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Phenolic compounds and cellular redox homeostasis: role in anti-oxidant protection and anti-tumoral effects

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“Everything in excess is opposed to nature.”

Hippocrates

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Abstract

Oxidative stress is linked to human disease and aging, which is characterized by an imbalance between the production and removal of reactive oxygen species (ROS) favouring the accumulation of ROS and consequently leading to oxidative damage of cellular structures. Living organisms evolved a group of anti-oxidant defences to maintain ROS under normal and sustainable levels. Those include superoxide dismutases, catalases, and the glutathione and thioredoxin systems. The maintenance of redox homeostasis in cells contributes to the normal function of cellular signalling pathways. In fact, ROS play a crucial role as second messengers, modulating the activity of proteins through redox regulation of cysteines. Therefore, ROS are implicated in the normal function of MAPK cascades, PI3K pathway as well as protein kinases and phosphatases and transcription factors, such as NF-kB, Nrf2, p53, FoXO, among others.

Cells have developed mechanisms to detect and repair molecular damages. However, under severe oxidative stress conditions, cellular damages accumulate causing aging and death. Oxidative stress is also related to cancer, a multifactorial disease and the major cause of death and disability worldwide. In addition, the presence of high levels of ROS in cancer cells is used to fuel proliferating and survival programs.

Phenolic compounds are important components in human health, being available through the intake of fruits and vegetables. They have anti-inflammatory and anti-tumoral properties, which may be mediated by anti-oxidant or pro-oxidant activities, by modulation of cell signalling pathways or both. The aim of this work was to clarify the effect of the phenolic compounds on cellular redox homeostasis. The eukaryotic model *Saccharomyces cerevisiae* was used to evaluate the effect of phenolics in oxidative stress resistance and chronological lifespan. Pyrogallol and myricetin treatment of *S. cerevisiae* increased the resistance to H_2O_2-induced oxidative stress and decreased intracellular ROS and protein carbonyls. Both compounds caused glutathione depletion but myricetin prevented H_2O_2-induced glutathione oxidation. In addition, myricetin increased chronological lifespan in *sod2Δ* mutant cells, which was related to a reduction in protein carbonyls.

The effect of phenolic compounds was also evaluated in cancer cells, which are characterized by having high basal levels of ROS. Many studies have related the anti-tumoral effects of phenolics to anti-oxidant and pro-oxidants mechanisms. However, it is still unclear how phenolics affect tumor cells. In this work the effect of phenolics in gastrointestinal cell lines proliferation, apoptosis and redox homeostasis, including intracellular ROS and anti-oxidant defences, was analysed *in vitro*. Phenolic compounds inhibited the proliferation of Caco-2, HT-29, MKN-28 and AGS tumor cells, for which the

tri-hydroxylation in the B-ring was an important factor. The anti-proliferative effect of all compounds, including myricetin, was partially related to the generation of H_2O_2 in culture medium, since the effect was reverted by the addition of catalase. Only quercetin effect was independent of H_2O_2 and $O_2^{\cdot -}$. Quercetin decreased ROS levels in AGS cells but increased them transiently in Caco-2 cells. Furthermore, quercetin induced cell cycle arrest and apoptosis and decreased glutathione levels in AGS cells, but did not affect thioredoxin oxidation levels and thioredoxin reductase activity. The results suggest that glutathione depletion contributes to the anti-tumoral effect of quercetin.

Resumo

O stress oxidativo está associado a diversas patologias humanas e ao envelhecimento, o qual é caracterizado pelo desequilíbrio entre a produção e a remoção de espécies reativas de oxigénio (ERO) favorecendo a sua acumulação e a consequente lesão de estruturas celulares. Os sistemas vivos desenvolveram um grupo de defesas anti-oxidantes para manter os ERO dentro de níveis normais. Essas defesas incluem dismutases do superóxido, catalases e os sistemas da glutationa e da tioredoxina. A manutenção da homeostasia redox nas células contribui para o normal funcionamento de vias de sinalização celular. Diversos estudos têm demonstrado que as ERO têm um papel crucial como mensageiros secundários, modulando a atividade de diversas proteínas através da regulação redox de cisteínas. Por esse motivo, as ERO estão implicadas no normal funcionamento das cascatas de MAP cínases, da via da fosfatidilinositol 3-cínase bem como das proteínas cínases e fosfatases e fatores de transcrição, como o NF-kB, Nrf2, p53, FoXO, entre outros.

As células desenvolveram mecanismos para detetar e reparar danos moleculares. No entanto, em condições de stress oxidativo severo, as lesões celulares acumulam-se levando ao envelhecimento e à morte. O stress oxidativo está também relacionado ao cancro, doença multifatorial e a maior causa de morte a nível mundial. Além disso, a presença de um elevado nível de ERO que normalmente se encontra nas células tumorais contribuem para a sustentação das vias proliferativas e de sobrevivência.

Os compostos fenólicos são componentes alimentares presentes em frutas e vegetais importantes para a saúde humana. Eles têm propriedades anti-inflamatórias e anti-cancerígenas, as quais podem ser mediadas por mecanismos anti-oxidantes ou pró-oxidantes, por modulação de vias de sinalização celular, ou ambos. Este trabalho teve como objectivo esclarecer o efeito dos compostos fenólicos na homeostasia redox celular. O modelo eucariótico *Saccharomyces cerevisiae* foi usado para avaliar o efeito dos compostos fenólicos na sua resistência ao stress oxidativo e na longevidade cronológica. O tratamento com pirogalol ou miricetina aumentou a resistência da levedura ao stress oxidativo induzido pelo H_2O_2, reduziu as ERO intracelulares e os níveis de proteínas carboniladas. Ambos os compostos provocaram uma depleção de glutationa e a miricetina preveniu a oxidação da glutationa provocada pelo H_2O_2. Além disso, a miricetina aumentou o tempo de vida cronológico em mutantes *sod2Δ*, o qual foi relacionado com uma redução dos níveis de proteínas carboniladas.

O efeito dos compostos fenólicos foi também analisado em células tumorais, as quais são caracterizadas por ter níveis elevados de ERO. Diversos estudos têm relacionado o efeito anti-cancerígeno dos compostos fenólicos a mecanismos anti-oxidantes e pró-oxidantes.

No entanto, é ainda necessário clarificar os mecanismos envolvidos no efeito desses compostos nas células tumorais. Neste trabalho o efeito de compostos fenólicos na proliferação, apoptose e na homeostasia redox, incluindo ERO intracelulares e defesas anti-oxidantes, foi analisado em diversas linhas celulares do sistema gastrointestinal *in vitro*. Os compostos fenólicos inibiram a proliferação celular das linhas tumorais do cólon Caco-2, HT-29, e do estômago MKN-28 e AGS, sendo a tri-hidroxilação do anel B um factor importante para o efeito. O efeito anti-proliferativo de todos os compostos, incluindo a miricetina, foi parcialmente relacionado com a geração de H_2O_2 no meio de cultura, indicado pela reversão do efeito pela adição de catalase. O efeito da quercetina foi independente do H_2O_2 e $O_2^{\cdot-}$. A quercetina reduziu os níveis de ERO intracelulares nas células tumorais gástricas AGS mas aumentou-os temporariamente nas células tumorais do cólon Caco-2. Além disso, a quercetina interrompeu a progressão do ciclo celular, induziu a apoptose e reduziu os níveis de glutationa nas AGS mas não afectou os níveis de tioredoxina oxidada e a atividade da tioredoxina redutase. Os resultados sugerem que a depleção da glutationa contribui para o efeito anti-tumoral da quercetina.

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CHAPTER 1.

INTRODUCTION

ROS in cell signalling and oxidative stress

Reactive oxygen species (ROS) are radical or non-radical oxygen species, such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) resulting from partial reduction of oxygen (O_2). In cells, ROS derive from normal cellular metabolism or can be formed by exposure to environmental toxic compounds (Holmstrom and Finkel, 2014). ROS may have different cellular effects depending on their type, signal intensity and signal duration. In moderate levels ROS serve as second messengers that regulate a variety of transcription factors (NF-κB, Nfr2, HIF-1, p53) and components of signalling pathways (Trachootham *et al.*, 2008a). The regulation of cell signalling is mediated through redox regulation of reactive cysteine residues in proteins, changing the structure and function of the protein. Oxidation of cysteine residues leads to the formation of reactive sulphenic acid derivatives (-SOH), which can form disulphide bonds with nearby cysteines (-S-S-) or undergo further oxidation to sulphinic (-SO_2H) or sulphonic (-SO_3H) acids. These oxidative modifications, except sulphonic acid and to a lesser degree sulphinic acid, can be reversed by cellular reducing systems such as thioredoxin (Trx) and glutaredoxin (Grx) (Miki and Funato, 2012). Furthermore, ROS can induce nitrosylation and glutathionylation of proteins (Popov, 2014; Sadowska-Bartosz *et al.*, 2014) (**Figure 1.1**).

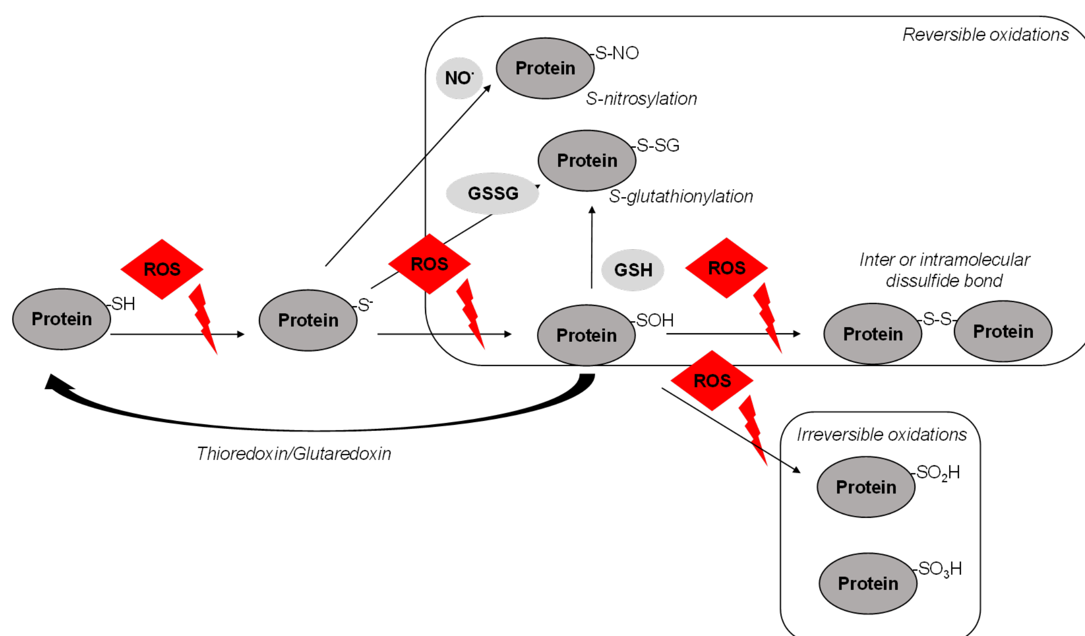


Figure 1.1 Redox regulation of proteins. Cysteine residues in proteins can be reversibly oxidized to sulphenic acid, which can be reduced by thioredoxin and/or glutaredoxin. Sulphenic acid derivatives can lead to intramolecular or intermolecular disulfide bond formation or modified by addition of GSH (*S*-glutathionylation). Reactive cysteines can also react with nitric oxide (*S*-nitrosylation). Further oxidation of sulphenic acid to sulphinic or sulphonic acid derivatives is irreversible.

Signalling pathways known to be redox regulated include the mitogen activated protein kinase (MAPK) cascades (Son *et al.*, 2011) and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway (Kitagishi and Matsuda, 2013). These pathways regulate important cellular processes, such as proliferation, growth, differentiation and apoptosis. However, above certain levels, ROS can cause oxidative stress leading to cellular damage. Basically, oxidative stress occurs when ROS overwhelm the cellular anti-oxidant defence system, due to an increase in ROS production and/or a decrease in cellular anti-oxidant capacity. This situation has been implicated in several age associated diseases and cancer (Kohen and Nyska, 2002).

Anti-oxidant defence mechanisms

To keep ROS at low levels, cells evolved several anti-oxidant defences, including detoxifying and repair enzymes, as well as small scavenger molecules. The enzymatic defences include superoxide dismutases (SOD), catalases, glutathione peroxidases (GPx) and the reducing systems glutathione (Yu, 1994), peroxiredoxins (Prx) and thioredoxins (Trx) (Hanschmann *et al.*, 2013) (**Figure 1.2**).

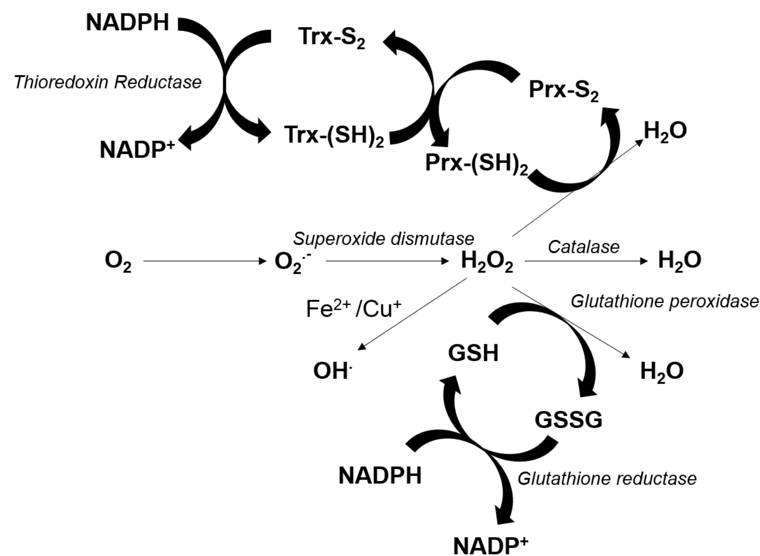


Figure 1.2 Cellular anti-oxidant defences. Superoxide radicals are converted into H_2O_2 by superoxide dismutases (Cu/ZnSOD in the cytosol or MnSOD in the mitochondria). H_2O_2 is reduced to water by catalases. Alternatively, H_2O_2 can be eliminated by the thioredoxin and glutathione systems, which depend on the NADPH for reducing equivalents. Transition metals, such as Fe^{2+} and Cu^{+}, can catalyse the formation of the reactive $OH^{·}$ from H_2O_2 through the Fenton reaction.

Superoxide dismutases and catalases

SODs are metalloenzymes that dismutate $O_2^{\cdot -}$ into H_2O_2 and O_2 (McCord and Fridovich, 1969). There are three main forms of SOD in eukaryotic cells: SOD1 (or CuZnSOD), a copper- and zinc-containing SOD present in the cytoplasm, mitochondrial intermembrane space and nucleus; SOD2 (or MnSOD), a manganese containing-SOD present in the mitochondrial matrix (Zelko *et al.*, 2002); and, in humans, the extracellular SOD (SOD3), a secretory glycoprotein containing copper and zinc and present in the interstitials spaces of tissues and in extracellular fluid (Fattman *et al.*, 2003). H_2O_2 is reduced to water and O_2 by catalases, heme containing proteins that in humans are present mainly in peroxisomes. Prx and GPx are also able to detoxify low concentrations of H_2O_2, however catalases play a pivotal role in the decomposition of high intracellular levels of H_2O_2 (Rhee *et al.*, 2005b).

Glutathione system

Glutathione (γ-glutamyl cysteinyl glycine) is the major cellular thiol anti-oxidant present at mM concentrations. Besides working as an anti-oxidant, it also participates in the detoxification of xenobiotics. In addition, glutathione participates in redox regulation of signalling pathways through thiol disulphide exchange reactions and post-translational modification of proteins by conjugation with reactive cysteines, a process known as *S*-glutathionylation (Anathy *et al.*, 2012; Schafer and Buettner, 2001; Sies, 1999). Almost 90% of glutathione is present in the reduced form (GSH) in cells, which is maintained by the *de novo* synthesis, enzymatic reduction of oxidized glutathione (GSSG) and uptake of exogenous GSH (Circu and Aw, 2010). The glutathione system consists of glutathione, GPx, glutathione reductase, glutathione *S*-transferase (GST), glutaredoxins (Grxs) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). GPx is a selenium containing enzyme that reduces H_2O_2 and other peroxides by using glutathione (GSH) in a NADPH dependent mechanism (Sies, 1999). Glutathione reductase is the enzyme responsible for the reduction of oxidized glutathione (GSSG) back to GSH. GSH in conjunction with GST participates in the detoxification of oxidized molecules and xenobiotics (Pastore *et al.*, 2003). Grxs catalyse the redox interactions between GSH and protein cysteines (Stroher and Millar, 2012). Changes in the intracellular balance of total glutathione levels and GSSG are considered major determinants in the redox status of the cell, and its maintenance is essential to support cell proliferation, survival and protection against apoptosis (Armstrong *et al.*, 2002).

Thioredoxin system

The Trx system acts together with glutathione to maintain intracellular ROS homeostasis. It differs from the glutathione system in that glutathione can be present up to 10 mM while Trx is present at approximately 1 mM in cells. However, the Trx system regulates a wider range of proteins compared to glutathione system (Le Moan *et al.*, 2006). The Trx family is a cellular disulphide reductase system, which catalyses the reduction of protein disulphide to dithiols regulating protein function. It is composed by the redox active Trx, a homodimeric seleno-protein Trx reductase (TrxR), Trx peroxidase/peroxiredoxin (Prx) and NADPH (Holmgren, 1985). Mammalian cells possess two Trx systems, the cytosolic Trx1 and the mitochondrial Trx2 system. Both of them have two catalytically active cysteines (Go and Jones, 2013) and Trx1 have three additional non-active cysteine residues at positions 62, 69 and 73 that are targets of glutathionylation, oxidation and *S*-nitrosylation, contributing to its own activity regulation (Haendeler, 2006). TrxR is a homodimeric flavoprotein containing selenium and has a broad range of substrates. Besides reduction of Trx, TrxR also reduces protein disulphide isomerase, Grx2 and small molecules, such as ebselen (Lu *et al.*, 2009).

Trx was first described as the reducing agent for ribonucleotide reductase, an enzyme involved in the synthesis of deoxyribonucleic acid (DNA) (Laurent *et al.*, 1964). Despite the presence of other cellular reducing agents, ribonucleotide reductase depends mostly of Trx, since the inhibition of TrxR causes a decrease in the activity of ribonucleotide reductase in human rhabdomyosarcoma A-204 cells (Mau and Powis, 1992). The role of Trx in DNA synthesis seems to be essential for fetal survival and development (Matsui *et al.*, 1996), however, disruption of hepatocyte specific TrxR in mice did not affect their survival (Rollins *et al.*, 2010), implying the existence of a backup system.

Besides the reduction of disulphide groups in proteins, the Trx system contributes also to the elimination of ROS as it reduces members of the Prx family, contributing to the degradation of H_2O_2 (Rhee *et al.*, 2005a), methionine sulfoxide reductase (Msr), which reduces oxidized methionine residues (Stadtman *et al.*, 2002) and Gpx3. The disulphide reductase activity of Trx targets multiple proteins, including phosphatase and tensin homolog (PTEN) and protein-tyrosine phosphatase 1B (PTP1B) (Schwertassek *et al.*, 2014), and in that way is involved in the redox control of multiple cellular pathways.

At physiological low levels, ROS act as messengers through modification of target proteins, such as kinases and phosphatases. Redox systems, such as glutathione and thioredoxin also participate in cell signalling. Several components of MAPK pathways and PI3K pathways, as well as cGMP and cAMP-dependent protein kinases (referred as PKG

and PKA respectively) and the PKC family of lipid-activated serine/threonine kinases, are redox regulated. Therefore, the redox state dictate cell fate decisions (survival or death).

Redox regulation of signalling pathways

MAPK pathways

MAPKs are protein kinases that phosphorylate the specific aminoacids serine, threonine and tyrosine in target proteins. Mammalian MAPKs include the extracellular signal-related kinases (ERK1/2), the c-Jun N-terminal kinases (JNK), the p38 kinase and the big MAP kinase 1 (BMK1/ERK5) (Raman *et al.*, 2007) **(Figure 1.3)**. They control several cellular processes, including proliferation, growth, differentiation and apoptosis. These pathways are composed of three connected kinases. MAPK kinase kinases (MAPKKK) is a serine/threonine kinase that activates MAPK kinases (MAPKK) by dual phosphorylation of serine and threonine residues within the activation loop; MAPKK in turn phosphorylate a threonine and tyrosine residue in the activation loop of MAPKs (Raman *et al.*, 2007). The threonine-x-tyrosine motif present in the activation loop is a common feature to most MAPKs and both residues need dual phosphorylation by MAPKK for proper activation. The ERK1/2, JNKs and p38 bear the activation motif TEY (Thr-Glu-Tyr), TPY (Thr-Pro-Tyr) and TGY (Thr-Gly-Tyr) in the activation loop respectively (Pearson *et al.*, 2001). The final MAPK within the cascade transmit the signal through phosphorylation of the target protein in a serine or threonine near a proline residue (Avruch *et al.*, 1994; Marshall, 1994).

MAPK phosphatases (MKPs), including serine/threonine, tyrosine and dual-specificity phosphatases (DUSPs), are crucial negative regulators of MAPK signalling (Keyse, 2000). These in turn are transcriptionally induced by the same stimuli that activates MAPK, suggesting the existence of a negative feedback loop that contribute to adaptation (Millar *et al.*, 1995). In addition, MAPK-mediated phosphorylation of some DUSPs and PTPs has been demonstrated to stabilize these proteins (Sohaskey and Ferrell, 2002). The balance between the activities of kinases and phosphatases controls the time and intensity of MAPK pathway activation determining the type of physiological response (Marshall, 1995). Therefore, the duration and magnitude of MAPK activation is a determinant factor to the biological outcome of signalling.

