results were expressed as the efficient dose ED_{50} given as the amount (μg) of product able to consume half the amount of the initial free radical.

Cell culture

Mouse hepatoma Hepa-1c1c7 cells (ECACC 95090613) were cultured in DMEM supplemented with 10 % heat-inactivated fetal calf serum and 0.1 % antibiotic. Cells were maintained as monolayer cultures at 37 °C in a humidified atmosphere with 5 % CO_2.

Cell growth inhibition

The assay was performed by a variation of the method described by Mosmann [38]. Samples containing 200 μl cell suspension (2 x 10^4 cells/ml) were plated in 96-well-flat-bottomed microtiter plates. After adherence of the cells within 24 h of incubation at 37 °C, different polyphenolic fraction dilutions on a scale of 10 μg/ml to 100 μg/ml were added separately. After incubation for 72 h at 37 °C in a humidified incubator with 5 % CO_2, MTT dissolved in PBS at 5 mg/ml and sterile filtered was added to all the wells at a final concentration of 0.5 mg/ml. Following 1 hour of incubation, the generated formazan was dissolved with 100 μl DMSO per well. The optical density was measured on a ELISA plate reader (Merck ELISA System MIOS version 3.2.) at 550 nm. Absorbance was proportional to the number of cells. The concentrations that caused 50 % of inhibition of cell growth (IC_{50}) were calculated.

Cell cycle analysis

Cell cycle was assessed through flow cytometry by using a fluorescence-activated cell sorter (FACS). Cells were cultured in 6-well flat-bottomed microtiter plates containing 2 ml of cell suspension. The number of cells [116400] was determined by the relationship number of cells/area wells, considering the 4000 cells that were cultured in 96-well plates. After 24 h of incubation at 37 °C with 5 % CO_2, polyphenolic fractions were added at their respective IC_{50} values and at a higher dose. Following 72 h of incubation, cells were harvested by mild trypsinization, collected by centrifugation and stained in Tris-buffered saline (TBS) containing 50 μg/ml PI, 10 μg/ml RNAse free of DNAse and 0.1 % Igepal CA-630 for 1 h at 4 °C. FACS analysis was carried out at 488 nm in an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA). Data from 12,000 cells were collected and analyzed using Multicycle program (Phoenix Flow Systems, San Diego, CA, USA). All experiments were performed in triplicate.

Assessment of apoptosis

Detection of apoptosis was performed using an Annexin V-FITC kit binding assay and analyzed by FACS. Cell culture, treatment with polyphenolic fractions and cell collection were carried out as was described in the preceding section. Thereafter cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC (Bender System kit) was added according to the product insert and incubated for 30 minutes at room temperature in the dark. One minute before FACS analysis PI was added at 20 μg/ml. Approximately 3 X 10^4 cells were measured for each histogram and experiments were performed in triplicate.

Results

Extraction and fractionation of polyphenolic compounds from a winery by-product was performed. The lyophilized fraction OW soluble in both ethylacetate and water (2 g) was further fractionated by RP-HPLC using the system described in the Methods. After loading and washing with water (1.7 l), fractions were eluted with

mixtures of CH_3CN in water: fraction I with 12 % CH_3CN (2 l) and fraction II with 24 % CH_3CN (1.6 l). The eluants were concentrated under vacuum and lyophilized to give slightly colored fluffy solids.

RP-HPLC analysis showed that the original fraction (OW) contained gallic acid (GA), (+)-catechin (Cat), (–)-epicatechin (Ec), glycosylated flavonols (F) and other peaks corresponding mainly to procyanidin oligomers. Fraction I was composed of monomers and small oligomers, whereas fraction II included flavonols and procyanidin oligomers of higher molecular weight. Table 1 summarizes the estimated values of mean size and composition of the catechin components (monomers and procyanidin oligomers) of fractions OW, I and II, obtained by thiolysis with cysteamine and RP-HPLC analysis. The fraction retained longer on RP-HPLC (II) presented the highest degree of polimerization and galloylation. Since the monomers were all included in fraction I, its mDP was the lowest.

The free radical scavenging efficiency of the fractions was evaluated by the DPPH radical method as described in the Methods section and compared with that of (–)-epicatechin and trolox (soluble analogue of vitamin E). The ED_{50} values obtained were the following: fraction OW, 12 µg; fraction I, 9 µg; fraction II, 11 µg; (–)-epicatechin, 13 µg; trolox, 15 µg. The three fractions, (–)-epicatechin and trolox presented similar ED_{50} values, with fraction I being the most potent of the products tested.

The antiproliferative effect of the three polyphenolic fractions (OW, I and II) was investigated. The concentration of fractions that caused 50 % inhibition of cell growth was obtained by determination of formazan dye uptake as explained in the Methods section. The fraction concentration was plotted against the percentage of relative cell proliferation after 72 h of treatment, which was calculated assuming that the cells cultured in the absence of the tested fraction had 100 % cell proliferation (Fig. 1). The IC_{50} values obtained were as follows: fraction OW, 43 µg/ml; fraction I, 61 µg/ml; fraction II, 81 µg/ml. The fraction with the highest degree of polimerization and galloylation (fraction II) caused 50 % inhibition of cell growth at a concentration approximately double the concentration required for fraction OW to produce the same effect.