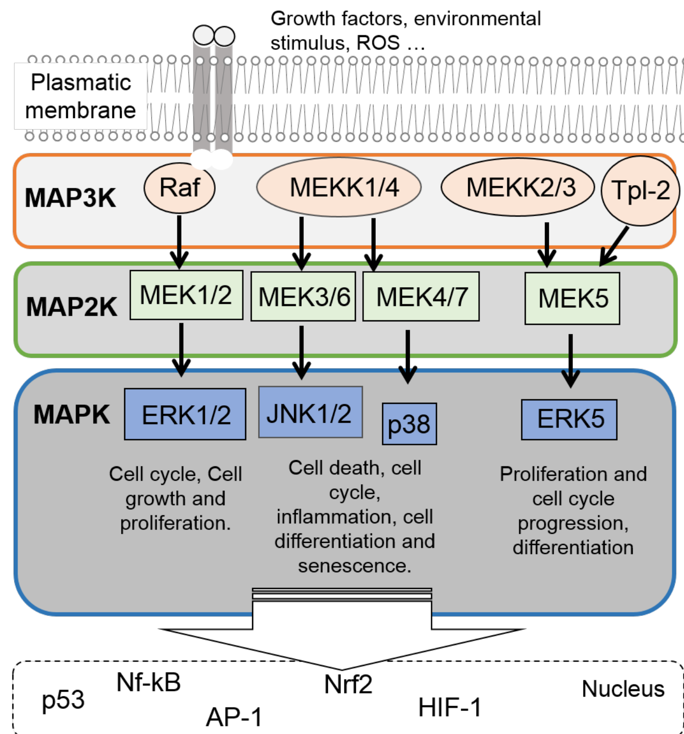


Figure 1.3 MAPKs pathways. Extracellular stimuli, including oxidative stress, activate several intracellular MAPK pathways through membrane receptors. Each MAPK pathway axis is composed of at least three tiered kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. Activated MAPKs phosphorylate various substrate proteins leading to activation of transcription factors and regulation of genes that control a variety of cellular activities, including cell proliferation, differentiation, survival, death and transformation.

Within the MAPK cascades, several protein kinases and MKPs are directly regulated by ROS (Winterbourn and Hampton, 2008) (Denu and Tanner, 1998; Lee *et al.*, 1998; Meng *et al.*, 2002). ERK1/2 phosphorylation is inhibited by S-nitrosylation in Cys183 in MCF-7 cells leading to cell apoptosis (Feng *et al.*, 2013). Upon oxidation, a disulphide bond is formed between Cys 109 and 196 in MKK6, which specifically phosphorylates and activates p38 MAPKs, leading to inactivation of the kinase activity. In addition, the two cysteines involved in the disulphide bond formation are conserved in all members of MAPKKs (Diao *et al.*, 2010).

Two cysteines residues (Cys153 and Cys158) in the Sty1 MAPK of the yeast *Schizosaccharomyces pombe* are specifically important for H_2O_2-induced gene expression and oxidative stress resistance (Day and Veal, 2010). The nitric oxide-mediated suppression of JNK1 is dependent on the redox regulation of Cys116 (Park *et al.*, 2006). TNF-α mediated ROS generation causes oxidation and inhibition of JNK inactivating phosphatases through oxidation of the catalytic cysteine into a sulphenic acid, resulting in sustained activation of JNK and promoting cell death (Kamata *et al.*, 2005). IL1β increases the production of H_2O_2 in rat primary glial cells, promoting the phosphorylation and activation of p38 due to the transient inactivation of protein

phosphatases (Robinson *et al.*, 1999). The production of ROS induced by transforming growth factor β contributes to oxidative modification and inactivation of MAPK phosphatase 1 (MKP-1), leading to sustained phosphorylation and activation of JNK and p38 MAPKs (Liu *et al.*, 2010). ERK activation is also promoted by the ROS-mediated inactivation of dual-specific phosphatase 3 (DUSP3) (Wentworth *et al.*, 2011). Oxidation of the Src homology 2 domain containing protein tyrosine phosphatase (SHP2) results in its inactivation and the consequent up-regulation of platelet-derived growth factor receptor (PDGFR) signalling (Meng *et al.*, 2002). Protein-tyrosine phosphatase 1B inactivation by H_2O_2 is required for sustained activation of epidermal growth factor receptor (EGFR) cell signalling (Lee *et al.*, 1998). Altogether, oxidation and inhibition of protein tyrosine phosphatases (PTP) by ROS appears to be one of the molecular mechanisms involved in the transduction and sustained activation of growth factor signals.

The apoptosis signal-regulated kinase-1 (ASK1) is an upstream MAPKKK that plays a pivotal role in promoting cell death under oxidative stress (Tobiume *et al.*, 2001) through the regulation of both JNK and p38 MAPK pathways (Ichijo *et al.*, 1997). ASK1 is regulated by Trx, which in the reduced form inhibits ASK1 oligomerization and consequent (Saitoh *et al.*, 1998) (**Figure 1.4**). Under the stimulation of oxidants or ROS, two cysteines

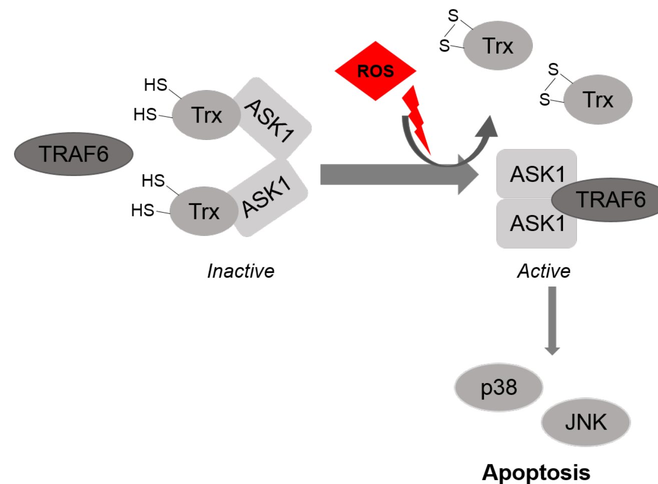


Figure 1.4 Regulation of ASK1 by Trx. The kinase activity of ASK1 is inhibited by the reduced form of Trx. Under several types of stress, Trx is oxidized and dissociates from ASK1, which is then activated by autophosphorylation and recruitment of TRAF6. Activated ASK1/TRAF6 phosphorylates the downstream MAPKs p38 and JNK leading to apoptosis.

residues in the redox center of Trx are oxidized forming a disulphide bond between Cys-32 and Cys-35 and resulting in the dissociation of the Trx from ASK1. This allows the complete oligomerization and the autophosphorylation of a conserved threonine residue (Thr838 in humans) in ASK1 (Saitoh *et al.*, 1998; Tobiume *et al.*, 2002).

Phosphoinositide 3-kinase (PI3K) pathway

The PI3K pathway is a key mediator of cellular responses to growth factors, hormones, and cytokines and is activated by receptor tyrosine kinases. Consisting of a p110 catalytic subunit and a p85 regulatory subunit, PI3K catalyses the synthesis of the second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3) from phosphatidylinositol 4,5 bisphosphate (PIP2). Within this signalling pathway, the PTEN dephosphorylates PIP3 back to PIP2. PIP3 recruits proteins containing the pleckstrin homology (PH) domain, including the serine/threonine kinases PDK1 (3-phosphoinositide-dependent protein kinase-1) and Akt, to the proximity of the cell membrane. For full activation, Akt is phosphorylated on a threonine residue by PDK1 and in a serine residue by the target of rapamycin (TOR) complex 2 (TORC2), a protein complex formed by at least the TOR protein and the rapamycin-insensitive companion of TOR, Rictor (Brown and Toker, 2015; Manning and Cantley, 2007) **(Figure 1.5)**. The active Akt phosphorylates a number of cytosolic and nuclear proteins regulating cell survival, cell cycle, protein translation, and glucose metabolism. Akt regulates the activity of glycogen synthase kinase 3 (GSK3) (Cross *et al.*, 1995), phosphorylates and inhibits the pro-apoptotic BAD proteins (Datta *et al.*, 1997), phosphorylates and blocks the interaction of FOXO transcription factors with target genes that promote apoptosis, cell cycle arrest and metabolic processes (Tran *et al.*, 2003) and phosphorylates E3 ubiquitin protein ligase (MDM2) leading to its nuclear translocation, where it down-regulates p53 functions associated to apoptosis (Zhou *et al.*, 2001).

The PI3K pathway is subject to redox regulation through oxidation of PTEN resulting in a disulfide bond formation between cysteine residues in the catalytic domain with consequent inactivation, which is reversed by peroxiredoxin II (Kwon *et al.*, 2004). Cellular receptors leading to activation of PI3K pathway may also be oxidized in cysteine residues for activation (Lee *et al.*, 2011).

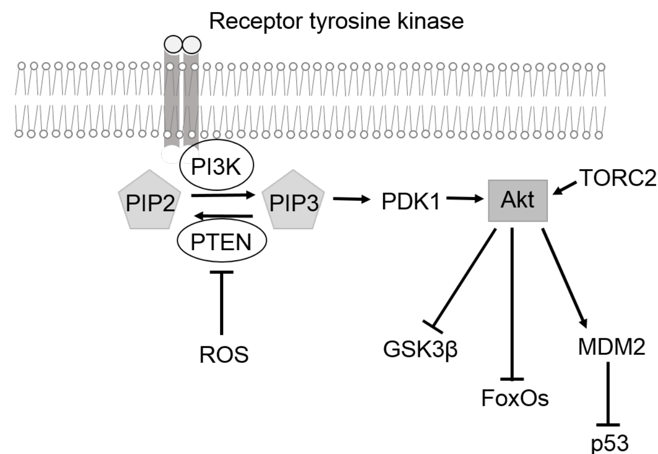


Figure 1.5 The PI3K pathway. The activation of the receptor tyrosine kinase results in PIP3 production by PI3K in the inner side of the plasma membrane. PDK1 and AKT interact with PIP3, being translocated to the inner membrane where Akt is activated by PDK1 and TORC2. AKT targets numerous substrates involved in the regulation of cell survival, growth and cell cycle progression. MDM2: E3 ubiquitin protein ligase; TORC2: target of rapamycin complex 2.

PKA, PKG and PKC

Oxidative modification can also occur in cGMP and cAMP-dependent protein kinases (referred as PKG and PKA respectively). In both cases there is an intermolecular disulphide bond formation between two dimeric subunits enhancing the activity of the kinases (Brennan *et al.*, 2006; Burgoyne *et al.*, 2007). The PKC is a family of lipid-activated serine/threonine kinases triggered by various ligands including hormones, neurotransmitters and growth factors. The PKCs are involved in cellular proliferation, differentiation, apoptosis and tumor promotion (Marengo *et al.*, 2011; Martiny-Baron and Fabbro, 2007; Steinberg, 2008). All PKC isoforms have two pairs of zinc fingers, each one containing six cysteines residues and two zinc atoms, making the regulatory domain susceptible to regulation by redox mechanisms (Cosentino-Gomes *et al.*, 2012). All these kinases have been implicated in MAPK signalling (Michel *et al.*, 2011; Sun *et al.*, 2014; Wang *et al.*, 2015).

Redox regulation of transcription factors

NF-κB

NF-κB is a transcription factor involved in the regulation of genes associated with inflammatory response. In absence of pro-inflammatory stimuli, NF-κB is kept latent in the cytosol bound to its inhibitors IκBs. Phosphorylation of IκBs by IκB-kinase (IKK complex) leads to proteassomal degradation of IκBs with consequent translocation of NF-κB to the

nucleus. The IKK complex is composed of IKK-α, IKK-β and a regulatory subunit IKKγ (also known as NEMO) (Hinz and Scheidereit, 2014). IKKγ forms dimers under H_2O_2 stimulation through the formation of disulphide bonds leading to the inhibition of IKK complex and consequent NF-κB activation (Herscovitch *et al.*, 2008). In addition, NF-κB can be redox controlled indirectly by PKA (Jamaluddin *et al.*, 2007).

NF-κB is sensitive to oxidation in its Cys62 of the p50 subunit, which results in inhibition of DNA binding (Matthews *et al.*, 1993). Therefore, once in the nucleus, NF-κB p50 subunit has to be reduced by reducing factor-1 (Ref-1) to enable DNA binding (Mitomo *et al.*, 1994; Pineda-Molina *et al.*, 2001). Ref-1 translocates to the nucleus under oxidative stress conditions where it acts as AP-endonuclease (Demple *et al.*, 1991; Tell *et al.*, 2009) and reduces cysteine residues in NF-κB and other target transcription factors, such as activator protein-1 (AP-1) (Xanthoudakis and Curran, 1992), p53 (Hanson *et al.*, 2005), and Nrf2 (Iwasaki *et al.*, 2006), enhancing DNA binding (Walker *et al.*, 1993). The oxidized Ref-1 appears to be reduced by nuclear translocated Trx (Wei *et al.*, 2000).

p53

p53 is activated in response to stress-related signalling, including DNA damaging agents and hypoxia, and is responsible for the transactivation of genes responsible for cell cycle arrest, apoptosis and DNA repair. The p53 cysteines are susceptible to oxidation (Sun *et al.*, 2003) and can be redox regulated by the reversible *S*-glutathionylation, (Velu *et al.*, 2007) which decreases the ability of p53 to bind to DNA. Both Ref-1 and Trx have been shown to reactivate oxidized p53 (Hanson *et al.*, 2005; Seemann and Hainaut, 2005).

Nrf2

Nuclear factor erythroid 2-Related Factor (Nrf2) is a cap´n´collar b-ZIP transcription factor that activates the transcription of several anti-oxidant genes via binding to the anti-oxidant responsive element (ARE). Under non-stress conditions, Nrf2 is sequestered in the cytoplasm by the association with an inhibitory protein, the Kelch-like ECH-associated protein-1 (Keap1) (Itoh *et al.*, 1999), which directs Nrf2 to ubiquitination and proteossomal degradation (Furukawa and Xiong, 2005). Upon oxidation of cysteines residues in Keap1, it dissociates from Nrf2 allowing the stabilization and translocation of Nrf2 to the nucleus (Zhang and Hannink, 2003).

FoxO

Forkhead box *O* is a family of transcription factors that function in tumor suppression (Paik *et al.*, 2007), stem cell maintenance (Tothova *et al.*, 2007) and regulation of longevity (Kenyon *et al.*, 1993). They also control the transcription of ROS-scavenging enzymes (de Keizer *et al.*, 2011). Several studies indicate that FoxOs can be directly regulated by ROS. FoxO4 activity is dependent on the formation of a cysteine-disulphide-dependent heterodimer with p300/CBP acetyltransferase (Dansen *et al.*, 2009). High levels of ROS induce disulphide dependent binding of FoxO4 to nuclear import receptor transportin-1 (TNPO1) leading to nuclear localization and extension of lifespan in *C. Elegans* (Putker *et al.*, 2013).

ROS imbalance in aging and cancer

The persistence of excessive levels of ROS have been linked to several pathological conditions, including neurodegenerative diseases (Cobb and Cole, 2015), metabolic disorders (Le Lay *et al.*, 2014; Maiese, 2015), cardiovascular diseases (Brown and Griendling, 2015) and cancer (Halliwell, 2007). It is also established that the accumulation of oxidative damage due to persistent oxidative stress is involved in the aging process (Wang *et al.*, 2013a).

Oxidative stress theory of aging

The oxidative stress theory of aging states that increases in ROS accompanies aging, leading to functional alterations and death (Hagen, 2003). During aging, there is an accumulation of oxidative damage to lipids (Sagai and Ichinose, 1980), proteins (Stadtman, 1992) and DNA (Agarwal and Sohal, 1994b). The respiratory chain in mitochondria is the main producer of cellular ROS (Chance *et al.*, 1979) and its production increases with aging (Sohal and Brunk, 1992). Also, mitochondrial DNA mutations have been implicated in human diseases. The age associated increase in oxidative stress may be related to an increase in the generation of ROS; a decline in cellular anti-oxidant defences (Agarwal and Sohal, 1996); or inefficiency to remove or repair damaged molecules (Agarwal and Sohal, 1994a). Accordingly, experimental elevation in enzymatic anti-oxidant defences reduce oxidative damage and increase lifespan. For example, simultaneous overexpression of *SOD1* and *CAT* genes decrease the amount of protein oxidative damage and extend *Drosophila melanogaster* lifespan (Orr and Sohal, 1994). Moreover, increase in the total cellular SOD activity delays the senescence of primary fibroblasts (Serra et al., 2003). Conversely, the inhibition of SOD induces premature

senescence in human fibroblasts (Blander et al., 2003). In contrast, other works suggest that oxidative stress contribute to increase lifespan through ROS mediated-activation of cellular anti-oxidant defences as an adaptative response of cells, a process called hormesis (Ristow and Schmeisser, 2011). For instance, lifespan is promoted in *Schizosaccharomyces pombe* through a ROS-dependent activation of the Sty1 MAP kinase stress pathway (Zuin *et al.*, 2010).

Saccharomyces cerevisiae as an aging model

The eukaryotic model *Saccharomyces cerevisiae* has been used as an organism to characterize the molecular mechanisms underlying oxidative stress and aging. Its similarities with mammalian cells, the short generation time and the simple genetic techniques have encouraged the use of yeast as a model to study human disease and aging (Karathia *et al.*, 2011).

In yeast, lifespan has been studied using two different approaches: replicative lifespan (RLS), which is based on the replicative potential of individual cells, and chronological lifespan (CLS), which is based on the survival of a cellular population in a non-dividing phase (Longo *et al.*, 2012). RLS is measured by the number of daughter cells produced by a mother cell prior to senescence and resembles the aging of mammalian cells that undergo a fixed number of population doublings. CLS is measured by culturing cells in liquid media, where they enter a non-dividing state once the glucose has been exhausted, and viability is measured over time. It is assumed that CLS resembles that of post-mitotic tissues. The relationship between RLS and CLS is poorly understood and several cellular factors involved in aging play opposite roles in each mechanism. One advantage of CLS over RLS is that CLS constitutes a model of organismal aging (Longo *et al.*, 2012). Using these models, several longevity pathways have been characterized.

Longevity pathways in Saccharomyces cerevisiae

Several signalling pathways involved in the response to nutrients have been implicated in the regulation of lifespan. In yeast, signalling pathways involved in nutrient response and aging includes the TOR/Sch9p and Ras/Cyr/PKA pathways, being both integrated by Rim15p. These pathways have roles in the regulation of cell growth, metabolism and stress resistance. Sirtuins have also been implicated in the regulation of processes important for cellular longevity. Sirtuins are a family of NAD^+-dependent deacetylases and most of them convert NAD^+ and the acetylated substrate into deacetylated products, nicotinamide and *O*-acetyl-ADP-ribose (Finkel *et al.*, 2009). Sirtuins react to changes in

metabolic activity and are regulators of proliferator-activated receptor coactivator-1α (PGC-1α) (Rodgers and Puigserver, 2007), acyl-CoA dehydrogenase (Hirschey *et al.*, 2010) as well as other enzymes involved in cellular metabolism.

TOR/Sch9 pathway

The TOR/Sch9 pathway is activated by amino acid-derived signals and is involved in the control of growth, metabolism and stress resistance (De Virgilio and Loewith, 2006). TOR proteins are serine/threonine kinases inhibited by the antifungal and immunosuppressive agent rapamycin (Schmelzle and Hall, 2000). The TOR proteins, Tor1p and Tor2p, form two distinct multimeric protein complexes known as the rapamycin sensitive TOR complex 1 (TORC1) and the rapamycin insensitive TORC2 (Schmelzle and Hall, 2000). In yeast, Tor1p or Tor2p associated with Kog1p, Tco89p and Lst8p form TORC1. Tor2p associated with Lst8p, Avo1p, Avo3p, Bit61p and Bit2p form TORC2 (Loewith *et al.*, 2002). In higher eukaryotes, insulin/PI3K signalling to TOR is mediated through Akt (also known as PKB), which alleviates the inhibition of TOR (Martin and Hall, 2005). In yeast, Sch9p shares a high degree of homology with the serine/threonine kinase Akt (Toda *et al.*, 1988). Several works demonstrate the role of TOR signalling and Sch9p in lifespan extension (Fabrizio *et al.*, 2001; Stanfel *et al.*, 2009). Decreased TORC1 activity has been found to increase lifespan in models of yeast (Powers *et al.*, 2006), nematodes (Vellai *et al.*, 2003), flies (Moskalev and Shaposhnikov, 2010) and mice (Harrison *et al.*, 2009).

Ras/Cyr/PKA pathway

The Ras/Cyr/PKA pathway responds to changes in glucose availability and plays a major role in the regulation of metabolism, stress resistance and cell cycle progression. In the active conformation, the G-proteins Ras stimulate Cyr1p activity which is responsible for the synthesis of cellular cAMP (Matsumoto *et al.*, 1982). CLS is extended by mutations that reduce the activity of Cyr1p and by deletion of the RAS2 gene (Fabrizio *et al.*, 2001).

Rim15p

Rim15p is a glucose-repressible protein kinase negatively regulated by TOR/Sch9p and Ras/Cyr/PKA pathways (Pedruzzi *et al.*, 2003). Lack of Rim15p abolishes the lifespan extension as well as the stress resistance phenotype of *sch9*Δ, *tor1*Δ and *ras2*Δ cells. The Rim15p regulates the stress response transcription factors Msn2/4p and Gis1p (Pedruzzi

et al., 2000) leading to the activation of several genes associated with oxidative stress responses, such as *SOD1* and *SOD2* (genes with stress response element in their promoter), and post-diauxic shift (genes with post-diauxic shift element in their promoter) (Cameroni *et al.*, 2004). Indeed, expression of Sod2p is necessary for the lifespan extension of *sch9*Δ mutants, which may be due to the protection of mitochondrial aconitase from age dependent inactivation. Furthermore, expression of mitochondrial Sod2p seems to be partially required for the longevity extension caused by mutations that decrease the activity of Ras/Cyr/PKA and TOR/Sch9 pathways (Flattery-O'Brien *et al.*, 1997; Pedruzzi *et al.*, 2000). The evidences indicate that extension of CLS seems to depend on the activation of stress resistance mechanisms.