Cell cycle analysis of Hepa-1c1c7 cells was performed 72 hours after treatment with fractions OW, I or II at concentrations equal to their respective IC_{50} values and
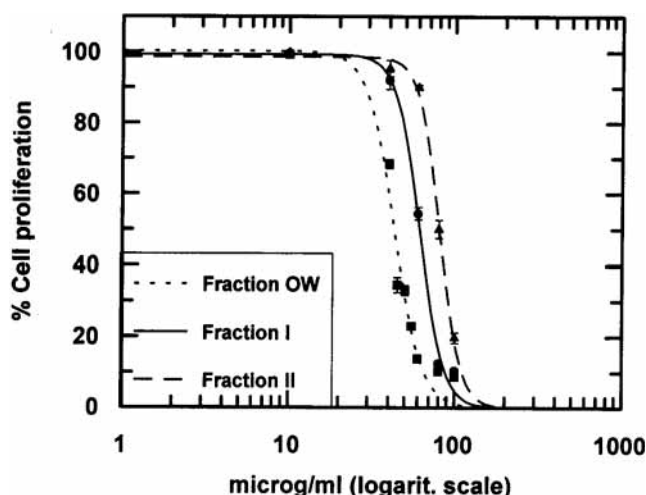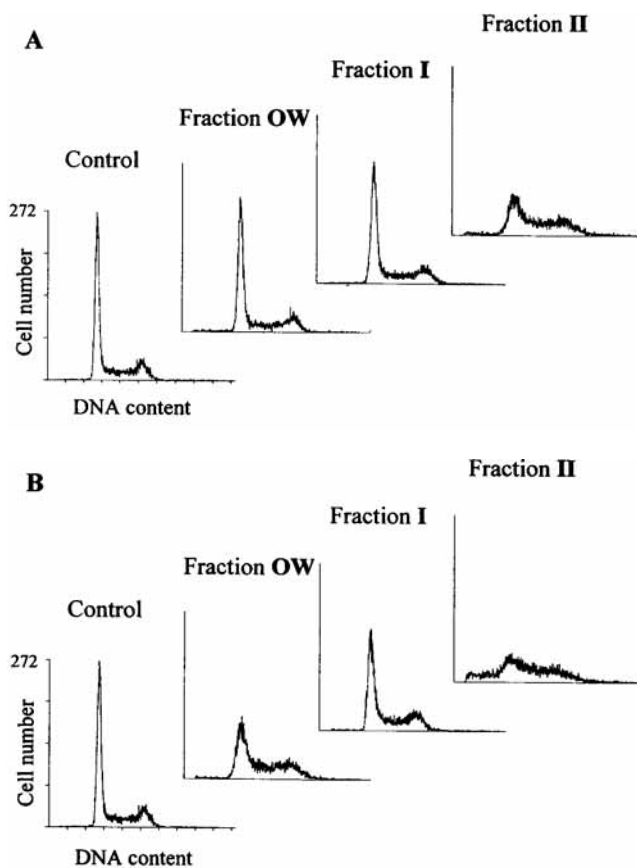

Fig. 1 Effect of polyphenolic fractions from grape (fractions OW, I and II) on cell proliferation of mouse hepatoma Hepa-1c1c7 after 72 hours of treatment. The relative percentage 72 hours, and relative percentage of cell proliferation was calculated considering untreated control cells after 72 hours as 100 % cell proliferation. Each point represented the mean of triplicates of experiments. Vertical bars indicate standard error of the means

at a higher dose which results in 80 % inhibition of cell proliferation, with respect to the untreated control cells (fraction OW, 60 µg/ml; fraction I, 80 µg/ml; fraction II, 100 µg/ml). Both analysis at the IC_{50} and at a higher dose showed that the fraction with the highest degree of polimerization and galloylation (fraction II) exerted the strongest effect on the cell cycle, producing an apparent aberrant cell cycle profile (Fig. 2. A). Compared to the untreated control cells, this fraction produced a decrease of cells in G0/G1 as well as an increase of cells in S and G2 phases. Neither fraction OW nor fraction I affected the cell cycle of Hepa-1c1c7 cells at concentrations equal to their respective IC_{50} values. However, when the concentrations were higher than their IC_{50} values, fraction OW increased the number of cells in S and G2/M phases by decreasing number of them in G1 and fraction I appeared to interfere with the Hepa-1c1c7 cell cycle by slightly arresting cells in G2/M phase (Fig. 2. B).

Assessment of apoptosis in Hepa-1c1c7 cells was performed 72 hours after treatment with fractions OW, I or II at the same concentrations mentioned above for the analysis of cell cycle. FACS analysis using Annexin V-FITC staining and PI accumulation was used to differentiate early apoptotic cells (Annexin V+ and PI–) from late apoptotic/necrotic cells (Annexin V+ and PI+). Results showed that fractions OW and I hardly induced apoptosis in Hepa-1c1c7 cells at either concentrations equal to their respective IC_{50} (Fig. 3. A) or higher than them (Fig. 3. B), whereas the treatment with fraction II generated 38 % apoptotic cells with respect to the untreated control (15 % early apoptosis plus 23 % late apoptosis/necrosis) when cells were treated with a concentration equal to its IC_{50} (Fig. 3. A) and 35 % apoptosis

Table 1 Chemical characterisation of the catechin components

Fraction	mDP	mMW	galloylation
OW	1.7	539	15 %
I	1.4	422	7 %
II	3.0	1005	31 %

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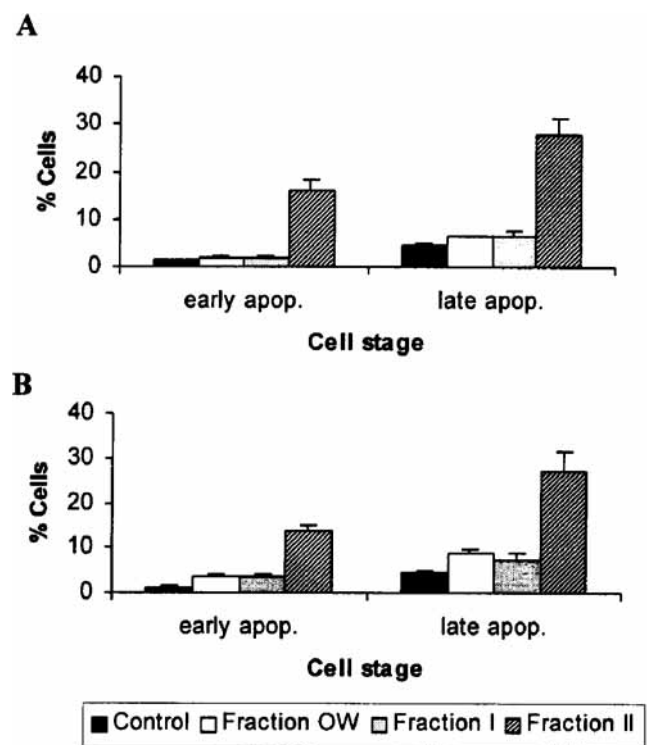
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Fig. 2 Cell cycle analysis of Hepa-1c1c7 cells untreated or treated 72 hours with polyphenolic fractions from grape, at concentrations equal to their respective IC_{50} values (fraction OW, 43 µg/ml; fraction I, 61 µg/ml; fraction II, 80 µg/ml) (**A**) and at a higher concentration (fraction OW, 60 µg/ml; fraction I, 80 µg/ml; fraction II, 100 µg/ml) (**B**) Experiments were carried out in triplicate

Fig. 3 Cytometric analysis of Annexin V-FITC staining and PI accumulation after exposure of Hepa-1c1c7 to polyphenolic fractions from grape. Each fraction was applied at a concentration equal to its IC_{50} value (fraction OW, 43 µg/ml; fraction I, 61 µg/ml; fraction II, 80 µg/ml) (**A**) and at a higher value (fraction OW, 60 µg/ml; fraction I, 80 µg/ml; fraction II, 100 µg/ml) (**B**) Early apoptotic cells were Annexin V^+ PI^-, whereas late apoptotic/necrotic cells were Annexin V^+ PI^+. Values are expressed as means ± sem of triplicates of experiments

(13 % early apoptosis plus 22 % late apoptosis) after treatment with a concentration higher than its IC_{50} (Fig. 3. B).