Sirtuins

The yeast silent information regulator 2 (sir2)-like proteins (sirtuins) are more associated with RLS. The *sir2*Δ mutant has a shorter RLS while overexpression of *SIR2* extends RLS (Kaeberlein *et al.*, 1999; Kennedy *et al.*, 1997). The involvement of Sir2p in the RLS extension may be due to its role in decreasing the levels of toxic repetitive extrachromosomal ribosomal DNA (rDNA) circles (ERCs) (Kennedy *et al.*, 1995). ERCs are individual or multiple rDNA genes that have been excised through unequal intra-chromatid exchange and its accumulation in cells promotes aging (Sinclair and Guarente, 1997). Accordingly, overexpression of Sir2p supresses rDNA recombination, ERCs formation and extends RLS (Kaeberlein *et al.*, 1999).

In contrast to RLS, *sir2*Δ mutant has a longer CLS than the wild type in caloric restriction conditions (Fabrizio *et al.*, 2005; Smith *et al.*, 2007; Wu *et al.*, 2011b). Also, deletion of *SIR2* in combination with mutations that reduce nutrient signalling increases CLS (Fabrizio *et al.*, 2005). This may be due to the absence of the repressive effects that Sir2p has on stress responsive genes. This negative regulation of stress responsive genes by Sir2p is controlled by the nicotinamide deaminase Pnc1p. In mutants of TOR signalling, the activity of Msn2/4p transcription factors is increased resulting in the induction of Pnc1p expression (Medvedik *et al.*, 2007). Pnc1p increases the flux through the NAD^+ salvage pathway stimulating Sir2p activity, which serves as a buffer system for the activation of cellular anti-oxidant defences (Anderson *et al.*, 2003).

ROS in the etiology of cancer

Carcinogenesis is a multistep process involving high genomic instability due to chronic oxidative stress leading to acquisitions of mutations and subsequent selective clonal expansion of the mutated cell (Hanahan and Weinberg, 2011). The evidence that ROS are involved in the initiation of cancer was first demonstrated in 1984 when exposure of mouse fibroblasts to ROS led to cellular transformation (Zimmerman and Cerutti, 1984). The absence of anti-oxidant defences, such as CuZnSOD and Prx1, in mice results in increased rates of cancer development (Egler *et al.*, 2005; Elchuri *et al.*, 2005). High ROS levels are associated to oxidative DNA damage and numerous studies have demonstrated that 8-hydroxydeoxy guanosine (8-OHdG), the most abundant DNA lesion, is elevated in numerous human cancers (Diakowska *et al.*, 2007; Miyake *et al.*, 2004; Tanaka *et al.*, 2008). A compilation of works suggests that not only ROS are involved in the carcinogenesis process but also regulate proliferative and apoptotic pathways in an aberrant way in cancer cells, promoting their survival (Sen *et al.*, 2012).

Pro-oxidant Environment in Cancer Cells

The persistent high levels of ROS induce the activation of key transcription factors that contribute to up-regulation of stress response genes, leading to cancer cell adaptation to the high levels of ROS. This favours the aberrant activation of several pathways by ROS, such as those involved in cellular migration, proliferation and survival. Indeed, it has been demonstrated that cancer cells have higher levels of ROS than normal cells *in vitro* (Szatrowski and Nathan, 1991) and *in vivo* (Trachootham *et al.*, 2008b; Zhou *et al.*, 2003). This increase in intracellular ROS levels potentiates cancer progression and promotes cell survival and proliferation (Radisky *et al.*, 2005) **(Figure 1.6)**. These effects are associated with the up-regulation of MAPK and PI3K/Akt pathways and anti-apoptotic genes and down-regulation of apoptotic genes (McCubrey *et al.*, 2007).
The effect of ROS depends on the type, concentration and time exposure. For instance, in MCF-7 cells, acute exposure (24 h) to 25 µM or 250 µM of H_2O_2 inhibits the growth in a dose-dependent manner, however, chronic exposure (3 months) to 25 µM results in increased cell growth and survival and higher tumorigenic potential (Mahalingaiah and Singh, 2014). This indicates that cancer cells are not only able to adapt to a chronic exposure to H_2O_2 but also use it to fuel signalling pathways contributing to their growth. Therefore, ROS are in part responsible for the cancer phenotype.

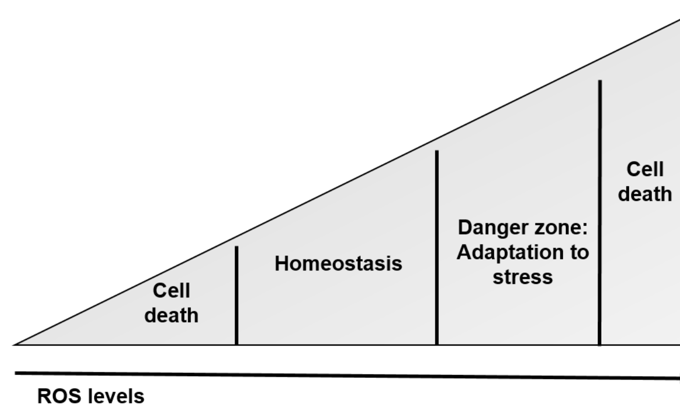


Figure 1.6 Control of ROS levels in cells. In moderate levels, ROS are involved in the control of cellular signalling and maintenance of homeostasis. Insufficient levels of ROS can result in impairment function of signalling pathways associated to survival. On the other hand, when ROS are elevated, cells respond through activation of mechanisms to adapt to the new levels of ROS. However, accumulation of oxidative damages and cell death occurs when a certain threshold is exceeded.

Anti-oxidant defences in cancer cells

Cancer cells seem to readjust its anti-oxidant defences and to up-regulate pro-survival signalling pathways in order to escape from severe oxidative damage caused by their intrinsic high redox state (Farber and Rubin, 1991). In general, higher levels of anti-oxidant defences are encountered in cancer cells in comparison to the normal counterparts (Hileman *et al.*, 2004; Hu *et al.*, 2005; Janssen *et al.*, 2000; Nakata *et al.*, 1992). However, the available literature on the activity and expression of anti-oxidant enzymes in cancer cells is highly controversial. For example, MnSOD activity has been postulated to be low in all malignant tumors (Amundson *et al.*, 1998; Kahlos *et al.*, 1998), but other studies have also shown high levels of MnSOD in malignant tumors (Janssen *et al.*, 2000; Li *et al.*, 2011).

Despite the controversy, many studies show that H_2O_2 levels are elevated in cancer cells and that those levels are controlled by variations in the balance between different anti-oxidant enzymatic defences and the levels of different ROS, such as H_2O_2 and $O_2^{\cdot -}$. This has been demonstrated in a number of cancer cell lines that have a low catalase activity together with high levels of MnSOD activity, which lead to high steady state levels of H_2O_2 and increase metastasis and resistance to apoptosis (Nelson *et al.*, 2003). Furthermore, the stable transfection of malignant MCF-7 cells with cDNA of human catalase inhibits proliferation and reverts the malignant features (Zhang *et al.*, 2002). All this data suggests a general role for H_2O_2 in tumor cell survival and proliferation.

Exploiting the redox environment in cancer cells for therapy

Based on the characteristics of the redox environment in cancer cells, two therapeutic approaches can be adopted: through anti-oxidant action, abrogating ROS signalling and suppressing tumor growth; or through pro-oxidant action, overwhelming the cellular anti-oxidant systems of cancer cells and increasing ROS levels above the threshold levels necessary to induce apoptosis. Indeed, it was demonstrated that excessive ROS basal levels render cancer cells more susceptible to ROS inducing drugs (Zhou *et al.*, 2003).

Phenolics in human health

Phenolic compounds in the human diet

Phenolics are compounds containing an aromatic ring with at least one hydroxyl group attached directly to a hydrocarbon group. They occur naturally in the plant kingdom as secondary metabolites that have several important functions, such as anti-microbial and protection against ultraviolet radiation (Crozier *et al.*, 2009). They are part of the human diet through ingestion of vegetables and fruits and several compounds are marketed as dietary supplements. Phenolics can be categorized depending on the number of phenol rings and the number and type of functional groups attached to the ring(s). They vary from single aromatic ring compounds, including simple phenols and phenolic acids, to more complex structures containing more than one aromatic ring. The last group is commonly called polyphenols (meaning compounds with more than one aromatic ring). Polyphenols can be further divided into several classes, ranging from stilbenes, lignans and flavonoids (Crozier *et al.*, 2009).

The basic structure of flavonoids consists of 2 aromatic rings (A and B) bound together by three carbon atoms to form the oxygenated heterocycle (C ring) **(Figure 1.7)**. Flavonoids can be further divided into several sub-classes, depending on the number, type and localization of functional groups attached to the molecules. Flavonols are the most abundant subclass of flavonoids present in the human diet. They are characterized by the presence of a 4-oxo function and a hydroxyl group in the carbon 3. Evidences indicate that the most abundant flavonols in the human diet are quercetin and kaempferol (Crozier *et al.*, 2009).

Anthocyanidins (aglycones) are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrilium (or flavylium) cation. Their consumption is increasing in the human

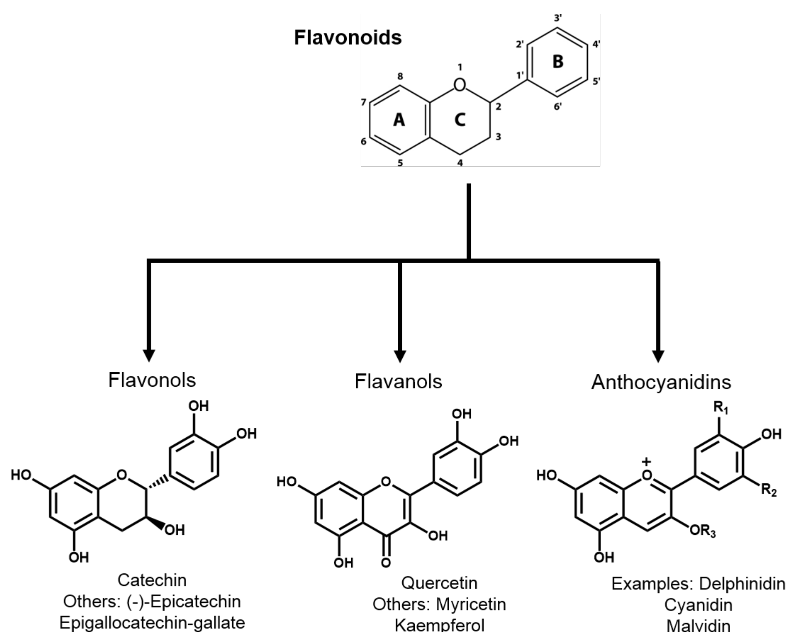


Figure 1.7 Basic flavonoids skeleton and overview of several sub-classes. At the flavonoid basic structure, marks for benzene rings are indicated.

diet through the consumption of berry fruits and, together with carotenoids, they are the most used colorants in food industry (International Food information Council and Foundation US Food & Drug administration, 2004). They are water soluble pigments responsible for the bright pink, red, violet and blue colours of flowers and fruits. More than 500 different anthocyanins and 23 anthocyanidins have already been identified.

Anthocyanidins have the characteristic of being in equilibrium between 4 different structures depending on the pH of the solution. The basic flavylium cation predominates at pH less than three (red colour); purple quinoidal bases are formed at pH between 2 and 3; at pH 5-6 a colourless carbinol pseudobase and a chalcone predominates (Crozier *et al.*, 2009; Flamini *et al.*, 2013).

Bioavailability of phenolics in the gastrointestinal system

Regarding the bioavailability of phenolics, we have to consider how they are consumed: through fruits and vegetables sources or dietary supplements. Considering fruits and vegetables, phenolics are predominantly present as glycoside conjugates (Flamini *et al.*, 2013). In this case, the bioavailability will depend on the food matrix. Due to the high complexity of the constituents of plant derived foods, some interfering substances may exist that can decrease the availability of some phenolics; however, it has also been

suggested that the mixture of several phenolics can exert a beneficial synergistic effects that may not exist in a supplement pill. Apart from the diet source of phenolics in human consumption, the inter-individual variability (variations in the colonic microflora, genetic variations in the enzymatic metabolism, age associated changes in the physiology of the intestinal tissue) also affects absorption in the gastrointestinal tract (Fang, 2014; Faria *et al.*, 2014).

Studies using mice and rat models and human intervention studies have provided information regarding the bioavailability of phenolics. In those works, a single supplement with purified compounds or a food component rich in a specific phenolic [example: wine, malvidin 3-glucoside (Bub *et al.*, 2001)] is given to the animals/subjects (Dang *et al.*, 2014; Felgines *et al.*, 2014; Kaushik *et al.*, 2012; Wruss *et al.*, 2015). The concentrations of the native form and respective metabolites that reach the blood plasma are at the nM range after an ingestion in the µM range. For instance, the ingestion of fried onions containing 275 µmol of flavonol glycosides (containing mainly quercetin glycosides) resulted in a maximum plasma concentration of metabolites between 62 nM (for quercetin-*O*-glucuronide) and 665 nM (for quercetin-3´-*O*-sulfate) (Mullen *et al.*, 2004). However, before absorption into the plasma, these compounds can reach reasonable concentrations in the gastrointestinal (GI) tissues, which are directly exposed to the diet. This is also demonstrated in studies using subjects with ileostomy, in which there is a large percentage of recovery of phenolics in the ileal fluid (Jaganath *et al.*, 2006). Also, in the first work using these subjects, the intake of a capsule supplement containing 100 mg of quercetin aglycone resulted in the recovery of 66 ± 9 mg of the parent compound in the ileostomy effluent (Hollman *et al.*, 1995). In another study, where the diet was not controlled (meaning that it was not encouraged to increase the intake of fruit and vegetables), quercetin was detected in human fecal water at µM range (0.77 – 2.04 µM) of one day in one subject male (Jenner *et al.*, 2005). Regarding anthocyanins, after the consumption of 350 ml of concord juice containing 528 ± 11 µmol of a phenolic mixture, of which 238 ± 6 µmol were anthocyanins, 40-45% (approximately 209 µmol) of intake was recovered in the ileal fluid of ileostomy volunteers. In the same study, using human healthy volunteers, anthocyanins were detected in the nM range in plasma (Stalmach *et al.*, 2012). Also, the presence of dietary supplements containing phenolics in the market increases the availability of these compounds in the human diet. For instance, myricetin supplements are marketed for human consumption recommending a daily dose of 300 mg (corresponding to 943 µmol). It has been demonstrated that flavonols are stable in gastric fluids (Hollman *et al.*, 1995; Kaushik *et al.*, 2012) and are found in considerable high levels in stomach tissue of rats after a chronic feeding diet rich in flavonols (Graf *et al.*,

2006). All these data clearly show the high concentrations of phenolics achieved in the GI tract.

Health benefits attributed to phenolics

The diet is a determinant factor for the overall health. Many pathological conditions, such as cancer, cardiovascular disorders and inflammatory associated diseases are highly influenced by the diet quality. Evidences from epidemiological data and animal studies indicate that phenolics of plant have beneficial effects in health (Babu *et al.*, 2013).
Phenolic compounds have been a subject of intense research. Initially it was though that their intrinsic anti-oxidant properties were responsible for the health benefits. However, the redox behaviour of a phenolic depends on its intrinsic physicochemical properties and concentration, as well as on external factors, such as pH, O_2 tension and presence of transition metals (Decker, 1997). Therefore, phenolic compounds have redox properties, behaving as anti-oxidants but also as pro-oxidants. In addition, some studies show that several phenolic compounds can interact directly with cellular components of signalling pathways (Vauzour *et al.*, 2010).

Anti-oxidant and anti-aging effects of phenolics in Saccharomyces cerevisiae

S. cerevisiae have been used to investigate the anti-oxidant properties of phenolic compounds and plant extracts, by analysing their effect in the cellular redox status. Furthermore, it has been a useful model to analyse the effect of those compounds in aging.
The most efficient mechanism to extend lifespan described until now is through caloric restriction. A reduction in calories intake by 30% to 75%, while maintaining all essential nutrients present in sufficient amounts in the diet, causes lifespan extension in several organism models (Taormina and Mirisola, 2014). In yeast cultures, the most common practice to mimic caloric restriction is through glucose limitation from 2% to 0.5% or 0.05% (Kaeberlein *et al.*, 2005; Smith *et al.*, 2007; Wei *et al.*, 2008). However, the balance of nutrients in the diet, such as proteins, lipids, carbohydrates, vitamins, minerals as well as phenolic compounds, may also have a crucial role in the regulation of lifespan and health-span (aging with minor age-associated pathologies).
The scientific community proposes that phenolic compounds are candidates to modulate lifespan due to their role in reducing oxidative stress and to their ability to modulate signalling pathways. Multiple phenolic compounds and plant extracts have been studied in the aging of *C. elegans* (Kampkotter *et al.*, 2007b), *Drosophila melanogaster* (Peng *et al.*,

2012), mice (Aires *et al.*, 2012), fish (Valenzano *et al.*, 2006) and in human cells (Chondrogianni *et al.*, 2010). However, evidence from humans is limited to data concerning effects in the improvement of aging associated pathologies.

In *S. cerevisiae* several plant extracts and isolated phenolic compounds protects from oxidative stress. A flavonoid enriched cocoa powder, gingko biloba leaves extract and phloridzin, a dehydrochalcone found in the skin of apples and in apple juice (Le Deun *et al.*, 2015), increases oxidative stress resistance against H_2O_2 (Marques *et al.*, 2011; Martorell *et al.*, 2011; Xiang *et al.*, 2011). Quercetin also increases the resistance against oxidative stress associated to a decrease in intracellular oxidation, glutathione oxidation, protein carbonylation and lipid peroxidation (Belinha *et al.*, 2007). Likewise, quercetin 3-*O*-β-D-glucopyranoside reduces lipid peroxidation, protein carbonylation and intracellular ROS levels induced by H_2O_2 and menadione, a source of superoxide radical (Silva *et al.*, 2009). Resveratrol and catechin increases the tolerance against H_2O_2 with a reduction in lipid peroxidation (Dani *et al.*, 2008).

Besides the normal cells, phenolics are also able to protect mutant strains in anti-oxidant defences against oxidative stress. The *Hibiscus tiliaceus* L methanolic flower extract, flowers widely used in birth control in Asia and Africa, increases the survival of several *S. cerevisiae* strains defective in anti-oxidant defences under H_2O_2 and *t*-BOOH treatment (Rosa *et al.*, 2006). Whole apple extracts or a polyphenolic extract from apple also increases the resistance against H_2O_2-induced oxidative stress in *kllsm4Δ1* mutants (Palermo *et al.*, 2012), which have a deletion in *LSM4* gene that encodes a subunit of the Lsm complex involved in mRNA degradation and the phenotype is characterized by a higher sensitivity to oxidative stress and premature aging (Cooper *et al.*, 1995; Mazzoni *et al.*, 2005). Ascorbic acid and caffeic acid improves the growth recovery under H_2O_2-induced oxidative stress of *sod1*Δ, *cta1*Δ and the double-knockout strain *oye2*Δ*glr1*Δ, a mutant that lacks the NADPH-oxidoreductase and the glutathione reductase enzymes, and which is characterized by having high levels of GSSG (Amari *et al.*, 2008).

The protective effects of phenolics against oxidative stress seems to be mediated through the activation of stress responsive pathways and cellular anti-oxidant defences. Those evidences are shown in works where the treatment of yeast cells with phenolics, in absence of an oxidative stress, results in an activation of cellular anti-oxidant defences. For example, transcription analysis showed that dp-3-gluc increases the transcription of the anti-oxidant genes, namely *TRX2* (mitochondrial Trx), *AHP1* (alkyl hydroperoxide reductase) and *CTT1* (cytoplasmic catalase). In addition, dp-3-gluc and petunidin-3-glucoside protects *S. cerevisiae* from the toxic effects of tert-butyl hydroperoxide (t-BOOH) in a Msn2-, Msn4-dependent manner (Jimenez *et al.*, 2010). Green tea extract,

catechin and EGCG also induces *TRX2* expression through the activation of the Yap1p transcription factor (Takatsume *et al.*, 2005).

The activation of stress responsive transcription factors may be indirectly mediated by the H_2O_2 formed in culture by the phenolic compounds. EGCG and green tea extract induce the nuclear localization of Yap1p and Msn2p correlated with production of H_2O_2 in the medium and this effect is reverted by the addition of catalase (Maeta *et al.*, 2007). Furthermore, the *yap1*Δ and *skn7*Δ mutants are sensitive to green tea extract, showing that the activation of stress responsive pathways is essential for a response towards the production of H_2O_2 in the medium by green tea extract (Takatsume *et al.*, 2005). Recently, it was shown that resveratrol causes ROS accumulation and induces the expression of Yap1p target genes, including *TRX2*, *TRR1* and *AHP1* (Escote *et al.*, 2012). Concluding, the increase in oxidative stress resistance achieved by *S. cerevisiae* treated with phenolic compounds may be mediated through a hormesis mechanism, in which phenolics induce cellular anti-oxidant defences through the generation of low levels of ROS.

Regarding the extension of lifespan, apple extract and phloridzin (Xiang *et al.*, 2011), quercetin (Belinha *et al.*, 2007), caffeine (Kuranda *et al.*, 2006; Reinke *et al.*, 2006; Wanke *et al.*, 2008) and resveratrol have been reported to do so in yeast. Phloridzin increase RLS in *S. cerevisiae* through an increase in the expression and activity of SOD, which is essential for the extending lifespan effects, as demonstrated by the absence of effect in *sod1*Δ and *sod2*Δ mutants (Xiang *et al.*, 2011). Whole apple extracts and the polyphenolic extract from apple prevents ROS accumulation during aging and prolongs CLS of *kllsm4*Δ1 mutants (Palermo *et al.*, 2012). Caffeine is a central nervous system stimulant present in several plants, such as in coffee and tea plant (Ahluwalia and Herrick, 2015) and is an inhibitor of TORC1. It has been reported that caffeine extends *S. cerevisiae* CLS via TORC1-Sch9p-Rim15p signalling (Kuranda *et al.*, 2006; Reinke *et al.*, 2006; Wanke *et al.*, 2008).

The important role of sirtuins in aging encouraged the search for phenolics able to activate sirtuins resulting in a delay of aging. In a compound screen analyses, it was shown that quercetin, piceatannol and resveratrol stimulate SIRT1 activity *in vitro*. However, only resveratrol increases RLS in *S. cerevisiae*, acting downstream of Pnc1p and requiring Sir2p for its effect. In contrast, resveratrol fails to extend CLS, indicating that its effect is mainly mediated by sirtuins instead of modulating oxidative stress responses in cells (Howitz *et al.*, 2003).

The anti-tumoral effects of phenolics: extrapolation from cell culture studies

Most of the data concerning biological effects of phenolics in cancer cells results from *in vitro* cell cultures. During research of redox events in cells in *in vitro* culture, it is important to consider potential artefacts. Cells in culture are exposed to a hostile environment, with a high tension of O_2 (160 mmHg) when compared to blood or tissues (<40 mmHg) (Sherwood, 2004). The O_2 tension will affect cellular responses to drugs, as reported for the anti-cancer drug triapazamine in A549 cells (Peng *et al.*, 2013) and the extend of activation of redox responsive transcription factors in cells, such as NF-κB (Grodzki *et al.*, 2013). Moreover, phenolics are unstable in commonly used culture mediums and can undergo auto-oxidation, generating ROS, quinone and semi-quinone species. The ROS and metabolites formed can in turn mediate the effects *in vitro* that normally are associated to the parent compounds (Long *et al.*, 2000). The instability of several phenolics and the generation of H_2O_2 in multiple culture mediums have been reported (Long *et al.*, 2010). The effects of the compounds and ROS will depend also on several factors, including cell type (Elbling *et al.*, 2010), culture medium (Liu and Sun, 2003) and other experimental conditions (Bellion *et al.*, 2009; Liu and Sun, 2003).

The (-)-epigallocatechin-3-gallate (EGCG), the most abundant and biologically active constituent in tea, has anti-proliferative and pro-apoptotic effects in several tumoral cell lines *in vitro*. However, EGCG has been demonstrated to be unstable on several culture mediums, such as McCoy's 5A, HBSS, Ham's H-12 (Hong *et al.*, 2002), DMEM (Weisburg *et al.*, 2004) and HAM's F12:RPMI 1640 (1:1) (Sang *et al.*, 2005). In McCoy's 5A, EGCG has a half-life of less than 30 min. Analysis of the medium overtime shows the formation of EGCG oxidation products, including theasinensin and H_2O_2. For example, 50 µM of EGCG generates 25 µM of H_2O_2 in culture medium (Hong *et al.*, 2002). It was suggested that EGCG is more stable in culture medium [HAM's F12 and RPMI 1640 (1:1)] supplemented with SOD, in a phosphate buffer flushed with nitrogen (N_2) or in a aqueous solution in the presence of EDTA, implicating metal-catalyzed auto-oxidation of EGCG and involvement of O_2. Under those conditions, (+)-gallocatechin-3-gallate (GCG), an epimer of EGCG, but not the oxidation products of EGCG (dimers), is detected. (Sang *et al.*, 2005). Regarding the cellular effects of EGCG in cells in culture, EGCG decreases the viability of human T-cell acute lymphoblastic leukemia jurkat cells, but this effect is reverted by the addition of catalase, SOD or an iron chelating reagent, suggesting that ROS and iron participates in the cytotoxic effect of EGCG (Nakagawa *et al.*, 2004). EGCG also induces cytotoxicity and decreases intracellular GSH levels in tongue carcinoma cell line CAL27 and in salivary gland carcinoma cell line HSG1 and the addition of catalase or an iron chelator reverts this effect. In this work, the authors used a high concentration of

EGCG (300 – 500 µM) which was certainly a determinant factor for auto-oxidation in culture medium (Weisburg *et al.*, 2004). Besides EGCG, other compounds, such as delphinidin, rosmarinic acid and hydroxytyrosol, have been found to disappear in different culture media (DMEM, RPMI and MEM) and generate significant amounts of H_2O_2. Some compounds, such as apigenin, tyrosol, resveratrol, hesperetin or curcumin, although unstable in culture medium, do not generate H_2O_2, (Long *et al.*, 2010). Addition of gallic acid to WME medium, but not to DMEM or DMEM/Ham F-12, results in a dose-dependent increase in H_2O_2 (Liu and Sun, 2003). The stability of phenolics is also pH dependent, as shown by the faster decomposition of curcumin (100 µM in RPMI 1640) in neutral-basic conditions (Wang *et al.*, 1997).

Two different groups found contradictory data concerning apple extracts. Liu and Sun did not detect H_2O_2 in three different culture media (DMEM, DMEM/Ham F-12 and WME) incubated with an apple extract, despite having anti-proliferative effect in HepG2 and Caco-2 cells. Accordingly, the addition of catalase did not revert the inhibitory effect of the apple extract in HepG2 and Caco-2 cells proliferation. However, it was observed that H_2O_2 is highly unstable in these culture media and therefore did not have any anti-proliferative or cytotoxic effect (under 100 µM) in those cell lines (Liu and Sun, 2003). Later, another group detected H_2O_2 in DMEM medium incubated with apple extracts, and also with the two most abundant phenolics present in the apple extract, quercetin and rutin. Treatment of HT-29 cells with the apple extracts or with quercetin or rutin led to an increase in intracellular ROS levels and in total glutathione levels. The addition of catalase, but not SOD, resulted in undetectable H_2O_2 levels and reverted the cellular effects of the compounds (Bellion *et al.*, 2009). The contrasting effects may arise from differences in the apple extract, from the use of a different cell line and conservation conditions of the culture medium. Attention must also be taken for the method used for the detection of H_2O_2. For instance, H_2O_2 levels in WME incubated with gallic acid were detected by the ferrous oxidation xylenol (FOX)-2 assay but not with horseradish peroxidase (HRPO)-mediated oxidation of phenol red assay (Liu and Sun, 2003).

The cytotoxicity of phenolic compounds and H_2O_2 is highly influenced by the cell anti-oxidant clearance system efficiency. EGCG is cytotoxic in a ROS dependent manner in the keratinocyte cell line HaCat and leukemia HL-60 cells (Elbling *et al.*, 2010). Interestingly, pre-exposure of cells to low doses of H_2O_2 before exposure to a high dose of EGCG, or vice versa, decreases the cytotoxicity of EGCG in the normal keratinocyte HaCat cells but not in the leukemia HL-60 cells, indicating that the normal cell line is more efficient in the activation of defence mechanisms (Elbling *et al.*, 2010).

Several studies addressed the role of ROS in the modulation of cellular signalling by phenolics. ROS have been implicated in curcumin induced histone hypoacetylation, which

is suppressed by the addition of SOD and catalase (Kang *et al.*, 2005b). In HT-29 cells, EGCG-induce ROS mediated JNK activation leading to cell death, an effect that is reverted by the addition of GSH and n-acetyl cysteine (NAC), a precursor of glutathione and an anti-oxidant due to the presence of a thiol group (Chen *et al.*, 2003). However, in addition to the anti-oxidant roles, NAC and GSH increase the ability of cells to detoxify compounds, promoting cell survival. GSH has also important functions in the modulation of cell signalling. For example, phenethyl isothiocyanate (PEITC) causes a decrease in the levels of Myeloid Leukemia Cell Differentiation protein (MCL1) conjugated with glutathione. Glutathionylation of the anti-apoptotic protein MCL1 confers resistance to proteolytic cleavage by caspases. Supplementation of NAC to the culture medium prevents PEITC-induced glutathione depletion, ROS increase and caspase 3 activation (Trachootham *et al.*, 2008b). This suggests that glutathione depletion is an event that precedes the increase in intracellular ROS and the decrease in cellular viability caused by phenolics.

In esophageal squamous cell carcinoma KYSE 150 and in epidermoid squamous cell carcinoma A431, EGCG reduces cellular proliferation, EGFR phosphorylation and EGFR protein expression. Although addition of SOD prevents the inactivation of EGFR mediated signalling, it enhances the anti-proliferative effect of EGCG. The analysis of metabolites in the medium revealed that EGCG forms dimers and H_2O_2. The presence of SOD increases the EGCG stabilization in culture medium causing epimerization of EGCG to GCG (Hou *et al.*, 2005). Therefore, the GCG is responsible for the inhibitory effect on cell growth but the auto-oxidation of EGCG is responsible for the inhibition of EGFR mediated signalling. This demonstrates that the effects may not be only attributed to the ROS generated during the degradation of the compounds in culture medium but may arise from resulting metabolites.

A recent study addressed the contribution of the oxidizing ability and H_2O_2 generation by caffeic acid to Nrf2 nuclear translocation. For that, the authors compared the effect of caffeic acid with that of two related compounds: dimethylcaffeic acid, which has only the electrophilic moiety (oxidizing moiety) of caffeic acid and cannot undergo auto-oxidation, and dihydrocaffeic acid, which has only the nucleophilic moiety of caffeic acid. The induction of Nrf2 signalling pathway in human ovarian carcinoma cells A2780 was analysed. Only dimethylcaffeic acid and caffeic acid causes nuclear translocation of Nrf2, confirming that the electrophilic moiety is necessary. In contrast to dimethylcaffeic acid, caffeic acid leads to an induction of mRNA-*NRF2* levels that is reverted by catalase and in oxygen free environment. Therefore, although H_2O_2 formation from caffeic acid oxidation contributes to the upregulation of *NFR2* expression, the oxidizing ability of caffeic acid also contributes to the oxidation of Keap1 and Nrf2 nuclear translocation. Of note, cells

treated with either compounds did not show alterations in intracellular ROS levels, meaning that ROS generated by these compounds may exist at levels that are not detectable by the available methods (Sirota *et al.*, 2015).

Some studies showed a relation between the levels of H_2O_2 generated in culture medium and intracellular ROS. The generation of H_2O_2 by EGCG in culture medium correlates with the increase in intracellular ROS and inhibition of cell growth in the HIT-T15 hamster pancreatic-β cell line. All these effects are prevented upon incubation with catalase, NAC, alpha lipoic acid, or iron chelators (Suh *et al.*, 2010). The cytotoxicity of EGCG in keratinocyte cell line HaCat and in the leukemia HL-60 cells is also associated to increased intracellular ROS levels (Elbling *et al.*, 2010). However, the generation of H_2O_2 in culture medium is not always associated to increases in intracellular ROS. Although quercetin and myricetin increases H_2O_2 generation in culture medium, intracellular ROS levels decrease in Caco-2 cells treated with these compounds (Yokomizo and Moriwaki, 2006).

Several components of the cell culture media and supplements can contribute to the instability of phenolics and consequent generation of ROS. Recently, sodium bicarbonate was identified as the component in cell culture media responsible for the generation of H_2O_2, at least from the olive polyphenols oleuropein and hydroxytyrosol. The decrease in cell viability caused by these compounds in a cancer (MDA-MB-231), normal (MCF-10A) and immortalized normal (STO) cells is prevented by the addition of sodium pyruvate. NAC also prevents oleuropein-induced death in MDA-MB-231 cells (Odiatou *et al.*, 2013).

Of note, the detection of H_2O_2 is not possible in the presence of cells (Hong *et al.*, 2002; Yokomizo and Moriwaki, 2006), probably due to the fact that H_2O_2 easily crosses cellular membranes and is decomposed by cellular anti-oxidant defences. Therefore, it is important to standardize experimental conditions, since the effects seem to be highly dependent on the ratio of cell number to working volume because of the cell mediated clearance of H_2O_2 from auto-oxidizing compounds (Elbling *et al.*, 2011). Novel methodologies, such as microfluidic cell culture systems, providing manipulation of O_2 tension in cell cultures in a practical and reproducible way will allow the evaluation of the anti-tumoral effect of phenolics under different O_2 tensions (Peng *et al.*, 2013).

Phenolics actions through anti-oxidant mechanisms

The anti-oxidant and reducing abilities of phenolics have been demonstrated in several *in vitro* assays, including the ferric reducing anti-oxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ABTS (2,2`-azinobis(3-ethylbenzothiazoline)-6-sulfonate radical cation assay) and the oxygen radical absorbance capacity (ORAC) assays (Prior *et al.*,

2005). All these methods indicate anti-oxidant activity (or reducing ability) for phenolic compounds in general. However, these chemical assays do not demonstrate anti-oxidant effects *in vivo*.

Besides scavenging ability, phenolics can inhibit pro-oxidant enzymes, such as xanthine oxidase (Dew *et al.*, 2005; Spanou *et al.*, 2012), lipoxygenase (Chen, 2011) NADPH oxidase (Maraldi, 2013) and cyclooxygenase (COX) (Gerhauser *et al.*, 2003) *in vitro*. Their anti-oxidant activity may also be mediated by the induction of cellular defences (Du *et al.*, 2007) with consequent decrease in intracellular ROS. For example, it was demonstrated that catechin and proanthocyanindin B4 at µM concentrations induce anti-oxidant enzymes in cardiac H9C2 cells in a concentration dependent manner (Du *et al.*, 2007). Epicatechin also enhances anti-oxidant enzymes and protects Ins-1E cells from *t*-BOOH induced stress (Martin *et al.*, 2014). In Sprague-Dawley rats, pre-treatment with quercetin 1 h before lipopolysaccharide challenge increases the activities of SOD, catalase and GPx in lungs (Huang *et al.*, 2015). However, it is not clear if the effect exerted by the phenolics in these studies is due to direct interaction with the enzymes or associated with cell signalling events that regulate the activity of the enzymes.

Phenolics actions through pro-oxidant mechanisms

Besides anti-oxidant properties, pro-oxidant effects have also been reported for phenolics. In the presence of high concentrations of transition metals or basic pH conditions, phenolics can act as pro-oxidants (Hastak *et al.*, 2003).

It has been reported that phenolics can have beneficial effects in normal cells through an hormetic effect, in which a mild oxidative stress is induced by the phenolic compound that in turn enhances the cellular anti-oxidant defences, protecting the cells against a subsequent challenge with higher doses of the same or similar agent (Gems and Partridge, 2008). This mechanism is frequently associated to induction of the Nrf2 and NF-κB transcription factors (Yang *et al.*, 2014).

On the other hand, the pro-oxidant properties of phenolics have been implicated in the induction of cell death in tumor cells. A recent study showed that several flavonoids inhibit the growth and increase cell death of HepG2 cells accompanied by an increase in intracellular ROS (Zhang *et al.*, 2015a). It is likely that cancer cells are more susceptible to ROS increases due to their higher basal levels of ROS in comparison to normal cells. In addition, the selective induction of cell death in tumor cells in comparison to non-tumoral cells has been attributed to the dual redox behavior of some phenolics. Several phenolics cause an increase in intracellular ROS in tumoral cells but have an opposite effect in normal cells, indicating that their redox behaviour also depend on the cell type. In

agreement, normal epithelial cells are more resistant to the cytotoxic effect of EGCG in comparison to oral carcinoma cells (Yamamoto *et al.*, 2004).
The redox behaviour may also depend on the concentration of the phenolic. For instance, quercetin at low concentrations stimulates cellular proliferation and increases the anti-oxidant capacity of lung tumor A549 cells, while treatment with high concentrations decreases cell survival and the anti-oxidant capacity of cells (thiol content, activities of SOD, catalase and GST). This pro-oxidant effect is related to the production of H_2O_2 in culture medium (Robaszkiewicz *et al.*, 2007). Exposure of human hepatoma cells to curcumin at low and high concentrations also diminishes or enhances ROS generation, respectively (Kang *et al.*, 2005b). The pro-oxidant cytotoxicity of phenolics may also arise from the formation of quinone or semi-quinone oxidation products that arylate GSH and other cellular nucleophiles, decreasing glutathione levels (Metodiewa *et al.*, 1999). Indeed, the depletion of glutathione in tumor cells treated with phenolics is frequently reported.

Modulation of signalling pathways and transcription factors by phenolics

In biological systems, the rate of free radical generation seems to be faster than the scavenging rates of phenolic compounds (Ali *et al.*, 2013). Therefore, it is unlikely that phenolic compounds mediate their effects through scavenging of ROS. Alternatively, mounting evidence suggests that several cellular signalling pathways and transcription factors can be modulated by phenolics. Since there is numerous data regarding many different phenolic compounds and plant extracts, only quercetin and myricetin studies will be specifically referred. At the end, works with other compounds that are pertinent to this work will be briefly introduced.

Direct interaction of quercetin with components of signalling pathways

Quercetin [IUPAC name: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one] is one of the most abundant flavonoid in plants. Many works have demonstrated that quercetin has anti-tumoral activity *in vivo* and *in vitro*. *In vitro* studies suggest that quercetin is internalized by cells and reaches the nucleus (Arredondo *et al.*, 2010; Granado-Serrano *et al.*, 2012). Thus, its effects can be mediated by modulation of signal transduction pathways or through regulation of transcription factors in the nucleus.

PI3K/Akt pathway. The biological activity of quercetin, including the anti-tumoral activity *in vitro*, has been associated with the modulation of MAPK and PI3K/Akt signalling pathways. Indeed, quercetin has been recognized to be a protein kinase inhibitor. Also it

was demonstrated that quercetin binds to PI3K. In ascite cells of Dalton´s lymphoma mice, quercetin down-regulates the PI3K/Akt/p53 pathways as well the glycolytic metabolism, suppressing tumor growth (Maurya and Vinayak, 2015). Quercetin decreases cellular viability and migration and promotes senescence and apoptosis in several glioma cell lines with concomitant suppression of PI3K/Akt signalling pathways (Pan *et al.*, 2015). Quercetin decreases cellular growth and down-regulates the expression of PI3K and the phosphorylation of Akt in HeLa cells (Xiang *et al.*, 2014). Quercetin inhibits the EGF-induced epithelial-mesenchymal transition via EGFR/PI3K/Akt/-ERK1/2 pathway (Bhat *et al.*, 2014). Quercetin and myricetin also inhibit the casein kinase 2 (CK2) (Li *et al.*, 2009; Lolli *et al.*, 2012), a serine/threonine protein kinase that is involved in the up-regulation of Akt and exerts anti-apoptotic effects (Guerra, 2006). Furthermore, it was demonstrated that quercetin inhibits H-ras induced invasion and migration of MCF10A cells and inhibits the PI3K activity in an *ex vivo* binding assay (Song *et al.*, 2014). A X-ray crystallographic structure revealed that quercetin fits into the adenosine triphosphate (ATP) binding site of PI3Kγ with slightly higher affinity than myricetin (Walker *et al.*, 2000). Furthermore, cell-based pull-down assays demonstrated that quercetin binds to and inhibits PI3K, decreasing the downstream signalling events (Hwang *et al.*, 2009).

MAPK pathway. Quercetin inhibits MEK1 kinase activity and, to a less extend, Raf1 *in vitro* and *ex vivo*. It directly interacts with MEK1, as demonstrated by pull down assays. In addition, a modelling study with the crystal coordinates of MEK1 suggests that quercetin C ring interacts with residues in the activation loop of MEK1 near to the ATP-binding site, in a similar fashion as observed for the complex between MEK1 and the specific inhibitor PD318088 (Lee *et al.*, 2008). Quercetin decreases cellular viability and migration and promotes senescence and apoptosis in several glioma cell lines with concomitant suppression of Ras/MAPK/ERK signalling (Pan *et al.*, 2015).

GSK-3β. Glycogen synthase kinase-3β (GSK-3β) is a proline directed serine/threonine kinase that regulates numerous signalling pathways associated to cell cycle, inflammation and proliferation, including Wnt signalling pathway (Shao *et al.*, 2005). Its inhibition in pancreatic cancer cells decreases proliferation and survival (Ougolkov *et al.*, 2005). Quercetin inhibits the activity of GSK-3β and molecular docking studies predict that quercetin fits in the binding pocket of GSK-3β with low interaction energies (Johnson *et al.*, 2011).

Quercetin effects associated to regulation of transcription factors

Nrf2. In general, quercetin is known to induce the Nrf2 transcription factor. In non-tumoral cell lines, quercetin has been described to increase the expression of anti-oxidant defences through Nrf2 activation. In normal neuronal cultures, treatment with quercetin causes Nrf2 nuclear translocation and increases the levels of γ-glutamate-cysteine ligase, the rate limiting enzyme of glutathione synthesis that is regulated by Nrf2, and increases cellular GSH levels (Arredondo *et al.*, 2010). The induction of Nrf2 by quercetin is also involved in the upregulation of genes encoding for the anti-oxidant proteins Prx3 and Prx5 in trabecular meshwork cells (Miyamoto *et al.*, 2011). In human hepatoma HepG2 cells, quercetin enhances ARE-binding activity and Nrf2-mediated transcription activity through induction of Nrf2 mRNA and protein expression and repression of its negative regulator, Keap1, which increases Nrf2 protein stability (Tanigawa *et al.*, 2007). In tumor cells, Nrf2 induction antagonizes the apoptotic effect caused by quercetin. In fact, it was demonstrated that knockdown of Nrf2 in tumor cells increases their sensibility to the apoptotic effect of quercetin (Lee *et al.*, 2015). Therefore, although quercetin induces apoptosis in tumor cells, the activation of Nrf2 gives advantage to tumor cells by increasing their resistance to apoptosis. Regulation of Nrf2 by quercetin may also have dual distinct effects, depending on the concentration and treatment time. For instance, quercetin treatment of HepG2 cells transiently stimulates Nrf2 (by activating the p38-MAPK) to later (18 h) have the opposite effect (Granado-Serrano *et al.*, 2012). More recently, it was shown that low doses of quercetin (20-30 μM) induces Nrf2 activation but higher doses do not induce Nrf2, and have cytotoxic effects (Lee *et al.*, 2015).