Discussion

Numerous studies have demonstrated the cancer protective effect of dietary polyphenols [7, 21, 22]. Nevertheless, antitumoral properties of these compounds have been a central point of discussions in the last few years [14].

A focus of our study points to a particular efficiency of grape polyphenolic compounds as antioxidants. The results of the DPPH assay clearly indicate a high antioxidant/antiradical activity of polyphenols from grapes, which is in line with previous studies [39, 40]. It has been described that the antioxidant activity depends on polymerization and increases with galloylation [41, 42]. However, although fraction II is the most galloylated it does not show the highest antioxidant/antiradical ca-

pacity. This is probably due to the fact that fraction II also contains glycosilated flavonols which are probably less potent antioxidants than gallate compounds. Moreover, our results show that the antioxidant capacity of the polyphenolic fractions does not correlate with the antiproliferative potency. Thus, even though the three fractions present similar antioxidant potency, the capacity to inhibit cell proliferation is higher for the original fraction OW (IC_{50}: 43 µg/ml) than for the two derived fractions I (IC_{50}: 61 µg/ml) and II (IC_{50}: 81 µg/ml), which is indicative of a synergistic effect on the polyphenolic compounds of the two fractions I and II. Interestingly, while fraction II showed the lowest capacity to inhibit cell proliferation, its effect on the cell cycle of Hepa-1c1c7 and its capacity to induce apoptosis in this cell line were stronger than those obtained for fractions OW or I. Thus, fraction II was the only one that affected the cell cycle and induced apoptosis in Hepa-1c1c7 at a concentration equal to its IC_{50} (81 µg/ml). However, fraction I at the same concentration induced neither apoptosis nor resulted in the apparent aberrant cell cycle profile described for fraction II, but produced a slight arrest of Hepa-1c1c7 cells in the G2/M phase.

Whereas there is general agreement on the beneficial effect of natural antioxidants in carcinogenesis prevention, some controversy exists on their effect on tumor proliferation. Thus, on the one hand, it has been described that antioxidants can prevent apoptosis in cancer cells and, on the other hand, that they can induce apoptosis in some systems [43]. The results obtained in the present study are in agreement with the induction of apoptosis in vivo by polyphenols described in colon tumors [22] and show that the degree of galloylation of polyphenolic fractions correlates with the capacity to alter cell cycle and induce apoptosis. These results, to-gether with the findings obtained from recent studies which demonstrate that galloyl esters of catechins such as (–)-epigallocatechin-3-gallate inhibit the activities of several key cell cycle regulatory proteins [28, 30] and trigger apoptosis in diverse cell lines [30–32], suggest that galloylation is important to consider in the study of the antitumoral properties of polyphenolic compounds. In conclusion the high antioxidant potency and capacity to induce apoptosis in cancer cells of fraction II makes it potentially interesting as a food supplement and deserves further characterization.

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Valorization of Grape (*Vitis vinifera*) Byproducts. Antioxidant and Biological Properties of Polyphenolic Fractions Differing in Procyanidin Composition and Flavonol Content

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Many byproducts and wastes generated by agroindustries contain polyphenols with potential application as food antioxidants and preventive agents against skin cancer and other diseases. The performance of polyphenolic fractions from Parellada grape (*Vitis vinifera*) pomace as antioxidants in different physicochemical environments was tested. Fractions containing oligomers with mean degree of polymerization between 3 and 4 and percentage galloylation ca. 30% were the most potent free radical scavengers and efficient antioxidants in an oil-in-water emulsion. A fraction including glycosylated flavonols was also efficient in the emulsion. All the fractions showed low aquatic toxicity and weak influence on proliferation of human melanoma cells.

INTRODUCTION

Agricultural byproducts contain a variety of biologically active species which mostly go to waste. Particularly, most plant-derived materials are rich in antioxidant polyphenols. An estimated 13% by weight of the grapes processed by the wine industry ends up as byproduct after pressing. This grape pomace, consisting of skins, seeds, and stems, is a rich source of polyphenols. These include catechins, namely monomeric and oligomeric flavan-3-ols (proanthocyanidins), and glycosylated flavonols (**Figure 1**). Catechins, as well as other polyphenols, are potent free radical scavengers (*1*, *2*). Since many undesired oxidation processes, including food decay and a variety of diseases, involve free radicals, polyphenols from agricultural byproducts are potential added value products with application as natural food additives and disease chemopreventive agents.

The widely used food antioxidants BHT, BHA, TBHQ, and alkyl gallates are synthetic compounds of phenolic nature (*3*, *4*). Consumer concerns and government regulations are fostering the research on alternative ingredients, mostly natural

polyphenols, as presumably safer food antioxidants with added beneficial functional properties (*5*). Since being natural is not necessarily equivalent to being safe, much more research on the biological effects of plant extracts and fractions is needed.

Apart from their free radical scavenging capacity, some catechins are inhibitors of key enzymes involved in the cell cycle (*6*−*8*). They also induce apoptosis in different cell lines (*9*−*11*) and inhibit the expression of certain tumor-related genes (*12*, *13*). All these activities make catechins candidates for preventive agents against cancer, cardiovascular diseases, and premature aging (*5*, *14*−*17*).

The size and composition of oligomeric proanthocyanidins appear to be related to their antioxidant activity, through differences in both the intrinsic scavenging capacity and the physicochemical properties governing their partition behavior within biological environments (*18*, *19*). Moreover, structural features such as the presence of gallate esters appear to be crucial for the cell-cycle-related enzyme inhibition activity of catechins (*6*, *20*). In food systems, partition phenomena also play an important part in the antioxidant preservative action of phenolic compounds (*21*, *22*).

We are primarily interested in possible applications of plant proanthocyanidin fractions in the fields of food preservation and skin protection. Polyphenolic mixtures have already been proposed as food antioxidants (*4*, *23*, *24*) and preventive agents against skin irritation and cancer (*25*, *26*). From an original white

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$R_1 = R_2 = H$, $R_3 = H$: kaempferol

$R_1 = OH$, $R_2 = H$, $R_3 = H$: quercetin

$R_1 = OH$, $R_2 = OH$, $R_3 = H$: myricetin

R_3 = saccharide: flavonol glycosides

A flavonols

B

R_4 = H: catechins, OH: gallocatechins

C

arrows indicate putative polymerization positions

R_4 = H: procyanidins, OH: prodelphinidins

R_5 = OH,   (gallates)

catechins (flavanols): B monomeric, C oligomeric (proanthocyanidins)
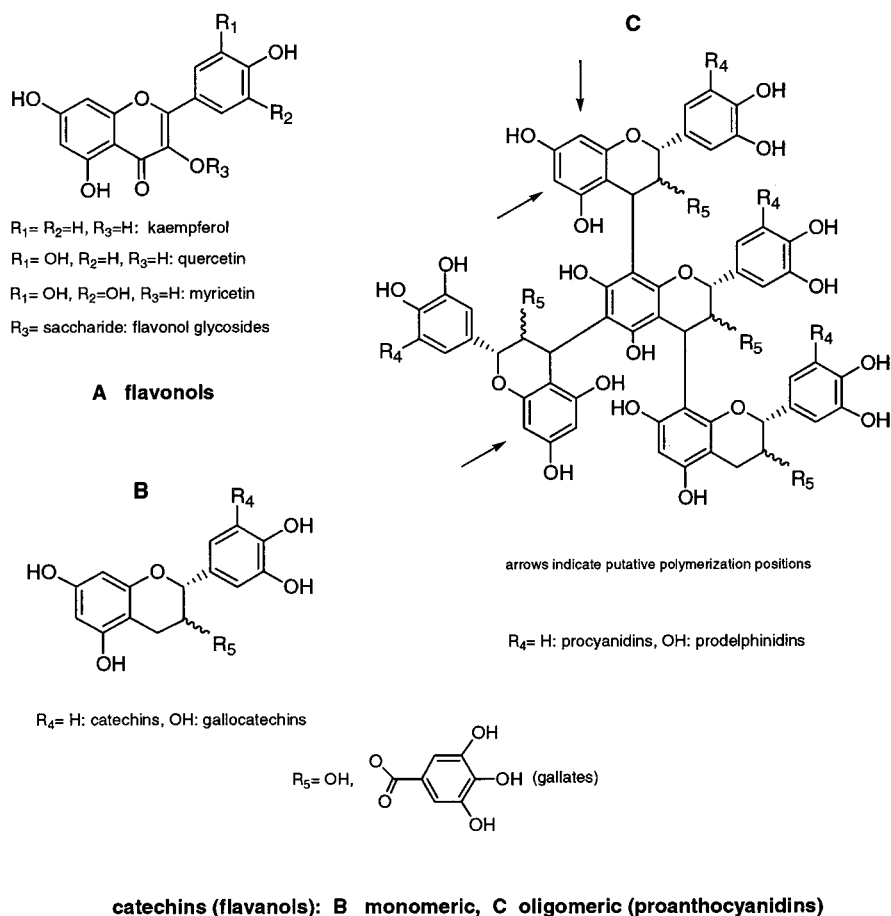
Figure 1. Structures of the major polyphenols found in white grape pomace.

grape pomace extract, we have generated fractions with different contents of monomeric catechins, oligomeric procyanidins, and glycosylated flavonols. The fractions have been evaluated using different assays pertinent to their putative applications: free radical scavenging, inhibition of lipid peroxidation of oil and oil-in-water emulsion, ecotoxicity, and antiproliferation on a melanoma cell line.

MATERIALS AND METHODS

Materials. The starting material, provided by Miguel Torres S.A. (Vilafranca del Penedès, Spain), was the byproduct from pressing destemmed Parellada grapes (*Vitis vinifera*) and consisted of skins, seeds, and a small amount of stems. This byproduct was collected in the month of October during the 1998 harvest, cooled immediately after pressing, and frozen. The polyphenolic fraction **OW**, soluble in both ethyl acetate and water, as well as preparative RP-HPLC-derived fractions **I** and **II** were obtained as described before (*27*, *28*). The following water and solvents were used: deionized water, analytical-grade MeOH (Panreac, Montcada i Reixac, Spain), and analytical-grade acetone (Carlo Erba, Milano, Italy) for semipreparative chromatography; Milli-Q water and HPLC-grade CH_3CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC; and analytical-grade MeOH (Panreac) for the DPPH assay. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK), biotech grade, was distilled in-house. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) was from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) from Aldrich (Milwaukee, WI), and (−)-epicatechin from Sigma Chemical (St. Louis, MO). Grapeseed oil was obtained from Borges Pont (Tàrrega, Spain) and corn oil stripped of tocopherols from Acros Organics (Fair Lawn, NJ). Soybean lecithin (L-α-phosphatidylcholine) was from Sigma Chemical. Dulbecco modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline were from

Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) from Invitrogen (Carlsbad, CA), and trypsin−EDTA solution C (0.05% trypsin−0.02% EDTA) from Biological Industries (Kibbutz Beit Haemet, Israel). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical.

Chromatography. Toyopearl TSK HW-40F (TosoHass, Tokyo, Japan), 32−63 μm particle size stationary phase, was packed into a flash chromatography type 240-mm × 25-mm-i.d. glass column and equilibrated with MeOH. Fractions **OW**, **I**, and **II** (6 mL of MeOH solution, 600 mg, two runs × 3 mL) were separately fractionated in two steps, MeOH (250 mL) and water/acetone (2:3) (250 mL) following elution protocols described in the literature (*29*, *30*). The flow rate was 12 mL/min (MeOH) and 3 mL/min (water/acetone). The solvent was then evaporated under vacuum, and the residues were dissolved in Milli-Q water (100 mL) and lyophilized to give slightly colored fluffy solids (from fraction **OW**, 293 mg **III** and 242 mg **IV**; from fraction **I**, 218 mg **V** and 178 mg **VI**; and from fraction **II**, 85 mg **VII** and 305 mg **VIII**). Alternatively, **OW** (four runs × 300 mg) was more extensively fractionated with MeOH and water/acetone (1:1) to generate a fraction of monomers, essentially equal to **III**, 100 mg fraction **IX**, 28 mg fraction **X**, and 243 mg fraction **XI**. The fractions were analyzed by RP-HPLC on a Smart System (Amersham-Pharmacia Biotech, Uppsala, Sweden) equipped with a μ Peak Monitor (Amersham-Pharmacia Biotech) and fitted with a μRPC C2/C18 SC 2.1/10 (100 mm × 2.1 mm i.d.) column (Amersham-Pharmacia Biotech). Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH_3CN 1:4, gradient 0 to 50% [B] over 38 min; flow rate, 200 μL/min; detection by triple-wavelength 214, 280, and 320 nm. Analytical runs were also performed at 365 nm to detect glycosylated flavonols.