NF-κB. Quercetin is also recognized to have anti-inflammatory properties frequently associated to the suppression of NF-κB, which plays a crucial role in the regulation of pro-inflammatory genes. Quercetin prevents 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced transformation of JB6 promotion-sensitive mouse skin epidermal (JB6 P+) cells by inhibiting the activation of activator protein 1 (AP-1) and NF-κB, associated with the downregulation of Raf/MEK1/ERK signalling (Lee *et al.*, 2008). In human cervical cancer HeLa cells, quercetin reduces cellular viability by mechanisms that involve NF-κB inhibition with simultaneous induction of p53 (Vidya Priyadarsini *et al.*, 2010). Quercetin also inhibits the growth of human salivary adenoid cystic carcinoma xenografts in nude mice through the suppression of NF-κB nuclear translocation and down-regulation of Akt and IKK-α (Sun *et al.*, 2010). Quercetin also inactivates the NF-κB pathway and activates the AP-1/JNK pathway to induce cell death in HepG2 cells (Granado-Serrano *et al.*, 2010). In i*n vitro* pulldown assay and *in vivo* chromatin immunoprecipitation assays

quercetin inhibits the binding of the transactivators CREB2, c-Jun, CCAAT/enhancer binding protein (C-EBP)-β and NF-κB to COX promoter in breast cancer MDA-MB-231 cells. Quercetin also inhibits the activity of p300 histone acetyltransferase, attenuating the p300-mediated acetylation of NF-κB p50 and binding to COX-2 gene promoter (Xiao *et al.*, 2011). Accordingly, quercetin induction of cytotoxicity in human oral cancer SAS cells is associated with blocking of MAPK and PI3K/Akt signalling pathways as well as NF-κB (Lai *et al.*, 2013). More recently, a microarray analysis showed that quercetin suppresses the expression of genes controlled by NF-κB and enhances the expression of genes associated with death receptor signalling and cell cycle inhibition in non-small cell lung cancer H460 cells (Youn *et al.*, 2013). Quercetin potently inhibits the IκB kinases IKK-α and –β (Peet and Li, 1999). Since these kinases phosphorylate the inhibitory subunit of NF-kB, IκB, to promote its degradation and activation of NF-κB (Hacker and Karin, 2006), their regulation by quercetin contribute to NF-κB inhibition.

FoxO. Quercetin biological effects are also associated to down-regulation of EGFR/Akt (Ganesan *et al.*, 2013; Huang *et al.*, 2013) and concomitant induction of FoxO transcription factors (Biggs *et al.*, 1999), which are involved in the regulation of cell growth, longevity and inflammation. For example, FOXO1 is crucial for the suppression of growth and induction of apoptosis by quercetin in two oral cancer cells, and this is mediated by the inhibition of EGFR/Akt signalling (Huang *et al.*, 2013). Consistently, the knockdown of FOXO1 attenuates its pro-apoptotic effects (Huang *et al.*, 2013). In a cell model of chronic obstructive pulmonary disease, quercetin increases nuclear FoxO3A leading to downregulation of NF-κB-mediated chemokine expression and reduction of inflammation (Ganesan *et al.*, 2013). Quercetin also induces the translocation of FOXO transcription factor DAF-16 to the nucleus in *C. elegans* (Kampkotter *et al.*, 2007a) inducing the expression of genes involved in lifespan and resistance to various stresses (Murphy, 2006).

p53. The tumor suppressor p53 is a transcription factor that has been regarded as the guardian of the genome due to its role in the prevention of DNA mutations (Baker *et al.*, 1990; Donehower *et al.*, 1992). p53 becomes activated by several stresses that compromise DNA stability (Vousden and Lu, 2002), including oxidative stress (Han *et al.*, 2008) and its activation leads to cell cycle arrest (Wang *et al.*, 2003) and activation of DNA repair proteins (Tanaka *et al.*, 2000). When DNA damage is severe and repair is impossible, activated p53 induces apoptotic cell death (Vousden and Lu, 2002). Quercetin up-regulates p53 expression in human cervical cancer (HeLa) cells related to its ability to intercalate in cellular DNA and to cause increases in intracellular ROS levels due to

depolarization of mitochondrial membrane potential, leading to cell cycle arrest and apoptosis (Bishayee *et al.*, 2013). The p53-inducible gene 3 (PIG3) is a NADPH:quinone oxidoreductase that participates in DNA damage response and whose activation is p53 dependent (Lee *et al.*, 2010a; Li *et al.*, 2013). More recently, it was shown that the cytotoxicity of quercetin in HepG2 is mediated by PIG3 together with increased intracellular ROS production. The activation of PIG3 is essential since the knockdown supresses the pro-apoptotic effect of quercetin (Zhang *et al.*, 2015a).

Direct Interaction of Myricetin with Components of Signalling Pathways

Myricetin [IUPAC name: 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone] has been reported to have anti-inflammatory (Fu *et al.*, 2013), anti-diabetic properties (Ong and Khoo, 2000) and cardioprotective effects (Angelone *et al.*, 2011). Myricetin also has anti-tumoral properties *in vitro* (Feng *et al.*, 2015) and *in vivo* (Nirmala and Ramanathan, 2011). For instance, myricetin supplementation in the diet decreases the incidence of tumors in rats exposed to 1,2-dimethylhydrazine (Nirmala and Ramanathan, 2011). It is reported to inhibit the activities of telomerase (Xue *et al.*, 2015), DNA topoisomerases (Lopez-Lazaro *et al.*, 2010) and multiple cellular receptors (Singh and Bast, 2015). Recently, myricetin has been shown to have anti-HIV activity *in vitro* (Pasetto *et al.*, 2014).

PI3K/Akt pathway. Myricetin was found to bind JAK1 from purified recombinant fusion GST-JAK1 (*in vitro*) or mouse JB6 cell lysates (*ex vivo*). Additional cell signalling data confirmed that the direct binding of JAK1 to myricetin inhibits downstream events including EGF-induced STAT3 (signal transducer and activator of transcription 3) phosphorylation and DNA binding and consequent cellular transformation (Kumamoto *et al.*, 2009b). The same authors demonstrated that myricetin also binds directly to the ATP-binding site of Akt, inhibiting the kinase activity and downstream associated events (Kumamoto *et al.*, 2009a). Myricetin also binds to PI3K and CK2 (Li *et al.*, 2009; Lolli *et al.*, 2012).

The ribosomal protein S6 kinase (RSK2) is a serine/threonine kinase and effector of mTOR and PDK1 (Pullen *et al.*, 1998; Saitoh *et al.*, 2002). Its mRNA and protein levels are highly elevated in primary breast cancers and correlate with poor patient survival (Segatto *et al.*, 2013). Its aberrant expression contributes to pathogenesis, metastasis, growth and invasion of gastric carcinomas (Xiao *et al.*, 2009). In a pull down assay it was demonstrated that myricetin can interact with the NH2-terminal kinase domain of RSK2, inhibiting its activity and the proliferation of gastric cancer cells (Feng *et al.*, 2015; Zang *et al.*, 2014). Through a molecular docking approach it was found that myricetin can easily

dock with and inhibit receptor tyrosine kinases, such as EGFR, insulin receptor (IS) and estrogen receptor (ER) (Singh and Bast, 2015), that are frequently up-regulated in cancers (Misawa and Inoue, 2015; Mujoo *et al.*, 2014; Salisbury and Tomblin, 2015).

MAPK pathway. Ex vivo binding data showed that myricetin binds to MEK1 without competing with ATP (Lee *et al.*, 2007b). Myricetin was also found to bind Fyn ATP-binding site (Jung *et al.*, 2008). Computer modelling studies revealed that myricetin fits easily onto the ATP-binding site of MKK4, and this was further supported by cell signalling data showing that this direct binding decreases TNF-α induced JNK and ERK phosphorylation, AP-1 activation and vascular endothelial growth factor expression in JB6P+ mouse epidermal cells (Kim *et al.*, 2009).

Myricetin effects associated to regulation of transcription factors

Nrf2. A DNA microarray analysis revealed that myricetin increases Nrf2-mediated ARE activation in HepG2 cells. It increases nuclear Nrf2 levels, enhances ARE-binding activity and reduces ubiquitination of Nrf2 and Keap1 levels (Qin *et al.*, 2013).

NF-κB. Like quercetin, myricetin decreases the promoter activity of COX-2 and protein expression by suppressing AP-1 and NF-κB activities in JB6 P+ cells exposed to UVB (Jung *et al.*, 2008). Myricetin also protects human umbilical vein endothelial EA.hy926 cells exposed to oxLDL through inhibition of NF-κB nuclear translocation and transcriptional activity and by decreasing oxLDL-induced ROS production (Yi *et al.*, 2012). Moreover, myricetin inhibits NF-κB related functions in mouse epidermal cells treated with acrylamide (Lim *et al.*, 2011), in mast RBL-2H3 cells treated with IgE or phorbol-12-myristate 13 acetate and calcium ionophore (Park *et al.*, 2008), in mouse macrophages stimulated with lipopolysaccharide (Kang *et al.*, 2005a), in mouse epidermal JB6 P+ cells treated with phorbol ester (Lee *et al.*, 2007a), in human bladder epithelial ECV304 cells treated with TNF-α (Tsai *et al.*, 1999) and in mouse bone marrow dendritic cells exposed to lipopolysaccharide (Fu *et al.*, 2013).

FoxO. The only study on the regulation of the FoxO transcription factor by myricetin was performed in *C. elegans*. Myricetin promotes nuclear translocation of the homologue of FoxO, DAF-16, leading to an increase in the lifespan of *C. elegans* (Buchter *et al.*, 2013).

P53. Myricetin triggers G2/M phase arrest in HepG2 cells associated with an increase in protein levels of the p53/p21 cascade (Zhang *et al.*, 2011b). The expression of p53 also increases in esophageal carcinoma EC9706 cells treated with myricetin (Wang *et al.*, 2014).

Many of the above mentioned proteins are also regulated by redox mechanisms. Thus, the contribution of the redox properties of phenolics for their bioactivity remains unclear. Also, several works showed that phenolics can alter intracellular ROS levels. However, there is a lot of controversy about the techniques more frequently used to analyse cellular ROS.

Effects of quercetin in intracellular ROS

Effects in normal cells. In excessive doses, several phenolic compounds can have cytotoxic effects in normal cells. In a study where the cytotoxicity of several flavonoids was tested, it was shown that quercetin decreases the survival of HUVEC cells (human umbilical vein endothelial cells) and human lung embryonic fibroblasts with an IC50 of 61 µM and 303 µM respectively. Furthermore, the cytotoxicity induced by the phenolics is mediated by increases in intracellular ROS (Matsuo *et al.*, 2005). High concentrations (between 100 and 200 µM) of quercetin triggers cellular necrosis in human retinal pigment epithelial cells *in vitro*. On the contrary, quercetin and other flavonoids exhibit no toxicity and even attenuate intracellular ROS and induce the expression of Nrf2-regulated genes in the HepG2-C8 cells (Saw *et al.*, 2014), and improve the endothelial function in human endothelial cells through the increase of NO production (Ugusman *et al.*, 2014).

Effects in normal cells subjected to oxidative stress conditions. The protective effect of phenolics in normal cells subjected to a stress condition (oxidative stress, hyperglycemia, hyperuricemia) has been widely studied. Quercetin inhibits the UV-B radiation mediated increase in intracellular ROS in corneal epithelial cell lines (Abengozar-Vela *et al.*, 2015), reduces intracellular ROS in human endothelial cells stimulated with lipopolysaccharide (Calabriso *et al.*, 2015), decreases intracellular ROS caused by hyperglycemia in endothelial progenitor cells (Zhao *et al.*, 2014), decreases ROS production in adipocytes stressed with tunicamycin (Nisha *et al.*, 2014), reduces apoptosis and ROS generation caused by doxorubicin in H9c2 cardiomyocytes (Dong *et al.*, 2014), decreases intracellular ROS in fructose exposed hepatocytes, abrogating the ROS-TXNIP induction (Zhang *et al.*, 2015b), reduces intracellular ROS in endothelial cells exposed to palmitate

(Wu *et al.*, 2014) and protects endothelial progenitor cells from glucose-induced impairment of cellular viability and from oxidative stress (Zhao *et al.*, 2014). A recent study showed that the treatment of human gastric epithelial cells (GES-1) with quercetin decreases the H_2O_2-induced loss of cell viability and intracellular oxidation. In the same work, a chemiluminescence imaging *in vivo* showed that quercetin attenuates ROS production and gastric damages upon gastric mucosal injury in mice (Hu *et al.*, 2015). This is the first report that provides data on the effect of phenolics in ROS levels *in vivo*.

Effects in cancer cells. The most frequent effect observed in tumor cells treated with quercetin is the induction of oxidative stress and apoptosis. Quercetin increases apoptotic cell death through generation of intracellular ROS and activation of the apoptotic pathway ASK1/p38 in a 5´AMP-activated protein kinase (AMPK)-α1 dependent manner in human breast cancer cells (MCF-7) (Lee *et al.*, 2010b). Quercetin also increases intracellular ROS and induces cell death in two human hepatoma cells (Chang *et al.*, 2006). In human colon carcinoma HCT116 cells, quercetin treatment causes a slow increase in MitoSOX Red fluorescence, a hydroethidine derivative targeted to the mitochondria, suggesting a mild pro-oxidant effect (De Marchi *et al.*, 2009).

Mounting evidence suggest that the disturbance of glutathione homeostasis in cancer cells seems to be a crucial event triggered by quercetin, since some of its effects are reverted by NAC. In MCF-7 breast cancer cells, quercetin increases intracellular ROS and activates AMPK which were reverted by the presence of NAC (Lee *et al.*, 2009). Treatment of colon cancer HCT116 cells with quercetin increases apoptosis through ROS-dependent activation of the AMPK/mTOR pathway and those events are reverted by co-incubation of quercetin with NAC (Kim *et al.*, 2013). In several human ovarian cancer cell lines, quercetin increases intracellular ROS and enhances TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through upregulation of death receptor 5 (DR5). Treatment of cells with NAC abolishes the potentiation effect of quercetin (Yi *et al.*, 2014). However, the protective effect of NAC may be associated with the increase in glutathione synthesis, rather than with its anti-oxidant properties, improving the capacity for cellular detoxification of quercetin.

In contrast, other reports indicate that quercetin leads to a reduction in intracellular ROS in Caco-2 cells (Yokomizo and Moriwaki, 2006) and ovarian cancer C13* cells (Li *et al.*, 2014). The upregulation of SOD1 and CAT gene expression probably contributes to the decrease in ROS levels induced by quercetin, which was associated with an increase in the resistance of tumor cells to cisplatin (Li *et al.*, 2014). Another study did not find the same association between intracellular ROS and the anti-tumoral effect of quercetin. In human cervical cancer cells (HeLa), pre-treatment with 80 μM of quercetin did not affect

intracellular ROS despite reducing cellular viability. Moreover, addition of GSH did not prevent quercetin induced loss of cell viability (Vidya Priyadarsini *et al.*, 2010).
Importantly, the effect of phenolics may depend on several factors, including treatment time. Intracellular ROS decreases after a short period of U937 cell line exposure to quercetin, but longer exposures lead to increased intracellular ROS, together with GSH depletion (Ferraresi *et al.*, 2005). A similar dual effect is observed in HepG2 cells (Kim and Jang, 2009).

Effects of myricetin in Intracellular ROS

Effects in normal cells subjected to oxidative stress conditions. Myricetin protects cells from H_2O_2 mediated increase in intracellular ROS and restores the expression and activity of cellular anti-oxidant defences such as SOD, catalase and GPx (Wang *et al.*, 2010). Myricetin also significantly attenuates 1-methyl-4-phenylpyridinium (MPP)(+)-induced cell death and suppresses the production of ROS in MES23.5 dopaminergic cells (Zhang *et al.*, 2011a). Myricetin cytotoxicity is mediated by ROS generation, since DTT was able to rescue the cells (Chen *et al.*, 2014).

Effects in intracellular ROS in cancer cells. Myricetin decreases intracellular ROS levels in Caco-2 cells (Yokomizo and Moriwaki, 2006). However, it induces apoptosis in human leukemia HL-60 cells without affecting intracellular ROS and the addition of SOD, catalase and NAC does not exert protective effects. Interestingly, the removal of the mitochondria by ethidium bromide treatment reduces the pro-apoptotic effects of myricetin, suggesting that mitochondria but not ROS are involved in myricetin-induced cell death (Ko *et al.*, 2005).

Modulation of intracellular ROS in cancer cells by other phenolics

Delphinidin and cyanidin, at concentrations between 25 and 100 µM, have cytotoxic effects in the highly metastatic colon cancer cell lines LoVo and LoVo/ADR but not in Caco-2 cells. Indeed, these compounds decrease intracellular oxidation induced by 2,2´-azobis(2-amidinopropane) dihydrochloride (ABAP) in Caco-2 cells, indicative of an anti-oxidant effect, but increase ROS levels in LoVo and LoVo/ADR cells. Furthermore, delphinidin and cyanidin treatment leads to glutathione depletion and decreases the activity of glutathione reductase in LoVo/ADR cells (Cvorovic *et al.*, 2010). In this study, the authors did not test if the apoptotic effect could be reversed by anti-oxidants. It should

be noted that the culture medium used for Caco-2 cells was different from that used for the other cells. Thus, the lack of cytotoxicity in Caco-2 cells may be due to the higher stability of the compounds on that medium.

Opposing effects have also been described in cancer vs normal cells (normal human PBMC) treated with cyanidin-3-rutinoside. Cyanidin-3-rutinoside causes ROS accumulation and induces apoptosis in leukemic HL-60 cells, however it decreases ROS levels in normal cells. Treatment of HL-60 cells with cyanidin-3-rutinoside results in the activation of p38/JNK and induction of apoptosis. These effects are reverted by NAC and catalase, implying that oxidative stress is involved in the mechanism of action. Interestingly, the authors used two different probes to analyse intracellular ROS, namely H_2DCF and dihydroethidium (DHE), and the increase in intracellular oxidation was only significant in cells labelled with H_2DCF. They further confirmed the increase in ROS accumulation with an enzyme based assay (HRPO-mediated oxidation of phenol red). Notably, pre-incubation with a low dose of H_2O_2 dramatically decreases the pro-apoptotic effect of cyanidin-3-rutinoside (Feng *et al.*, 2007). This result indicates that cyanidin-3-rutinoside induces a type of stress similar to H_2O_2, since a pre-incubation with low levels of H_2O_2 is known to induce cellular anti-oxidant defences (Schreck *et al.*, 1991).

In human glioblastoma A172 cells, treatment with capsaicin, a homovanillic acid derivative from hot chilli peppers (Cordell and Araujo, 1993), induces apoptosis. In addition, as early as after 1 h of treatment, capsaicin reduces intracellular ROS and the extend of lipid peroxidation in comparison to the control cells. Interestingly, previous addition of H_2O_2 (100 μM) for 1 h protects A172 tumor cells from capsaicin-induced apoptosis. Pre-treatment with NAC also reduces cellular ROS levels and apoptosis (Lee *et al.*, 2000). In HepG2 cells, capsaicin induces apoptosis and increases intracellular ROS levels and these events are suppressed by tocopherol, inhibitors of NADPH oxidase and in cells expressing Rac1N17, a dominant negative mutant of Rac1 involved in the activation of NAPDH oxidase. This suggests that generation of ROS by NADPH oxidase is essential for apoptosis induction by capsaicin. However, in this work authors did not analyse the auto-oxidation of capsaicin in the culture medium (Lee *et al.*, 2004).

β-phenylethyl isothiocyanate (PEITC) is a sulfur containing phenolic present in cruciferous vegetables, such as watercress (Chung *et al.*, 1992). Exposure of primary chronic lymphocytic leukemia cells to PEITC leads to ROS accumulation, oxidation of mitochondrial cardiolipin and glutathione depletion. Normal lymphocytes are less sensitive to PEITC, which causes a moderate increase in ROS levels in these cells. Supplementation of the culture medium with NAC prevents the PEITC-induced glutathione depletion, ROS increase and caspase 3 activation in lymphocytic leukemia cells (Trachootham *et al.*, 2008b).

Treatment of human lung adenocarcinoma H661 cells with EGCG and EGC also causes intracellular accumulation of ROS and induction of apoptosis, which is completely abolished by the addition of catalase but not of SOD (Yang *et al.*, 1998).

The variability of data is certainly associated to the use of different cell lines, culture mediums, type, treatment time and concentration of phenolics, and the methodology used to analyse intracellular ROS levels.

Scope of this thesis

Oxidative stress is associated to the aging process. Cells are programmed to undergo apoptosis as a consequence of the accumulation of oxidative damage. However, the occurrence of cellular DNA transformations can lead cells to circumvent apoptosis. Moreover, transformed cells can adapt to the new environment and proliferate, leading to cancer. Recently, the oxidative stress concept has evolved, in which ROS and RNS are tightly controlled and play crucial roles in cellular signalling, contributing for survival, proliferation, differentiation and apoptosis. Therefore, ROS may trigger a variety of biochemical reactions and biological responses, making it necessary to review the available data on intracellular redox status and cellular anti-oxidant defences in cells treated with phenolics. This work aimed to clarify the effect of phenolic compounds on anti-oxidant defences and intracellular ROS environment and their contribution for the anti-aging and anti-tumoral effects frequently associated to phenolics. To that end, two biological models were chosen: the yeast *Saccharomyces cerevisiae*, to study oxidative stress resistance and aging, and human cancer cells, to analyse the effect of phenolics in an already compromised redox environment.