Thiolysis with Cysteamine and RP-HPLC. The size and composition of the procyanidins within the fractions were estimated from the HPLC analysis of the depolymerized fractions, essentially as described (*31*, *32*), except that cysteamine was used instead of toluene-α-thiol

(*33*). Briefly, the terminal flavan-3-ol units were released as such by acid cleavage in the presence of cysteamine, whereas the extension moieties were released as the cysteamine derivatives on the fourth position of the flavanoid system. The resulting mixtures were submitted to analytical RP-HPLC (μRPC column, gradient 8−18% [B] over 30 min), and the molar amount (nanomoles) of all the released moieties was calculated from the peak areas and calibration curves obtained with pure samples:

mean degree of polymerization (mDP) =

$$\text{total nmol/nmol terminal units}$$

mean molecular weight (mMW) = total mass/nmol terminal units

% galloylation = 100 × ([nmol of (−)-epicatechin-gallate
(EcG) + nmol of cysteamine-EcG]/total nmol)

Free Radical Scavenging Activity. The antiradical activity of the fractions was evaluated by the DPPH method (*34, 35*). The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with DPPH (4.8 mg) in MeOH (200 mL), and the mixture was incubated for 1 h at room temperature. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 515 nm of standard samples of DPPH at different concentrations. The equation of the curve was $\text{Abs}_{517\,\text{nm}} = 11345 C_{\text{DPPH}}$, as determined by linear regression. The results were plotted as the degree of absorbance disappearance at 515 nm (($1 - A/A_0$) × 100) against the amount of sample divided by the initial concentration of DPPH. Each point was acquired in triplicate. A dose−response curve was obtained for every fraction. ED_{50} corresponds to either micrograms or micromoles of product able to consume half the amount of free radical divided by micromoles of initial DPPH. The molecular amounts (micromoles) of procyanidin mixtures were calculated with the mean molecular weights (mMW) estimated by thiolysis with cysteamine. The results are expressed as antiradical power (ARP), or $1/\text{ED}_{50}$.

Inhibition of Lipid Peroxidation in Oil by Differential Scanning Calorimetry (DSC). A Mettler Toledo (Greifensee, Switzerland) DSC 20 differential scanning calorimeter, calibrated with indium, was used. The samples were prepared essentially as described (*36*). Briefly, controls or fractions (ca. 1 mg) were dissolved in acetone (100 μL), and grapeseed oil (Borges Pont S.A., Tàrrega, Spain) (500 μL) was added. Acetone was then eliminated under vacuum, and the samples were immediately subjected to DSC analysis. The oil samples were weighed on an aluminum pan which was placed on the DSC cell without sealing. The measurements were performed isothermically at 150 °C with an oxygen flow rate of 200 mL/min. The onset point was calculated as the time when the exothermic peroxidation process was started, as recorded from a sudden enthalpy variation.

Inhibition of Lipid Peroxidation in an Oil-in-Water Emulsion. Lipid peroxidation in corn oil emulsion was monitored by measuring UV absorbance at 234 nm, corresponding to the formation of conjugated dienes upon air oxidation (*21, 37−39*). The emulsion was formed essentially as described (*40*). A mixture of corn oil stripped of natural antioxidants (10% w/w) and soybean lecithin (1% w/w) in 25 mM potassium phosphate pH 5 buffer was mixed with an Ultra-turrax T25 (Ika-Labortechnik, Staufen, Germany) at 9000 rpm for 2−3 min until the complete emulsification. The emulsion was then homogenized with a Microfluidics Corp. (Newton, MA) 110L high-pressure homogenizer in six cycles at 300 bar. Polyphenolic fractions dissolved in buffer (100 μL) were added to the emulsion (10 mL) to obtain initial concentrations ranging from 1 to 20 μg/mL. The samples (2.5 mL) were placed into 10-mL screw-capped test tubes and oxidized at 50 °C in a shaker bath for 4 days. Every day, aliquots (50 μL) were taken and dissolved in MeOH (10 mL) to obtain absorbance values in the linear range, and UV absorbance at 234 nm was recorded as a measure of the formation of conjugated diene hydroperoxides. The lipid peroxidation was calculated as millimoles of hydroperoxides per kilogram of oil using an absorptivity of 26 000 for linoleate hydroperoxides (*41*) and plotted against oxidation time. The experiments were done in triplicate.

Aquatic Toxicity Assay. A biosensor-based measurement system, Microtox test system (Azur Environmental, Carlsbad, CA), was used for determining the potential toxicity of the different fractions in aqueous solution. Microtox (DIN 38412-34) (*42*) employs a marine luminescent bacteria, specifically the strain *Vibrio fischeri* NRRL B-11177 (Azur Environmental), to measure toxicity from aqueous samples. These bacteria liberate energy in the form of visible light (intensity maximum at 490 nm) as a consequence of the series of metabolic reactions. On exposure to toxic substances, the light output is reduced, and this reduction is proportional to the toxicity of the sample. The concentration of an aqueous solution of a chemical that causes a 50% reduction of the light emitted by the bacteria (EC_{50}) is calculated from a concentration−response curve by regression analysis. Toxicity data obtained in the present work were based on a 30-min exposure of bacteria to the sample solution at 15 °C.

Proliferation Assay on SK-Mel-28 Human Melanoma Cell Line. SK-MEL-28 adherent cells (ATCC HTB-72) were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), in the presence of 0.1% (v/v) antibiotics (10 000 U/mL penicillin, 10 000 μg/mL streptomycin), at 37 °C in a humidified environment with 5% CO_2. The cells were split (ratio 1:2 to 1:5) by mild trypsinization every 4−5 days, and the medium was changed every 2−3 days. The cell culture used in this study was free of mycoplasm infection, as shown by the EZ-PCR Mycoplasm test kit (Biological Industries) prior to the treatment with the samples.

Cell growth was determined using the Mosmann assay (*43*) with some modifications. Cells were seeded into 96-well plates at 2×10^4 cells/mL density, 200 μL/well, and incubated for 24 h in the culture medium prior to addition of the samples dissolved in DMEM. Control wells were treated with an equal volume of DMEM as the test cultures. After 72 h of culture, the supernatant was aspirated, and 100 μL of sterile filtered MTT (0.5 mg/mL in DMEM) was added to each well. The plates were incubated at 37 °C, 5% CO_2 for 1 h. The supernatant was removed, and the blue MTT formazan that precipitated was dissolved in DMSO (100 μL) and its optical density (OD) measured at 550 nm on a multiwell reader (Merck ELISA System MIOS).

The inhibitory effect of the fractions at each concentration was expressed as a percentage [(mean OD of treated cells after 72 h of incubation with the product/mean OD of control cells after 72 h of incubation with extra medium instead of product) × 100]. The IC_{50}, or sample concentration causing a 50% reduction in the mean OD value relative to the control at 72 h of incubation, was estimated using GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd., Microsoft Corp., Surrey, UK), curve option IC_{50} curve start at 0.

RESULTS AND DISCUSSION

Characterization of the Fractions and Free Radical Scavenging Activity. The total extract **OW** contained mainly catechins, both monomeric and oligomeric, and glycosylated flavonols. From this mixture, a set of fractions differing in composition and procyanidin structure has been generated using a combination of chromatographic techniques (**Scheme 1**). RP-HPLC discriminates among solutes by hydrophobicity, while Toyopearl HW-40 has been shown to separate flavonoids in order of increasing size (*29, 44*) by the combined action of gel permeation and adsorption phenomena. The gallate esters of the procyanidin dimers elute together with bulkier oligomers (*29*). From a total extract **OW**, soluble in both ethyl acetate and water, which was obtained by liquid−liquid extraction (*27, 28*), we have generated fractions containing only catechin or glycosylated flavonol monomers (**V** and **VII**, respectively), procyanidin dimers with low gallate content (**IX**), procyanidin oligomers with variable galloylation (**IV, VI, VII, X, XI**), and different combinations of monomers and oligomers (**I, II, III**). **Table 1** summarizes the qualitative composition of the fractions and their corresponding antiradical power by the DPPH assay. Procyanidin size and composition were estimated by thiolysis with cysteamine, and glycosylated flavonols were detected by analytical RP-HPLC at 365 nm.
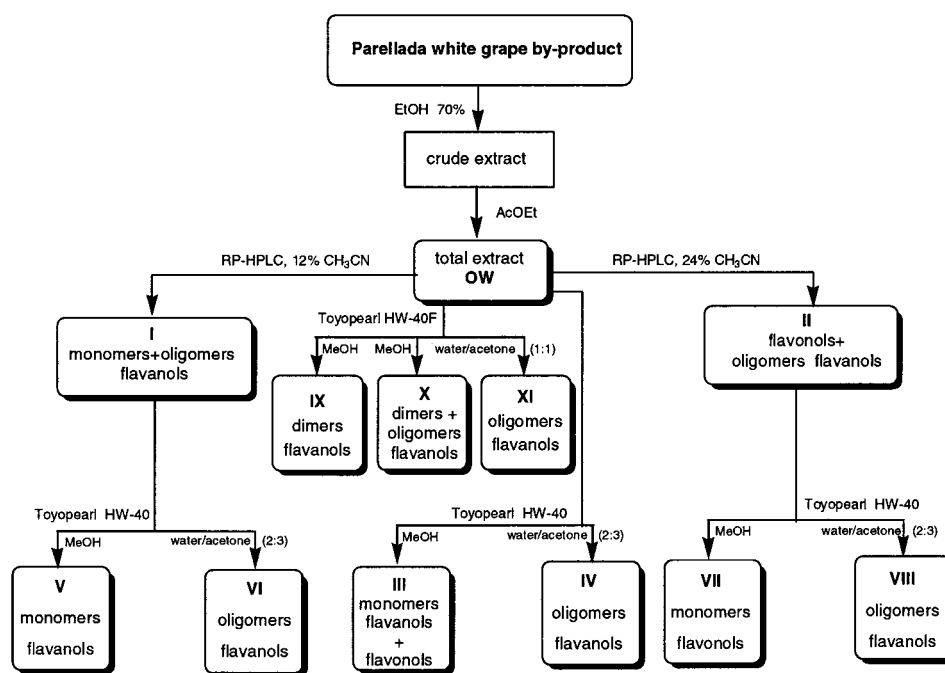
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Scheme 1

Table 1. Size, Composition,[a] and Antiradical Power[b] of Polyphenolic Fractions from Parellada White Grape Pomace

fractions	mDP	mMW	galloylation (%)	ARP[c] (1/ED$_{50}$) × 10^3	molar ARP[d] (1/ED$_{50}$)
OW	1.7	552	15	19	–
I	1.4	422	7	26	11
II	3.0	1005	31	22	–
III	1.0[e]	–	<1	10	–
IV	2.7	880	25	27	24
V	1.0[e]	290	<1	19	6
VI	2.4	751	16	24	18
VII	1.0[e]	–	0	7	–
VIII	3.4	1160	34	27	31
IX	2.0	624	12	23	14
X	2.2	759	30	30	22
XI	3.7	1232	31	28	35

controls	DP	MW	galloylation	ARP[c] (1/ED$_{50}$) × 10^3	molar ARP (1/ED$_{50}$)
(−)-epicatechin	1.0	290	0	21	6
Trolox	1.0	250	–	16	4

[a] Mean of two independent thiolysis experiments with RP-HPLC replicate injections. [b] Mean of three experiments. [c] ED$_{50}$ in microgram fraction or control/ micromoles DPPH. [d] ED$_{50}$ in estimated micromoles fraction/micromoles DPPH only for procyanidin fractions devoid of flavonols. [e] Not calculated.

The DPPH assay provides information about the intrinsic free radical scavenging power in solution irrespective of the physicochemical environments encountered in biological systems (*35, 45, 46*). The antiradical power (ARP) is expressed in relation to both mass (micrograms) and molecular amount (micromoles). The former provides an idea of the weighed amount of fraction needed to exert a given antiradical effect, and the latter carries information on the efficiency of the procyanidin components of that fraction. There was a correlation between procyanidin mean degree of polymerization and antiradical power for the fractions devoid of flavonols. At equal galloylation (**VIII, X, XI**), molar ARP was roughly proportional to mDP. The results

also indicate that the more galloylated a fraction, the higher its ARP was. This is consistent with the fact that the pyrogallol moiety provides more hydrogen atoms or electrons than the catechol group, as proven under different experimental setups (*47–49*). Glycosylated flavonols (fraction **VII**) were less efficient than the non-galloylated monomeric flavanols (**V**, mostly (+)-catechin). The presence of glycosylated flavonols, which are less efficient scavengers than the aglycons (*1, 50*), lowered the overall antiradical power of fractions **OW, II**, and **III**. All the fractions, except those consisting of glycosylated flavonols (**VII**) or a mixture of glycosylated flavonols and flavanol monomers (**III**), were more effective than Trolox. In conclusion, the highest antiradical power corresponded to the mixtures of compounds with the highest degree of polymerization and galloylation and no glycosylated flavonols.