CHAPTER 2.

Effect of myricetin, pyrogallol and phloroglucinol on yeast resistance to oxidative stress

Results included in:

Mendes V, Vilaça R, de Freitas V, Ferreira PM, Mateus N, Costa V (2015) Oxid. Med. Cell Longev. AID 782504

Summary

The health beneficial effects of dietary phenolic compounds have been attributed to their intrinsic anti-oxidant activity, which depends on the structure of the compound and number of hydroxyl groups. In this study, the protective effects of pyrogallol, phloroglucinol and myricetin on the yeast *Saccharomyces cerevisiae* was investigated. Pyrogallol and myricetin, which has a pyrogallolic structure in the B ring, increased H_2O_2 resistance associated with a reduction in intracellular oxidation and protein carbonylation, whereas phloroglucinol did not exert protective effects. The acquisition of oxidative stress resistance in cells pre-treated with pyrogallol and myricetin was not associated with an induction of endogenous anti-oxidant defences as assessed by the analysis of superoxide dismutase and catalase activities. However, myricetin, which conferred greater stress resistance, prevented H_2O_2-induced glutathione oxidation. Moreover, myricetin increased the chronological lifespan of yeast lacking the mitochondrial superoxide dismutase (Sod2p), which exhibit a premature aging phenotype and oxidative stress sensitivity. These findings show that the presence of hydroxyl groups in the ortho position of the B ring in pyrogallol and myricetin contributes to the anti-oxidant protection afforded by these compounds. In addition, myricetin may alleviate aging-induced oxidative stress, particularly when redox homeostasis is compromised due to downregulation of endogenous defences present in mitochondria.

Introduction

Oxidative stress is a hallmark of human disorders such as cancer and age-associated diseases (Halliwell and Gutteridge, 2007). It results from an unbalance between the levels of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and cellular anti-oxidant defences. The toxicity of high levels of ROS and RNS is associated with the accumulation of damaged molecules, including proteins, lipids and nucleic acids (Halliwell and Gutteridge, 2007). Under normal physiological conditions, ROS are kept at low levels by anti-oxidant defences such as superoxide dismutases (SOD) that catalyse the dismutation of superoxide radicals into hydrogen peroxide (H_2O_2), catalases or peroxidases that reduce H_2O_2 into water, as well as non-enzymatic defences, including glutathione that plays critical roles in redox homeostasis and cellular detoxification (Valko *et al.*, 2006). In addition, anti-oxidants obtained in the diet, such as vitamins C and E and phenolic compounds, play essential roles in cellular protection (Jacob and Burri, 1996).

Phenolic compounds are natural anti-oxidants present in the human diet through the consumption of fruits, vegetables and drinks such as juice, tea, coffee and wine (Del Rio *et al.*, 2013; Stevenson and Hurst, 2007). Structurally, these compounds are characterized by having one or more hydroxyl groups attached to at least one aromatic ring (Del Rio *et al.*, 2013). The number and position of hydroxyl groups are important features that affect the anti-oxidant activity of phenolic compounds (Crozier *et al.*, 2009). These compounds possess anti-proliferative, pro-apoptotic and anti-inflammatory properties and they have been associated with the prevention of cancer and cardiovascular, neurodegenerative and metabolic disorders (Seifried *et al.*, 2007; Sies, 2010). The protective effects of these compounds have been attributed not only to their intrinsic anti-oxidant activity but also to the modulation of cell signalling pathways, including mitogen-activated protein kinase cascades, that regulates oxidative stress responses (Kong *et al.*, 2000; Ramos, 2008; Son *et al.*, 2011).

The budding yeast *Saccharomyces cerevisiae* has been used as an eukaryotic model organism to characterize the molecular mechanisms underlying oxidative stress resistance and to evaluate the anti-oxidant potential of dietary extracts and phenolic compounds (Wu *et al.*, 2011a). We have previously reported that quercetin, the most common flavonol in the diet, increases yeast oxidative stress resistance (Belinha *et al.*, 2007) and exerts its protective effects against oxidative stress by inducing the biosynthesis of trehalose, a stress protectant disaccharide, and the activation of the cell wall integrity pathway (Vilaca *et al.*, 2012). Other studies have shown that resveratrol and catechin increase oxidative stress resistance in yeast by mechanisms associated with the

activation of catalase (Dani *et al.*, 2008), whereas delphinidin 3-glucoside and petunidin 3-glucoside protect yeast through activation of the stress response regulators Msn2p and Msn4p (Jimenez *et al.*, 2010). Moreover, the sirtuin Hst3p has been implicated in oxidative stress protection afforded by a polyphenol-enriched cocoa powder (Martorell *et al.*, 2011).

Pyrogallol and phloroglucinol are simple phenols that contain three hydroxyl groups in the ortho and meta position, respectively, of a benzene ring (**Figure 2.1 (a)**). Humans are exposed to pyrogallol through ingestion of tea and coffee (Muller *et al.*, 2006) but also from degradation of gallic acid in colon (Yasuda *et al.*, 2000). Phloroglucinol is found as a monomer of phlorotannins in brown algae, which is increasing in the human diet (Montero *et al.*, 2014). Myricetin is a naturally occurring flavonol characterized by having a pyrogallol structure in the B ring as well as a 4-oxo function with an unsaturated bond between the 2 and 3 carbons within the C ring and the presence of hydroxyl groups at C3 and C5 (Crozier *et al.*, 2009) (**Figure 2.1 (a)**). In the human diet, myricetin is commonly found in tea, berries and red wine (Hertog *et al.*, 1993). In this study, we investigated the effect of myricetin, pyrogallol and phloroglucinol on yeast resistance to oxidative stress.

Materials and Methods

Reagents.

All reagents and chemicals used were of analytical grade. Sodium or potassium phosphates, riboflavin and H_2O_2 were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), myricetin, pyrogallol, phloroglucinol and nitro blue tetrazolium were purchased from Sigma (Sintra, Portugal). Phenolic compounds were dissolved in DMSO at a 200 mM stock concentration and stored at -80ºC. Solutions were prepared in ultrapure water (Milli-Q).

Yeast strains and growth conditions.

Saccharomyces cerevisiae cells (Euroscarf, Germany) used in this study were BY4741 (Matα, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0; parental strain), *sod1*Δ (BY4741 *sod1*Δ::*KanMX4*) and *sod2*Δ (BY4741 *sod2*Δ::*KanMX4*). Yeast cells were grown in YPD medium [1% (w/v) yeast extract, 2% (w/v) bactopeptone and 2% (w/v) glucose] or in synthetic complete (SC) drop-out medium containing 2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids supplemented with the appropriate amino acids (80 mg His L-1, 400 mg Leu L-1, 80 mg trp L-1) and nucleotides (80 mg Ura L-1). Cultures were maintained in an orbital shaker, at 26ºC and 120 rpm, with a ratio of flask volume/medium volume of 5:1.

Oxidative stress resistance assays.

Yeast cells were grown to the exponential phase (OD600 = 0.5 - 0.6) in YPD medium, pre-treated with phenolic compounds (myricetin, pyrogallol or phloroglucinol at 300 µM) or equal volume of DMSO (vehicle) for 15 min and subsequently exposed to 1.5 mM H_2O_2 for 1 hour. Cell viability was determined by dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26ºC for 3 days. Viability was expressed as the percentage of colony-forming units (CFU).

Intracellular oxidation.

The oxidant-sensitive probe 2´,7´-dichlorodihydrofluorescein (H_2DCF-DA) (Molecular Probes) was used to measure intracellular oxidation. Yeast cells grown to the exponential

phase in YPD medium and pre-treated with phenolic compounds for 15 min were subsequently exposed to 1.5 mM H_2O_2 for 1 hour in the absence or presence of 25 μM H_2DCF-DA. Cells were spun down (4,000 rpm, 4 min), washed twice and suspended in filtered phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Fluorescence was measured in FL-1 channel (excitation and emission wavelength at 488 nm and 525 nm respectively) in a Becton-Dickinson FACSort flow cytometer. Auto-fluorescence was analyzed in cells untreated with H_2DCF-DA. Data was acquired from a total of 10,000 events/sample. BDCellQuest Pro Software was used for data acquisition and FlowJo Software for data analysis.

Protein carbonylation.

Protein extracts were prepared in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Roche Applied Science), by vigorous shaking of the cell suspension, in the presence of glass beads, for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Protein content was estimated by the Lowry method, with bovine serum albumin as a standard. Protein carbonylation assays were performed by slot-blot analysis, as previously described (Belinha *et al.*, 2007), using rabbit IgG anti-dinitrophenyl (DNP) (Sigma) at a 1:5,000 dilution as the primary antibody and goat anti-rabbit IgG-peroxidase (Sigma) at 1:5,000 as the secondary antibody. Immunodetection was performed by chemiluminescence, with a kit from GE Healthcare (RPN 2109). Quantification of bands was performed by densitometry.

Glutathione levels and enzymatic activities.

All the procedures were carried out at 4ºC. Yeast cells were harvested by centrifugation. Glutathione levels were measured by the method of Tietze (Tietze, 1969), as described in previous work (Belinha *et al.*, 2007). For enzyme activities, yeast extracts were prepared as described for the analysis of protein carbonylation. The activity of catalase and SOD was analysed *in situ*, after separation of proteins (50 μg) by native polyacrylamide gel electrophoresis (PAGE), as described previously (Conyers and Kidwell, 1991; Flohe and Otting, 1984). Quantification of bands was performed by densitometry.

Chronological lifespan.

Overnight cultures in SC medium were diluted to OD600 = 0.5 and grown to the stationary phase for 3 days (in the case of BY4741 and *sod1*Δ cells) or for 1 day (in the case of *sod2*Δ cells). Then, the compounds (300 μM myricetin or pyrogallol or phloroglucinol) or DMSO (vehicle; volume identical to compounds) were added to the cultures (day 0). These cells were kept in culture media at 26ºC and viability was analysed at indicated times by standard dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26ºC for 3 days and viability was expressed as the percentage in CFU relative to day 0.

Statistical analysis.

Analysis was performed in GraphPad Prism. Data are expressed as the mean values ± standard error of the mean (SEM) of at least three independent experiments. The 0.05 probability level was selected as the point of statistical significance. Values of oxidative stress resistance assays were analysed by one-way ANOVA and compared by Dunnett´s multiple comparisons test. Intracellular ROS and protein carbonyls were analysed by two-way ANOVA and compared by Sidak´s multiple comparisons test. Statistical analysis of total and oxidized glutathione levels and the ratio $GSSG/GSH_T$ was performed by two-way ANOVA, Sidak´s multiple comparisons test (*$p<0.05$) for comparison of values between treatments in each condition (control or with H_2O_2) and multiple *t*-tests, using the Holm-Sidak method for corrections (*$p<0.05$) for comparison of values between control and H_2O_2 for all treatments. Lifespans were compared by Student´s *t*-test.

Results

Myricetin and pyrogallol increase hydrogen peroxide resistance in Saccharomyces cerevisiae.

To assess the effect of myricetin, pyrogallol and phloroglucinol on oxidative stress resistance, exponential phase yeast cells were pre-treated with these compounds individually (300 μM) or DMSO (control) for 15 min and subsequently exposed to 1.5 mM H_2O_2 for 1 hour. The presence of phenolic compounds *per se* (in the absence of H_2O_2) did not affect cell viability, intracellular oxidation or protein oxidation. Myricetin and pyrogallol, in contrast with phloroglucinol, increased cell viability in cells exposed to H_2O_2 from 33% (in control cells) to 64% and 51%, respectively (**Figure 2.1 (b)**).

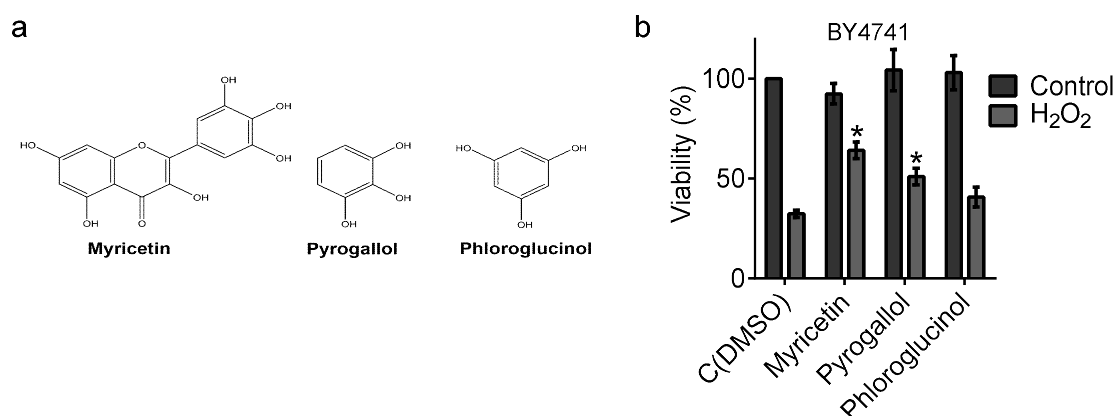


Figure 2.1 (**a**) Chemical structure of the phenolic compounds used in this work. (**b**) Effect of myricetin and simple phenols (pyrogallol and phloroglucinol) on oxidative stress resistance. Yeast cells were grown to the exponential phase in YPD medium, pre-treated with compounds (300 μM) or equal volume of DMSO (control) for 15 min and subsequently treated with 1.5 mM H_2O_2 for 1 h. Viability is expressed as the percentage of the CFU. Values are mean ± SEM of at least 3 independent assays. Values were compared by one-way ANOVA, Dunnett´s multiple comparisons test (*$p<0.05$).

To investigate if H_2O_2 resistance induced by these phenolic compounds was correlated with a decrease in oxidative stress markers, intracellular ROS levels were measured by flow cytometry using cells labelled with an oxidant sensitive probe, H_2DCF-DA (**Figure 2.2 (a-b)**), and protein oxidation was assessed through the analysis of protein carbonyl content (**Figure 2.2 (c)**). In control cells, exposure to H_2O_2 caused a 10-fold increase in intracellular ROS and a 3-fold increase in protein carbonylation. Myricetin and pyrogallol significantly decreased H_2O_2-induced intracellular oxidation and protein carbonylation.

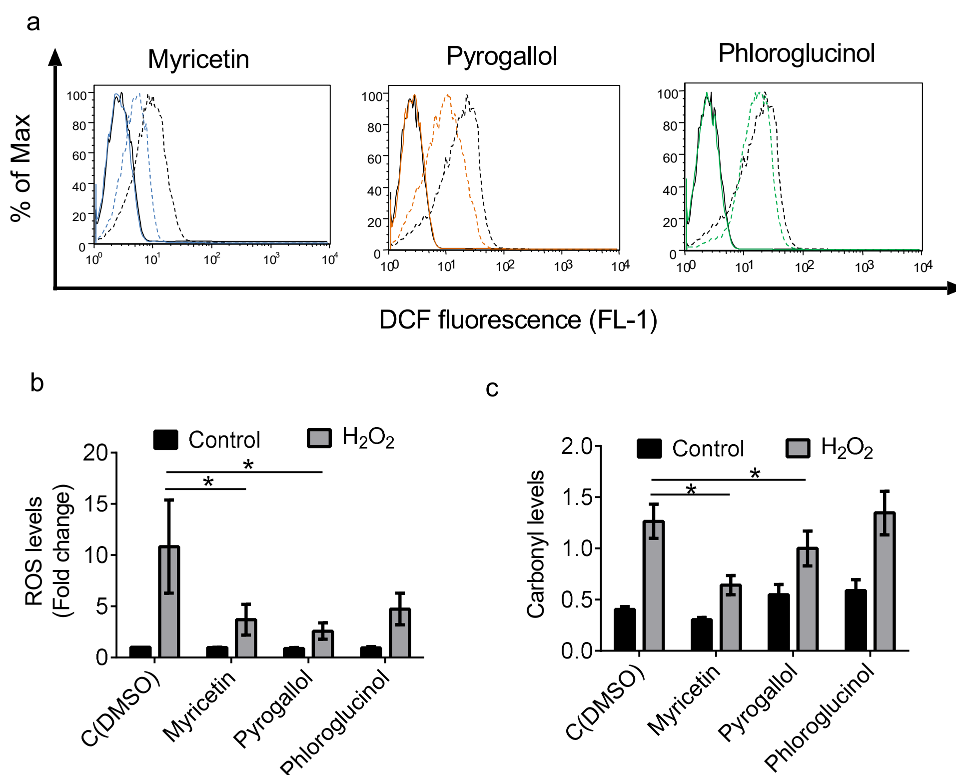


Figure 2.2 Effect of myricetin, pyrogallol and phloroglucinol on intracellular oxidation and oxidative damage. Yeast cells were grown in YPD medium to the exponential phase and pre-treated with compounds (300 µM) or equal volume of DMSO (control) for 15 min and subsequently treated with 1.5 mM H_2O_2 for 1 h. **(a)** Representative histograms of intracellular ROS analyzed by flow cytometry using H_2DCF-DA as a probe. Black: C(DMSO); blue: myricetin; orange: pyrogallol; green: phloroglucinol; undashed: unstressed; dashed: exposed to 1.5 mM H_2O_2 for 1 h. **(b)** Quantification of intracellular ROS expressed by mean fluorescence intensity values/10,000 cells/sample (arbitrary units) from at least 3 independent assays. **(c)** Quantitative analysis of protein carbonyl content performed by densitometry using data taken from the same membrane. Proteins were derivatized with DNPH and slot-blotted into a PVDF membrane. Immunodetection was performed using an anti-DNP antibody. Values are mean ± SEM of at least 3 independent assays. Values compared by two-way ANOVA, Sidak´s multiple comparisons test (*p<0.05).

Myricetin and pyrogallol do not affect the activity of superoxide dismutase or catalase.

To investigate if the protective effect of myricetin or pyrogallol was associated with an induction of anti-oxidant defences, the activity of SOD and catalase was determined. Consistent with published data (Salo *et al.*, 1990), SOD activity decreased 31% in control cells (DMSO-treated) exposed to H_2O_2 (**Figure 2.3 (a)**). Pre-treatment with the phenolic compounds did not affect basal SOD activity or prevent its decrease upon exposure to H_2O_2. Catalase is not expressed in exponential phase cells (Belazzi *et al.*, 1991) and, therefore, its activity was not detected in control cells. Moreover, catalase was not induced in cells treated with the tested compounds (data not shown). These results indicate that the increase of oxidative stress resistance in cells pre-treated with myricetin or pyrogallol do not result from the induction of SOD and catalase.

Myricetin suppresses H_2O_2-induced glutathione oxidation.

The tripeptide glutathione (GSH) is the most abundant low-molecular weight thiol that serves to maintain a reduced intracellular environment (Meister and Anderson, 1983). To assess the effect of myricetin, pyrogallol and phloroglucinol on redox homeostasis, glutathione levels were determined in cells exposed to H_2O_2 (**Figure 2.3 (b-d)**). In control cells, after exposure to H_2O_2, total glutathione levels (GSH_T) decreased 37% whereas GSSG levels increased 70%, increasing the ratio between GSSG and GSH_T. Similar results were observed in cells pre-treated with phloroglucinol, which is consistent with the fact that this compound did not affect oxidative stress resistance. Myricetin and pyrogallol *per se* (in the absence of H_2O_2) decreased GSH_T levels. However, H_2O_2-induced glutathione depletion was lower in cells pre-treated with these compounds, comparing with DMSO-treated cells. Moreover, the increase of GSSG levels and GSSG/GSH_T ratio

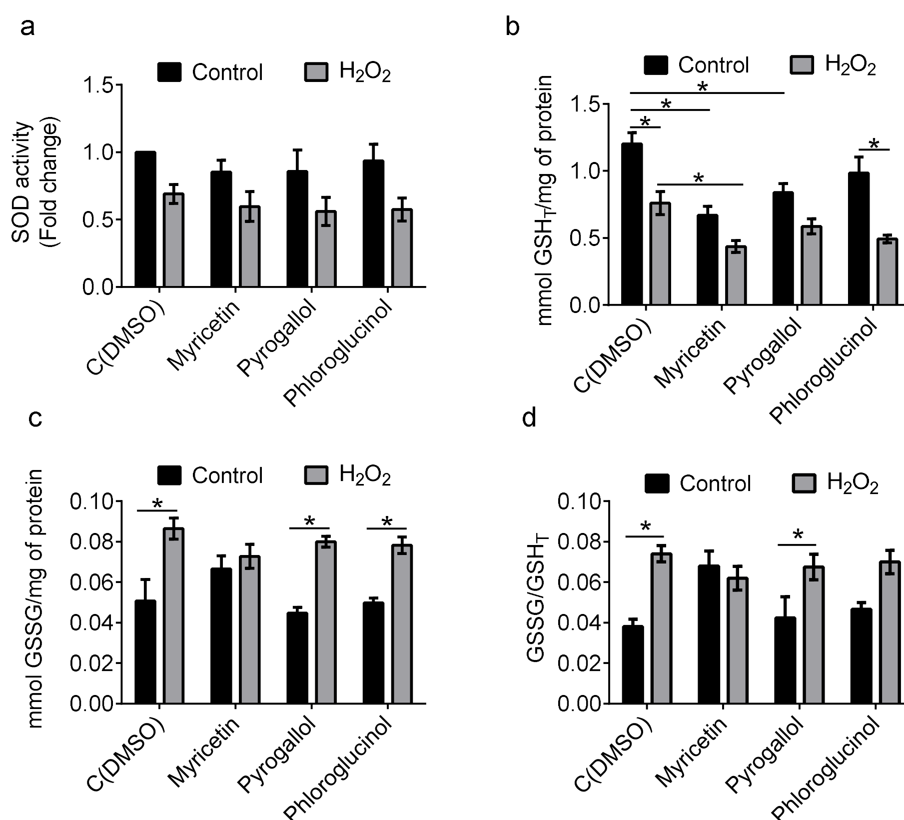


Figure 2.3 Effect of myricetin, pyrogallol and phloroglucinol on anti-oxidant defences. Yeast cells were grown in YPD medium to the exponential phase and pre-treated with compounds (300 μM) or equal volume of DMSO (control) for 15 min and subsequently treated with 1.5 mM H_2O_2 for 1 h. **(a)** SOD activity was assessed *in situ* after native PAGE. Band intensities were measured by densitometry using data taken from the same membrane; **(b)** GSH_T levels; **(c)** GSSG levels and **(d)** ratio between oxidized glutathione and total glutathione levels. Values are mean ± SEM of at least 3 independent assays. GSH_T and GSSG levels were compared by two-way ANOVA, Sidak´s multiple comparisons test (*p<0.05) and the ratio by Student´s *T* test (*p<0.05).

induced by H_2O_2 was suppressed by myricetin, but they were not attenuated by pyrogallol. This is consistent with our data showing that oxidative stress resistance in cells pre-treated with myricetin was higher than in pyrogallol pre-treated cells.

Myricetin increases the chronological lifespan of sod2Δ mutant cells.

Aging has been associated to an increase in intracellular oxidation and accumulation of oxidative damages (Dai *et al.*, 2014). Mitochondria are a major source of ROS and its dysfunction has been implicated in aging (Longo *et al.*, 1996; Ziegler *et al.*, 2015). Mitochondria contain several anti-oxidant enzymes, including the superoxide dismutases Sod1p (CuZnSOD) that is present in the mitochondrial intermembrane space (and cytosol), and Sod2p (MnSOD) located in the mitochondrial matrix. Cells lacking Sod1p or Sod2p exhibit a decreased CLS associated with the accumulation of oxidative damages (Demir and Koc, 2010).
The protective effect of myricetin and pyrogallol against oxidative stress caused by H_2O_2 led us to assess its effect on the chronological lifespan (CLS) of parental cells as well as of *sod1Δ* and *sod2Δ* mutant cells. Parental cells showed a time-dependent loss of cell viability, which was not affected by pre-treatment with myricetin, pyrogallol or phloroglucinol (**Figure 2.4 (a)**). These phenolic compounds also did not affect the lifespan of *sod1Δ* cells (data not shown). However, myricetin significantly increased the CLS of *sod2Δ* cells (**Figure 2.4 (b)**), suggesting that this compound exerts a protective effect that is particularly relevant in cells that have a decreased capacity to scavenge superoxide radicals within mitochondrial matrix. Consistently, myricetin decreased protein carbonylation in aged *sod2Δ* cells, although it had a modest effect in parental cells (**Figure 2.4 (c, d and e)**). In contrast, pyrogallol and phloroglucinol did not extend the CLS of *sod2Δ* cells (**Figure 2.4 (b)**).
Mitochondria play an important function during oxidative stress. Indeed, $\rho 0$ petite strains, which lack mitochondrial DNA and have deficiencies in electron transport chain function, are sensitive to H_2O_2 (Grant *et al.*, 1997; Thorpe *et al.*, 2004). A recent study showed that H_2O_2 increases the mitochondrial production of superoxide radicals, which have a protective effect at low concentrations (Thorpe *et al.*, 2013). However, high concentrations of superoxide radicals are detrimental. In agreement, *sod2Δ* cells were sensitive to H_2O_2 (**Figure 2.5**). We also assessed the effect of phenolic compounds in *sod2Δ* cells exposed to H_2O_2. The results show that pyrogallol pre-treatment slightly increased H_2O_2 resistance

of *sod2*Δ cells, although to levels lower to that observed in parental cells. In contrast, myricetin and phloroglucinol did not affect H_2O_2 resistance in these mutants (**Figure 2.5**).

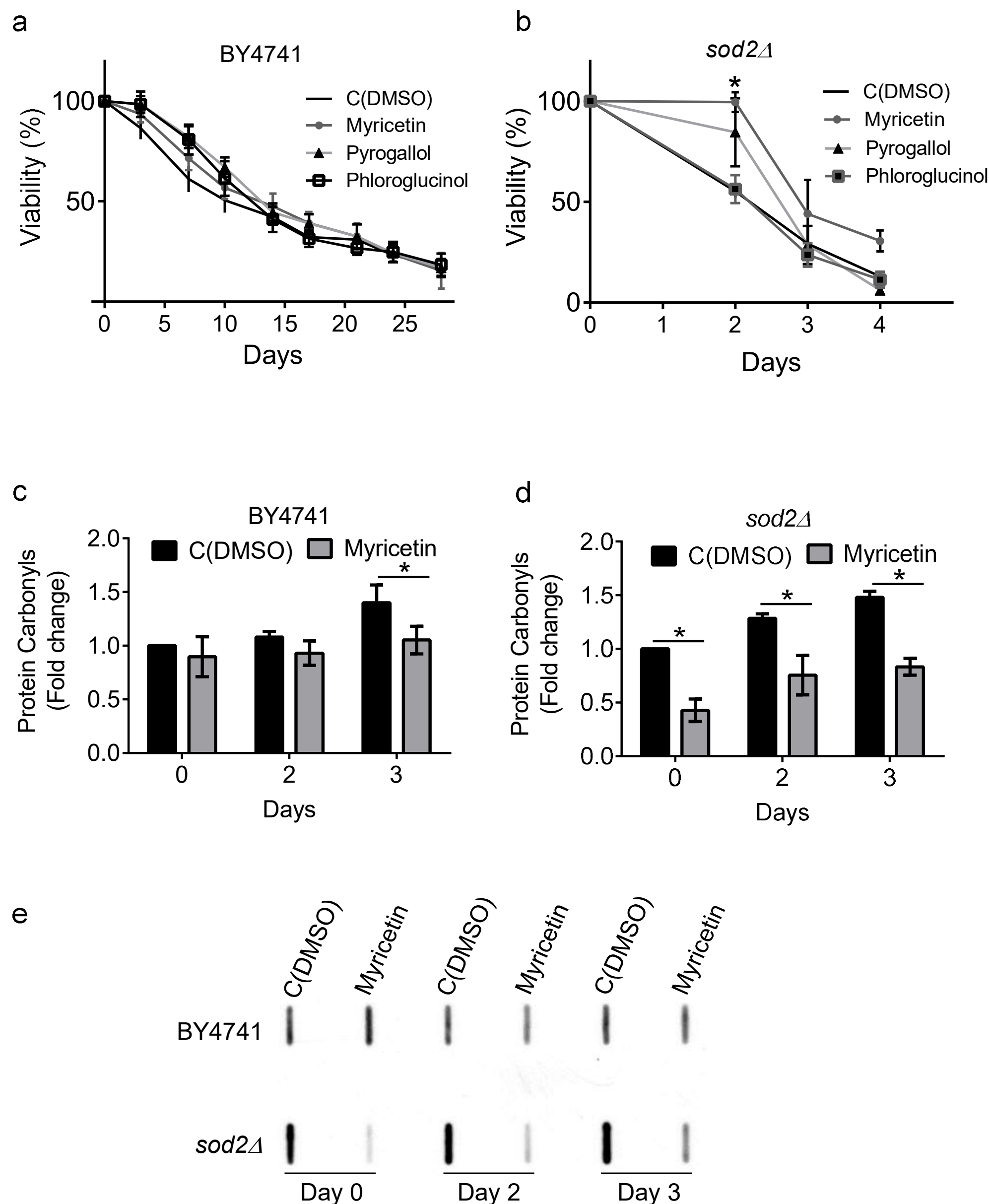


Figure 2.4 Effect of myricetin, pyrogallol and phloroglucinol on **(a)** BY4741 and **(b)** *sod2*Δ cells CLS. Cells were grown in SC-glucose medium to the stationary phase and treated with myricetin or pyrogallol or phloroglucinol (300 µM). Viability was measured by standard dilution plate counts which were considered 100% on day 0 (first treatment day). **(c-d)** On the indicated days, the levels of protein carbonyls were analysed during aging of cells pre-treated with myricetin. Values are mean ± SEM of at least 3 independent assays. **(e)** Representative blot of protein carbonyls during CLS. Viability values were compared by Student´s *T* test (*$p<0.05$) and protein carbonyl values were compared by two-way ANOVA, Sidak´s multiple comparisons test (*$p<0.05$).

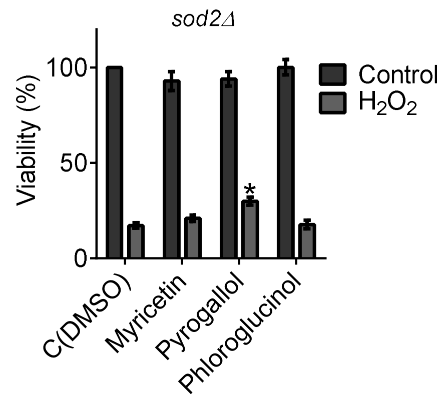


Figure 2.5 Effect of myricetin, pyrogallol and phloroglucinol on the oxidative stress resistance of *sod2*Δ cells. Yeast cells were grown to the exponential phase in YPD medium, pre-treated with compounds (300 μM) or equal volume of DMSO (control) for 15 min and subsequently treated with 1.5 mM H_2O_2 for 1 h. Viability is expressed as the percentage of the CFU. Values are mean ± SEM of at least 3 independent assays. Values were compared by one-way ANOVA, Dunnett´s multiple comparisons test (**p*<0.05).

Discussion

The increased production of ROS and RNS together with the decrease of anti-oxidant defences have been implicated in the pathogenesis of numerous diseases and aging (Dai *et al.*, 2014). Thus, a diet containing natural compounds with anti-oxidant properties, such as phenolic compounds, may be beneficial to human health. The anti-oxidant activity of these compounds is determined by structural features, including the number and position of hydroxyl groups, polarity, solubility and reducing potential (Bors *et al.*, 2001; Menezes *et al.*, 2011). In this study, we used the yeast *Saccharomyces cerevisiae* to assess *in vivo* the anti-oxidant capacity of the flavonol myricetin and two simple phenols, pyrogallol and phloroglucinol. Myricetin was the most effective in increasing H_2O_2 resistance in yeast, whereas phloroglucinol had no protective effect. Consistently, H_2O_2-induced intracellular oxidation and protein carbonylation decreased in cells pre-treated with myricetin and pyrogallol but not with phloroglucinol. Pyrogallol and phloroglucinol contain three hydroxyl groups in the ortho and meta position, respectively, of a benzene ring. The vicinal positions of hydroxyl groups in pyrogallol results in a lower bond dissociation energy of O-H, facilitating the donation of hydrogen to free radicals (Thavasi *et al.*, 2006). In accordance, our results show that pyrogallol, in contrast with phloroglucinol, increased the viability of yeast cells exposed to H_2O_2. Myricetin, which contains a pyrogallol structure in the B ring, conferred an even higher resistance. Our results are in accordance with data demonstrating the importance of the pyrogallol structure for the bioactivity of phenolic compounds (Mitsuhashi *et al.*, 2008). Our data is also consistent with several reports showing a protective effect of myricetin against oxidative stress in mammalian cells. For instance, myricetin decreases H_2O_2-induced DNA damage in Caco-2 and HepG2 cells (Aherne and O'Brien, 1999) and decreases *t*-BOOH-induced protein oxidation and lipid peroxidation in erythrocytes from type 2 diabetes mellitus patients (Pandey *et al.*, 2009).

Being redox-active compounds, phenolic compounds can also act as pro-oxidants and, therefore, induce stress responses leading to an increase in the levels of cellular anti-oxidant defences (Calabrese *et al.*, 2012; Kessler *et al.*, 2003). Our results indicate that this mechanism does not contribute to the protective effects of myricetin and pyrogallol in yeast, since these compounds did not increase intracellular oxidation or affect catalase and SOD activities under the conditions used in this study. We have previously observed that H_2O_2 resistance in yeast incubated with quercetin is also not associated with pro-oxidant effects or modulation of anti-oxidant defences (Belinha *et al.*, 2007). In contrast, other reports showed that catalase activity increases in yeast treated with resveratrol and catechin, enhancing cellular resistance to oxidative stress (Dani *et al.*, 2008).

Glutathione is an important cellular small molecule responsible for the maintenance of redox homeostasis (Meister and Anderson, 1983). The reduced form (GSH) mediates H_2O_2 decomposition catalyzed by GPx (Meister and Anderson, 1983) giving rise to oxidized glutathione (GSSG) which is then reduced to GSH by glutathione reductase (Mullineaux *et al.*, 1994). Glutathione has also important functions in detoxification of toxic compounds (St-Pierre *et al.*, 1994) and in the protection of proteins from oxidation through glutathionylation (Gallogly and Mieyal, 2007). Thus, glutathione oxidation is a biomarker of oxidative stress. In control (DMSO-treated) cells, exposure to H_2O_2 led to an increase in GSSG levels that, concomitantly with glutathione depletion, resulted in a higher GSSG/GSH_T ratio. In cells pre-treated with myricetin, H_2O_2-induced glutathione oxidation and the increase in the ratio GSSG/GSH_T were suppressed, which is consistent with the reduction of intracellular oxidation. Pre-treatment with pyrogallol, which had a lower protective effect comparing with myricetin, did not prevent glutathione oxidation. These results suggest a correlation between the protective effect of myricetin and maintenance of glutathione redox status. Treatment with both myricetin and pyrogallol *per se* led to a decrease in total GSH levels, which may result from the formation of GS-compound adducts mediated by glutathione *S*-transferases (GST). Indeed, these adducts have been reported for quercetin (Kessler *et al.*, 2003; Spencer *et al.*, 2003) and GST activity may be induced by these phenolic compounds, similarly to the effects of coumarin (Higgins and Hayes, 2011).

High levels of ROS have been implicated in aging in yeast and higher eukaryotes (Bitterman *et al.*, 2003; Fabrizio *et al.*, 2004). The accumulation of oxidative damages leading to neuronal death is associated with age-related diseases such as Alzheimer and Parkinson diseases (Ho *et al.*, 2012). Therefore, a diet replete in phenolics with anti-oxidant activity, reduces the functional decline associated with aging and age-related disorders, increasing healthspan (Alcalay *et al.*, 2012; Vassallo and Scerri, 2013). Several studies showed an increase of yeast lifespan incubated with phenolic compounds. Resveratrol and phloridzin, a major apple compound, increase yeast replicative lifespan by mechanisms associated with the activation of the sirtuin Sir2p (Howitz *et al.*, 2003; Xiang *et al.*, 2011). Moreover, quercetin and apple polyphenolic fractions increase yeast CLS (Belinha *et al.*, 2007; Palermo *et al.*, 2012). Here, we report that, although myricetin does not affect the CLS of parental and *sod1*Δ cells, it extends the lifespan of yeast cells lacking the mitochondrial superoxide dismutase, which are known to exhibit a very short lifespan (Longo *et al.*, 1996). In contrast, pyrogallol did not extend the CLS of *sod2*Δ cells. These results suggest that myricetin may be more effective in protecting aged cells that have high intracellular ROS levels and oxidative damage, especially in the mitochondria. In agreement, several reports show protective effects of myricetin in mitochondria. For

instance, myricetin decreased the generation of H_2O_2 in isolated mouse skeletal muscle mitochondria (Grunz *et al.*, 2012), decreased the depolarization of the inner mitochondrial membrane potential in C6 glial cells exposed to oxygen-glucose deprivation (Panickar and Anderson, 2011) and it was the most efficient among other phenolic compounds in the protection of mouse brain mitochondria against toxicity induced by methyl mercury (Franco *et al.*, 2010). Notably, myricetin was unable to protect *sod2*Δ cells against high doses of H_2O_2 whereas pyrogallol slightly increased the oxidative stress resistance of these mutants. It is likely that the excessive oxidative stress in *sod2*Δ cells treated H_2O_2 overwhelms the protective effects of these compounds.

In summary, our data show that myricetin and, to a lesser extent, pyrogallol, increased yeast resistance to H_2O_2. This protective effect was correlated to a reduction in intracellular oxidation and protein carbonylation and a maintenance of GSSG/GSH_T ratio. However, changes in catalase or SOD activities were not associated to the protective effects. Furthermore, myricetin attenuated the shortened CLS of yeast cells lacking the mitochondrial superoxide dismutase (*sod2*Δ mutants).

CHAPTER 3.

Effects of phenolics in gastrointestinal tumor cell lines

Results included in:

Mendes V, Costa V, Mateus N (2015) RSC Advances 5, 1-9.

Summary

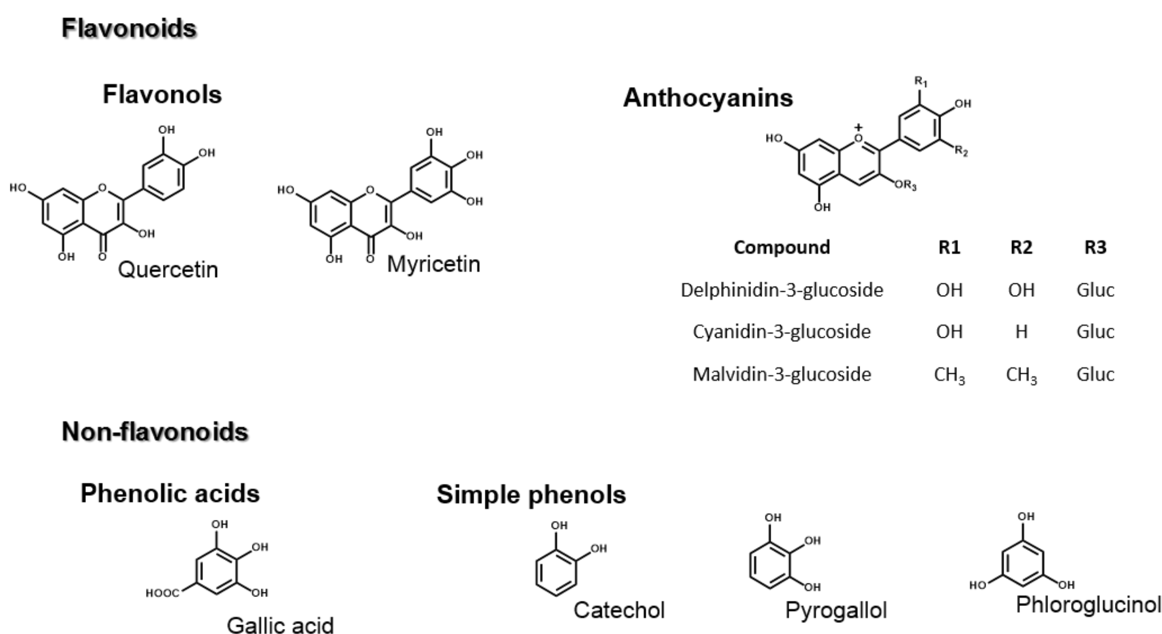
Several studies demonstrate the anti-tumoral potential of food phenolics. However, this effect has been attributed to an artefact resulting from the H_2O_2 generated by phenolic compounds auto-oxidation in culture medium in *in vitro* cell cultures. In this work, the contribution of H_2O_2 for the anti-proliferative effect of phenolic compounds in gastric and colon cancer cell lines was analysed. Tri-hydroxylation on the B-ring was correlated with higher levels of H_2O_2 in culture medium and higher anti-proliferative potential. The inhibitory effect of the phenolics, except quercetin, was partially dependent on H_2O_2 generation. Quercetin effect was also not mediated by $O_2^{\cdot -}$. Quercetin, in contrast with other phenolics, affected intracellular oxidation, decreasing ROS levels in AGS cells but transiently increasing them in Caco-2 cells. Myricetin effect was only associated to a reduction in proliferation and increase in apoptosis in AGS cells. Quercetin induced cell cycle arrest, increased apoptosis and decreased glutathione levels in AGS cells. Further analyses revealed that neither quercetin nor myricetin affected the thioredoxin system in AGS cells. These results suggest that glutathione depletion contribute to the anti-tumoral effect of quercetin.

Introduction

Multiple lines of evidence suggest that oxidative stress induced by reactive oxygen species (ROS) is involved in the multistage carcinogenesis process. Oxidative stress has been defined as an imbalance in the pro-oxidant/anti-oxidant equilibrium in favor of the pro-oxidants. However, recent evidence showing signalling properties of ROS suggests that oxidative stress is better defined as a disruption of redox signalling and control (Jones, 2006). Indeed ROS have been implicated in signalling through modulation of the redox states of protein and transcription factors (Liu *et al.*, 2000; Wu *et al.*, 2000). As so, the maintenance of an appropriate level of intracellular ROS is crucial for proper redox balance and signalling in the control of cellular proliferation (Murrell *et al.*, 1990). Cells have different responses to ROS, depending on the species type, concentration, and stimulus time. Under mild ROS levels, cells activate a variety of adaptation mechanisms, including redox buffering systems, such as the glutathione and thioredoxin systems, and several anti-oxidant enzymes, such as catalase and superoxide dismutase (SOD) (Pelicano *et al.*, 2004). Growing evidence suggests that cancer cells exhibit high constitutive intrinsic ROS levels in comparison to normal cells, due in part to oncogenic transformation (Vafa *et al.*, 2002), increased metabolic activity, and mitochondrial malfunction (Warburg, 1956). These high constitutive levels of ROS in cancer cells seem to sustain proliferative signalling (Policastro *et al.*, 2004).