Inhibition of Grapeseed Oil Peroxidation. The polyphenolic fractions were assayed for their ability to inhibit grapeseed oil peroxidation using differential scanning calorimetry (DSC) in the isothermal mode. DSC measurements on oil peroxidation correlate with the results obtained by other procedures, such as Rancimat (*51*), and have been used to test the antioxidant protection exerted by natural and synthetic compounds (*52, 53*). α-Tocopherol and Trolox were used as controls. To set up the working conditions for testing the fractions in less than 60 min, peroxidation was triggered at different temperatures between 135 and 175 °C in the presence and in the absence of Trolox. At 150 °C, the onset time for grapeseed oil peroxidation was ca. 6 and ca. 50 min in the presence and in the absence of Trolox (2 mg/mL), respectively. Thermogravimetric analysis showed that the tested polyphenols (fraction **OW**) were stable at this temperature in the presence of oxygen. The protective performance of α-Tocopherol, Trolox, and the polyphenolic fractions was then tested by DSC at 150 °C. α-Tocopherol exerted weak protection against peroxidation, whereas Trolox retarded the process by ca. 50 min (**Figure 2**). These results are in agreement with those obtained by measuring the initiation of oil peroxidation by UV spectrometry at 234 nm (*21, 38*). Frankel and colleagues suggested that the less lipophilic Trolox might better protect lipids against oxidation by being located at oil−air
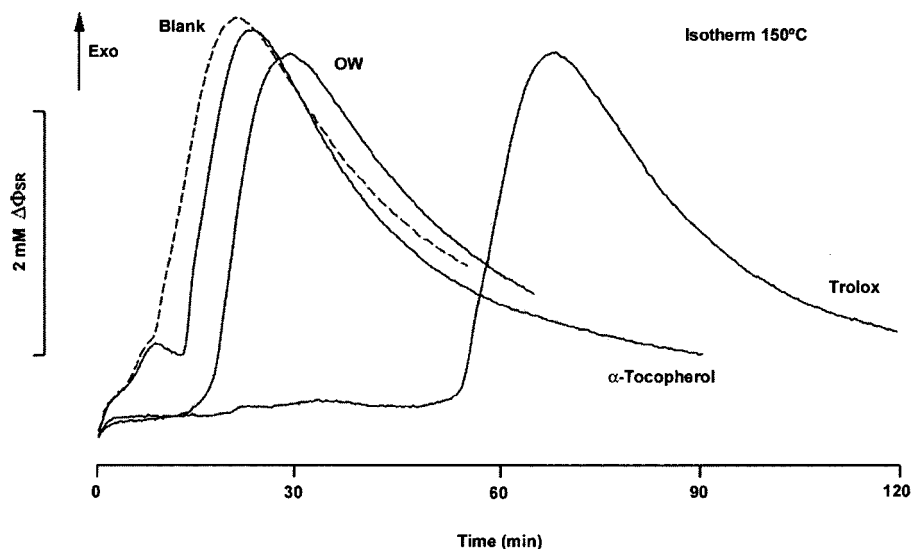
Figure 2. DSC curves of grapeseed oil peroxidation. Φ_{SR}, heat flow rate. Concentrations: Trolox and **OW**, 2 mg/mL; α-tocopherol, 4 mg/mL.
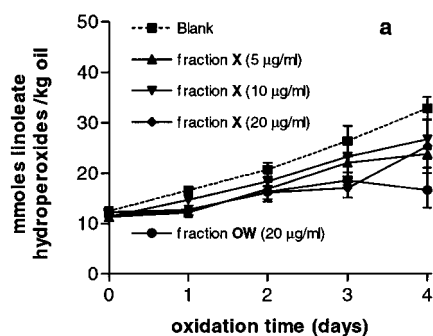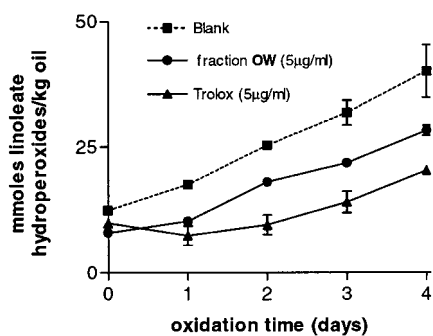

Figure 3. Inhibition of corn oil oxidation in an oil-in-water emulsion. Comparison between extract fraction **OW** and Trolox. Bars represent SEM (standard error of the mean) confidence limits.
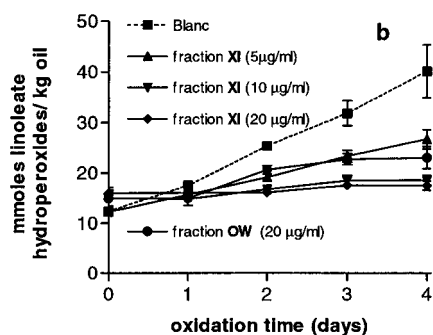

Figure 4. Inhibition of corn oil oxidation in an oil-in-water emulsion. Comparison between fractions with equal galloylation and different degree of polymerization. Bars represent SEM (standard error of the mean) confidence limits.

interfaces or at the oil surface. All the fractions generated in this work (**Scheme 1**) were tested and were less efficient than Trolox. The total extract **OW** as well as the fractions containing monomers and/or dimers showed some protective action (**Figure 2**), whereas fractions with higher degree of polymerization were completely inactive. The reason for these results might be that grape oligomeric procyanidins are poorly soluble in the oil triglycerides. In fact, they formed hazy mixtures. Curiously, trimers and bulkier oligomers are able to establish more hydrophobic interactions than monomers and dimers in certain nonpolar environments, as suggested by their higher retention on reversed-phase HPLC. This does not seem to be the case with oils. In any case, polyphenolic fractions from the grape byproduct showed low capacity to protect oil from oxidation.

Inhibition of Lipid Peroxidation in an Oil-in-Water Emulsion. Since most food and living systems are colloids rather than solutions in solvents of a given polarity, assays in models with interfaces (e.g., lipid–water) must be part of the evaluation of antioxidants (*54*). The antioxidant performance of the fractions and Trolox has been tested in an oil-in-water emulsion made up with corn oil stripped of natural antioxidants under the conditions described in the Materials and Methods. **Figure 3** compares the efficiency of the total extract **OW** with Trolox. **OW**, (−)-epicatechin, and most of the fractions were less efficient than Trolox as antioxidants in the emulsion. Since Trolox is less water soluble than **OW** (soluble in both ethyl acetate and water) and its fractions, the results are in agreement with the so-called polar paradox (*55*, *56*), i.e., the apparent

contradiction that oil-soluble antioxidants perform better in emulsions than in oils and water-soluble antioxidants are better in oils than in emulsion systems. Interestingly, fractions **IV**, **VIII** (data not shown), and **XI** (**Figure 4b**), with mean degree of polymerization around 3, were very efficient in this system. Since these fractions are clearly more water soluble than Trolox, the results appear not to be in complete agreement with the polar paradox. If the protection against lipid peroxidation in oil-in-water emulsions depends on the tendency of an antioxidant molecule to be located at water–oil interfaces, as suggested by Frankel and co-workers (*21*, *22*, *54*, *57*), it could very well be that oligomeric polyphenols show such a phase behavior to some extent, regardless of their poor oil solubility. Bulky procyanidins might be able to establish hydrophobic and/or hydrophilic

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Table 2. Acute Aquatic Toxicity of Polyphenolic Fractions from Parellada White Grape Pomace and Trolox

fraction	toxicity (Microtox)		
	EC_{50} (mg/mL)	EC_{50} (μM)	$\log(1/EC_{50}\ (\mu M))$
OW	52	94	1.03
III	85	293	0.55
VIII	45	39	1.41
IX	65	105	0.98
X	60	81	1.09
Trolox	38	152	0.82

interactions, depending on the environment. They may be a peculiar kind of flexible molecules with hydrophilic hydroxyl groups linked to a hydrophobic core (aromatic rings) with a capacity to expose both or any of the two regions, resulting in surfactant-like oil−water interface active conformations.

Galloylation does not appear to be crucial for the efficacy of procyanidins in the oil emulsion. **Figure 4** shows that fraction **X**, with high galloylation (30%) and relatively low mDP (2.2), was less efficient than fraction **XI** (mDP 3.7), which is equally galloylated (31%).

Interestingly, glycosylated flavonols (fraction **VII**), which were less potent free radical scavengers than monomeric catechins (fraction **V**), were equally efficient in the emulsion. Fractions that included flavonols (**II**, **III**) were more efficient than expected from their ARPs in the DPPH assay. Again, these molecules may be favorably located at the oil−water interface.

Ecotoxicity. We have tested the sensitivity of marine luminescent bacteria toward selected polyphenolic fractions (**OW**, **III**, **VIII**, **IX**, **X**) as a first approximation to the study of their putative toxicity. The influence of the fractions on the bacterial metabolic activity is recorded as changes in light emission. Microtox is a quick, simple, and very reproducible test that presents good correlations with other bacteria and with fish (*58*). Acute toxicity of the fractions is summarized in **Table 2**. All the fractions tested showed low toxicity, similar to that of Trolox. When the values are expressed in molar concentration, the results suggest that some correlation exists between bacterial sensitivity and structure (mDP, galloylation) of the added procyanidins (**Figure 5**).

Proliferation of SK-Mel 28 Human Melanoma Cells. Selected fractions (**OW**, **III**, **IV**, **V**, **VI**, **VIII**) showing different procyanidin polymerization, galloylation, and flavonol content were assayed for their influence on the proliferation of melanoma cells. The fractions exerted a weak antiproliferative effect on this tumoral cell line. The IC_{50} values obtained ranged from 70 (**OW**) to 213 μg/mL (**VIII**). IC_{50} values expressed in molar concentrations showed that the fractions containing oligomeric procyanidins of different mean sizes were equally potent, with values between 124 and 184 μM, whereas the monomers (**V**) were clearly less efficient (655 μM). The results reported here are in overall agreement with those obtained with SK-MEL-1 and/or SK-MEL-28 cells after treatment with flavonoids of the flavonol and flavone type (*59*, *60*). All the polyphenols tested so far have proved to be less potent than the clinically used drug melphalan, which shows IC_{50} values lower than 10 μM (*59*).

In conclusion, fraction **OW**, which contains components that are soluble in both ethyl acetate and water, was a potent antiradical mixture which was also effective in an oil-in water emulsion and showed some activity in oil. The main components of this fraction are catechin monomers, procyanidin oligomers, and flavonol derivatives. Due to concerns about side effects attributed to high doses of catechin (*61*, *62*), the monomeric
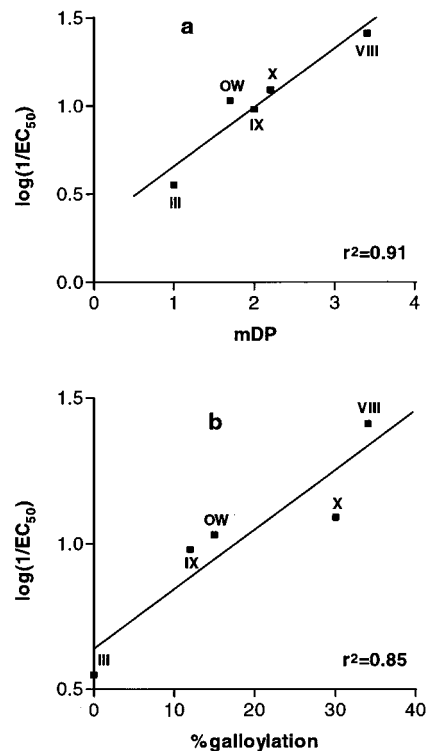
Figure 5. Correlation between sensitivity of luminescent bacteria toward polyphenolic fractions and mean degree of polymerization (a) or percentage galloylation (b).

species are usually removed from polyphenolic preparations of grape origin (*63*, *64*). Fractions (**VIII**, **XI**) devoid of catechin monomers and containing oligomers of relatively high degree of polymerization (between 3 and 4) and galloylation (ca. 30%) were the most potent free radical scavengers and efficient antioxidants in the oil-in-water emulsion. Flavonol derivatives were also efficient in the emulsion, despite their low antiradical power. Fractions such as **II**, with high mDP and flavonol content, together with **VIII** and **XI,** appear to be efficient lipid protecting agents in emulsions and candidates for natural food antioxidants and skin protecting agents.

Since galloylation did not seem to be necessary for lipid protection in the emulsion and gallates have been shown to influence intracellular events (*6*, *65*, *66*), mixtures with high mDP and low galloylation might be even better options than the fractions described here for products designed to offer protection by exclusively scavenging radicals.

ABBREVIATIONS USED

BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DMEM, Dulbecco modified Eagle's medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; FCS, fetal calf serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; THBQ, *tert*-butylhydroquinone; Trolox, 2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

ACKNOWLEDGMENT

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JF025868I