Natural phenolic compounds are found in plants resulting from their secondary metabolism. Phenolics can be divided into several classes, such as simple phenols, phenolic acids, flavonols and anthocyanins, among others (**Figure 3.1**). They differ on the number and arrangement of carbon atoms, hydroxylation and methylation pattern and presence of attached sugars. They are part of the human diet through the consumption of plant derived foods and it is believed that they contribute to the health benefits associated to a diet rich in vegetables, fruits and grains (Stevenson and Hurst, 2007). Population based studies have shown that high dietary intake of fruits and vegetables is associated to a reduced risk of several types of cancer (Riboli and Norat, 2003), including those located in the GI tract, where phenolics can be found in high concentrations and in direct contact with epithelium cells (Stalmach *et al.*, 2012). Being redox active compounds, phenolics can act not only as anti-oxidants but, depending on several factors, also as pro-oxidants. Their redox behavior is determined by several factors such as pH, presence of oxygen and transition metals, temperature, concentration and also the number of hydroxyl groups in the structure (Cao *et al.*, 1997) and their redox potential (Bors *et al.*, 1995). Most of the evidence for the molecular mechanisms mediating the effects of phenolics is based in *in*

vitro studies using cell lines. These mechanisms have been associated with their intrinsic anti-oxidant characteristics and to indirect anti-oxidant effects through modulation of pro-oxidant enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase inhibition by resveratrol in endothelial cells (Chow *et al.*, 2007), and interaction with signalling pathways (Kumamoto *et al.*, 2009a), which are also regulated by redox mechanisms. However, many of the previously reported data was recently attributed to an artefact derived from *in vitro* cell cultures. The addition of phenolics to commonly used cell culture mediums results in their auto-oxidation and consequent formation of ROS, such as $O_2^{\cdot -}$ and H_2O_2, and of *o*-semiquinones and *o*-quinones that are usually cytotoxic (Long *et al.*, 2010) and mediate the changes on cell signalling pathways, proliferation and apoptosis erroneously attributed to phenolics. This work aimed to analyse the dependency on phenolics auto-oxidation in culture medium for their anti-proliferative effect in cancer cells. Several structurally related phenolic compounds, including simple phenols, phenolic acids, flavonols and anthocyanins, were tested in four human cancer cell lines derived from the GI tract: two human adenocarcinoma gastric (AGS and MKN-28) and two colon carcinoma (Caco-2 and HT-29) cell lines. Also, cellular adaptation to alterations in redox homeostasis was studied through analysis of cellular anti-oxidant defences in treated cells, including glutathione and the thioredoxin system.



Compound	R1	R2	R3
Delphinidin-3-glucoside	OH	OH	Gluc
Cyanidin-3-glucoside	OH	H	Gluc
Malvidin-3-glucoside	CH_3	CH_3	Gluc

Figure 3.1 Classes and sub-classes of phenolics and respective structures used in this work.

Materials and methods

Phenolic compounds.

Quercetin, myricetin, pyrogallol, gallic acid, catechol and phloroglucinol were obtained from Sigma-Aldrich (Madrid, Spain). Delphinidin-3-glucoside (Dp-3-gluc), malvidin-3-glucoside (Mv-3-gluc) and cyanidin-3-glucoside (Cy-3-gluc) were obtained from Extrasynthese (France). All phenolics were dissolved in DMSO (Fluka, Madrid, Spain) at a stock concentration of 200 mM and aliquots were stored at -20ºC.

Cell lines and growth conditions.

Caco-2 cells [HTB-37 from American Type Culture Collection (ATCC)], were grown in Minimum Essential Medium Eagle (MEME) supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) antibiotic/antimycotic solution (100 units/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B) and 4 mM of L-alanyl-L-glutamine. AGS (CRL-1739 from ATCC), MKN-28 (JCRB0253 from JCRB Cell Bank, Nibio) and HT-29 (HTB38 from ATCC) cells were grown in Roswell Park Memorial Institute Medium (RPMI)-1640 AQmedia supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic/antimycotic solution. All cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2. For all experiments, 24 h before treatment, all cell lines, except AGS, were seeded at a cell density of 4.84 x 10^4 cells/cm^2. AGS cell line was seeded at 2.42 x 10^4 cells/cm^2. All cell culture medium components were obtained from Sigma-Aldrich (Madrid, Spain).

SRB assay.

To determine the anti-proliferative potential of phenolics, cell growth was assessed through quantification of whole protein in culture using the protein binding sulforhodamine B (SRB; Sigma-Aldrich) (Skehan *et al.*, 1990). The treatment conditions were as follows: cells were seeded in 96 well plates (100 µl/well) and after 24 h they were treated with increasing concentrations of phenolics (or DMSO; control). In the case of co-incubations with enzymatic anti-oxidants, culture medium was supplemented with 10 U/ml of catalase (from bovine liver, Sigma-Aldrich) and/or 15 U/ml of SOD (from bovine erythrocytes, Sigma-Aldrich) before incubation with phenolics. After treatment, cells were fixed with 25% (w/v) trichloroacetic acid and incubated for 1 h at 4ºC. Cells were then washed 5

times with deionized water and plates were air-dried before staining for 30 min with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid. SRB was removed and cells were washed 5 times with 1% acetic acid to remove the unbound dye. After drying, the dye in culture plates was solubilized with 100 μl of Tris-HCl (10 mM, pH 10.5) and the absorbance was determined at 492 nm on a plate reader. Growth inhibition was determined as percent of cell density in treated over control cells corrected for the optical density at time zero. IC50 (growth inhibition by 50%) was obtained from the interpolation of dose-response curves.

BrdU assay.

To analyse DNA synthesis, the cell proliferation 5-bromo-2'-deoxyuridine (BrdU) colorimetric kit from Roche was used. Cells were treated as for SRB assay. The BrdU labelling solution was added at a final concentration of 10 μM to each well and left at 37°C for 2 h. After removing the cell culture medium, cells were fixed for 30 min and the anti-BrdU-antibody conjugated to horseradish peroxidase was added for 90 min at room temperature. After washing 3 times each well, tetramethylbenzidine (TMB) was added and the reaction was stopped with 1M sulphuric acid after 10 min. Absorbance was measured at 450 nm.

Quantification of H_2O_2 in the culture medium.

The ferrous oxidation in xylenol orange (FOX) assay was used to determine the H_2O_2 generated in culture medium by phenolics (Elbling *et al.*, 2010). The compounds (or DMSO; control) were added to culture medium (MEME and RPMI medium) and incubated under cell culture conditions (96 well plates, without cells). After 1 h, 6 h and 24 h, aliquots of the sample medium (20 μL) were added to 180 μL solution of xylenol orange, sorbitol and Fe (II) in sulfuric acid prepared freshly before the assay [1 volume of 25 mM ammonium ferrous (II) sulfate, 2.5 M H_2SO_4 for 100 volumes of 100 mM sorbitol, 125 μM xylenol orange]. After 15 min at room temperature, absorbance was measured in a microplate reader at 595 nm. The concentration of H_2O_2 was quantified using a H_2O_2 (Merck, Darmstadt, Germany) standard curve (0 - 200 μM). In parallel, blank controls (only solution reagent) and reagent controls (solution without ammonium ferrous (II) sulphate) were used, the last to exclude the interference of transition metals present in the samples. In addition, samples with 40 U/well (200 μl of final volume) of catalase were prepared to analyse the specificity of the reaction.

Intracellular ROS levels.

The oxidant-sensitive probe H_2DCF-DA (Molecular Probes) was used to measure intracellular ROS levels. For that, 24 h after seeding in 6 well plates, cells were treated with phenolics (or DMSO; control). After 1 h and 24 h, the medium was removed and cells were washed twice with sterile phosphate buffered saline (PBS; Sigma-Aldrich) and incubated with 1 µM of H_2DCF-DA in culture medium without FBS for 30 min at 37ºC. Then, cells were washed with PBS and detached with trypsin/EDTA (Sigma-Aldrich), ressuspended in 500 µl of culture medium, centrifuged at 1700 rpm for 5 min, ressuspended in PBS and filtered. Propidium iodide (PI; Molecular Probes) was added at a final concentration of 1 µg/ml to exclude death cells. Cells auto-fluorescence was analysed using cells untreated with H_2DCF-DA and PI. Fluorescence for DCF signal was analysed in FL-1 channel (excitation and emission wavelength at 488 nm and 525 nm respectively) and for PI signal in FL-3 channel (excitation and emission wavelength at 536 nm and 617 nm respectively) of a Becton-Dickinson FACSort flow cytometer. Data was acquired from a total of 10 000 events/sample. BDCellQuest Pro Software was used for data acquisition and FlowJo Software for data analysis.

Protein carbonyls, SOD and catalase activities.

Cells were seeded in 60 cm^2 plates and after 24 h were treated with phenolics (or DMSO; control). After 1 h and 24 h, the medium was removed, cells were washed twice with ice cold PBS, scrapped into PBS, spun down at 1200 rpm for 10 min, ressuspended in 1 ml of PBS and centrifuged at 13000 rpm for 1 min. The supernatant was rejected and cell pellets were stored at - 80ºC. At the day of the enzymatic assays, cell pellets were ressuspended in 50 mM potassium phosphate buffer pH 6.7 containing 0.1% Triton-X100 and protease inhibitors (Complete, EDTA-free, Roche, Germany) and cells were lysed through sonication with intermittent resting of cells on ice. After that, samples were centrifuged at 13000 rpm for 15 min at 4ºC. Cellular extracts were recovered to measure protein content, catalase and SOD activities. Protein content was measured by the Lowry method (Lowry *et al.*, 1951). SOD activity was determined *in situ* after native gel (10% acrylamide) electrophoresis using 20-30 µg of protein, as previously described (Flohe and Otting, 1984). Bands intensities were measured using QuantityOne software. Catalase activity was measured spectrophotometrically by following the decomposition of H_2O_2, as previously described (Aebi, 1984). Protein carbonyls were analysed after derivatization with 2,4-dinitrophenylhydrazine (DNPH), as described (Costa *et al.*, 2002). The protein sample was transferred to a nitrocellulose membrane by slot-blot and protein

carbonylations was measured using rabbit IgG anti-dinitrophenyl (DNP) (Sigma) at a 1:5000 dilution as the primary antibody and goat anti-rabbit IgG-peroxidase (Sigma) at 1:5000 as the secondary antibody. Immunodetection was performed by chemiluminescence, with a kit from GE Healthcare (RPN 2109). Quantification of bands was performed by densitometry.

Glutathione levels.

After treatments, cells were scrapped from the cell plate over ice and immediately centrifuged (1200 rpm, 4ºC). The cell pellet was lysed by sonication in 100 µl of 100 mM potassium phosphate pH 7.4 containing 2 mM EDTA and protease inhibitors and in 100 µl of 2 M $HClO_4$. The supernatant was collected and the pH was neutralized by adding 2M KOH 0.3M MOPS. The precipitate was removed and one part of the supernatant was incubated with 2-vinylpyridine to analyze reduced glutathione. The rest of the sample was used to analyze total levels of glutathione. Glutathione levels were analyzed spectrophotometrically through the enzymatic recycling assay described by Tietze (Tietze, 1969).

Thioredoxin and thioredoxin reductase activities.

After treatment, cells were washed with PBS, harvested and lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 0.5% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.2% (w/v) SDS, 150 mM NaCl and 1 mM EDTA] containing protease inhibitors. The end-point insulin assay was used for measurement of thioredoxin and the activity of thioredoxin reductase was analysed according to Arnér *et al*. (Arnér ES, 2005).

Redox state of Trx1/2.

Treated cells were washed with PBS and lysed in TEU buffer [50 mM Tris-HCl (pH 8.2), 1 mM EDTA, 8 M Urea] containing 30 mM of iodoacetic acid (IAA) for the first alkylation step of free thiols and incubated at 37ºC for 30 min. IAA introduces a negative charge for each thiol. Samples were precipitated and washed with ice cold acetone/HCl (98:2, v/v), reduced with 3.5 mM DTT for 30 min and subjected to a second alkylation step with 30 mM iodoacetamide (IAM) for another 30 min. The mobility standards were prepared as described in Zhang *et al.* (Zhang *et al.*, 2014). To prepare mobility standards, cellular extracts were dissolved in TEU buffer and incubated with 3.5 mM DTT for 30 min to be

fully reduced. After, the sample was divided into four aliquots and then treated with reagents containing varying molar ratios of IAA/IAM (concentrations in mM: 30/0, 30/10, 10/30, 0/10) yielding the mobility standards containing Trxs with different patterns of IAA/IAM labelling on their thiols. Protein was quantified by the Lowry method. Proteins were separated on urea-gel (12% acrylamide (stacking gel 2.5%) with 8 M Urea) and transferred to a nitrocellulose membrane. Thioredoxins were detected with rabbit anti-Trx1 and anti-Trx2 (Sigma Aldrich, 1:1000) and the secondary antibody anti-rabbit IgG-peroxidase (Sigma Aldrich, 1:5000). Immunodetection was performed by chemiluminescence, with a kit from GE Healthcare (RPN 2109). Quantification of bands was performed by densitometry.

Cell cycle.

After treatment for 24 h, cells were washed with PBS, trypsinized and ressuspended in culture medium. After centrifugation and rejection of the supernatant, cells were fixed in 70% (v/v) ethanol (in PBS) overnight at 4ºC. After fixation, cells were centrifuged and incubated for 2 h - 3 h with 50 μg/ml PI and 20 μg/ml RNase in the dark. Cell cycle was analysed in a Becton-Dickinson FACSort flow cytometer using the data from FL-3 channel (excitation and emission wavelength at 536 nm and 617 nm respectively). BDCellQuest Pro Software was used for data acquisition and Mod Fit LT Software for data analysis.

Apoptosis.

Cells were treated for 24 h and harvested. For flow cytometry analysis, cells were incubated for 15 min with annexin V-FITC (Immuno Tools) in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4). Before performing the flow cytometry analyses, 1 μg/ml PI was added to each sample. Fluorescence for annexin V-FITC was analysed in FL-1 channel (excitation and emission wavelength at 488 nm and 525 nm respectively) and for PI signal in FL-3 channel (excitation and emission wavelength at 536 nm and 617 nm respectively) of a Becton-Dickinson FACSort flow cytometer. Data was acquired from a total of 10 000 events/sample. BDCellQuest Pro Software was used for data acquisition and FlowJo Software for data analysis. For protein expression analysis, cell pellets were ressuspended in a lysis buffer [20 mM HEPES, 350 mM NaCl, 20% glycerol (v/v) and 1% NP-40 (v/v)] supplemented with protease inhibitors, 0.5 mM EDTA and 0.1 mM DTT and kept on ice for 30 min with intermittent vortexing. Protein quantification was performed with the Bradford method (BioRad Protein Assay Cat no

500-0006). Proteins (40-50 µg) were separated by electrophoresis in a 15% SDS polyacrylamide gel and blotted into a nitrocellulose membrane. Pro-caspase-3 and caspase-3 subunits were immunodetected using a rabbit anti-caspase-3 (Santa Cruz Biotech, 1:400) as primary antibody and the goat anti-rabbit IgG-HRP (Sigma-Aldrich, 1:5000) as a secondary antibody. Glyceraldehyde-3-phosphatase dehydrogenase (loading control) was immunodetected using a mouse anti-GAPDH (Santa Cruz Biotech, 1:1000) as primary antibody and the goat anti-mouse IgG-HRP (Invitrogen, 1:3000) as a secondary antibody. Immunodetection was performed by chemiluminescence, with a kit from GE Healthcare (RPN 2109).

Statistical analysis.

Data are expressed as the mean values ± standard error of the mean (SEM) of at least three independent experiments. Statistical analysis of SRB assay data was performed using GraphPad Prism, for which an analysis of variance (one-way ANOVA) with Dunnett´s multiple comparison test was applied to determine the differences between control and treatment means. All other data were compared by Student´s *t*-test.

Results

Effect of phenolics on AGS and Caco-2 proliferation.

Several structurally related phenolic compounds were used in this study: simple phenols (catechol, pyrogallol and phloroglucinol); phenolic acids (gallic acid); flavonols (quercetin and myricetin); and anthocyanins (dp-3-gluc, mv-3-gluc and cy-3-gluc). To investigate their effect on cellular proliferation, tumor cells were treated with increasing concentrations of the phenolics for 48 h (**Figures 3.2 and 3.3**).

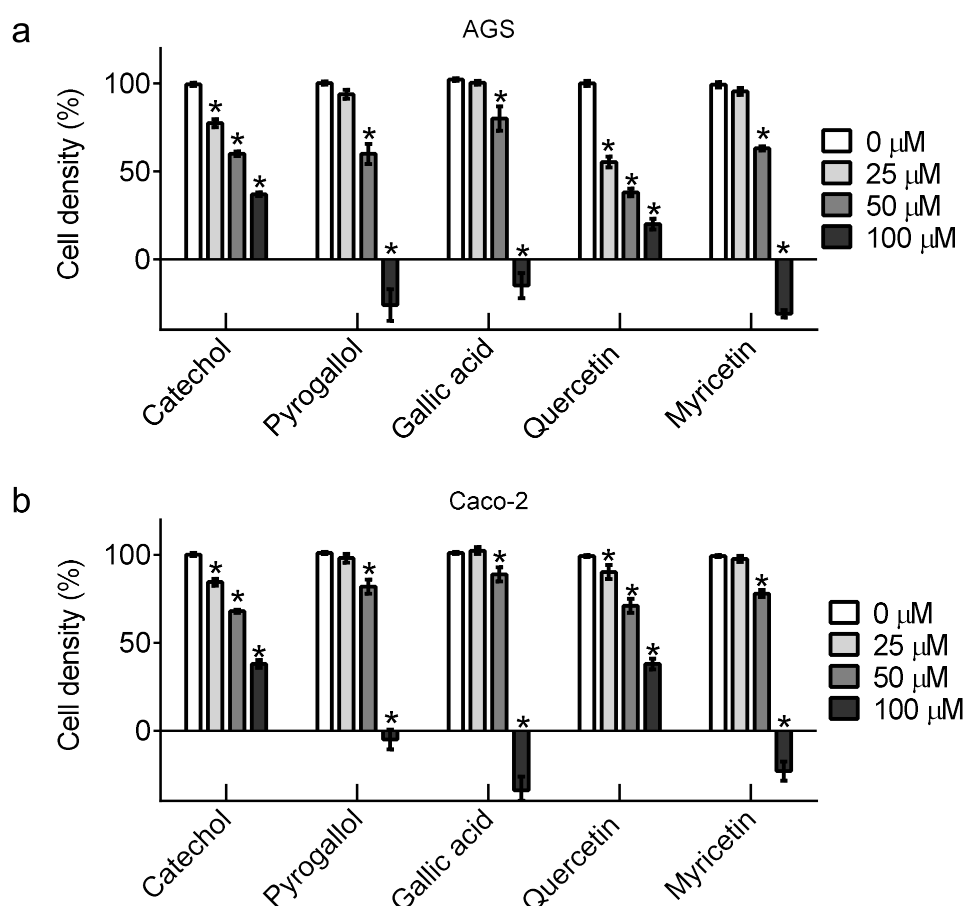


Figure 3.2 Effect of phenolic compounds on **(a)** AGS and **(b)** Caco-2 cellular proliferation. Cells were treated with the indicated concentrations (or DMSO; control) for 48 h. Cell density was determined by the quantification of protein in culture through SRB assay and is expressed as percent of treated over control cells corrected for the optical density at time zero. Bars indicates means ± SEM. *$p < 0.05$ compared to vehicle control.

Catechol, pyrogallol, gallic acid, quercetin, myricetin and dp-3-gluc were the most effective in inhibiting cellular proliferation. The potential of phenolics to inhibit proliferation was similar between several tumor cell lines tested, including the gastric cancer cell line MKN-

28 and the colon cancer cell line HT-29 (**Figure S1**). Interestingly, Caco-2 cells were less sensitive to quercetin, showing an IC50 two fold higher (69.9 ± 5.9 μM) than that observed for AGS cells (32.3 ± 2.8 μM) (**Table 1**). In the AGS cell line, the compound exhibiting the lowest IC50 was quercetin, followed by myricetin, pyrogallol, gallic acid and catechol.

Table 1. Phenolics concentration that inhibits 50% of cell growth (IC50) after 48 h.

IC50 (μM) (Mean ± SEM)		
Compound	**AGS**	**Caco-2**
Catechol	73.1 ± 2.2	76.6 ± 1.6
Pyrogallol	58.4 ± 0.9	61.1 ± 2.0
Gallic acid	63.9 ± 1.0	54.3 ± 5.7
Quercetin	32.3 ± 2.8	69.9 ± 5.9
Myricetin	54.2 ± 0.5	59.3 ± 0.9

However, myricetin, pyrogallol and gallic acid were cytotoxic at higher concentrations, as demonstrated by the decrease in the number of cells. Anthocyanins were the least effective in inhibiting proliferation. Among these compounds, dp-3-gluc was the most potent, with a 150 μM treatment decreasing cell density to approximately 50% in Caco-2 and AGS. Caco-2 cells were more sensitive to cy-3-gluc and mv-3-gluc than AGS cells. These compounds (at 150 μM) decreased Caco-2 cell density to 63.0% ± 4.5 and 70.2% ± 0.9, respectively, but had minor effects on AGS cells.

Similar results were observed when cellular proliferation was analysed by measuring the incorporation of the pyrimidine analog, BrdU, into the newly synthesized DNA of proliferating cells, providing data more directly related to cellular proliferation (**Figure 3.4**).

The analysis of potential cytotoxicity of the phenolic compounds in a normal cell line was also tested (**Figure S2**). Treatment of human foreskin fibroblasts (HFF-1) for 48 h with the compounds resulted in mild cytotoxicity at higher concentrations (above 100 μM). Therefore, the concentrations able to inhibit tumor proliferation do not affect the proliferation of the normal cell line.

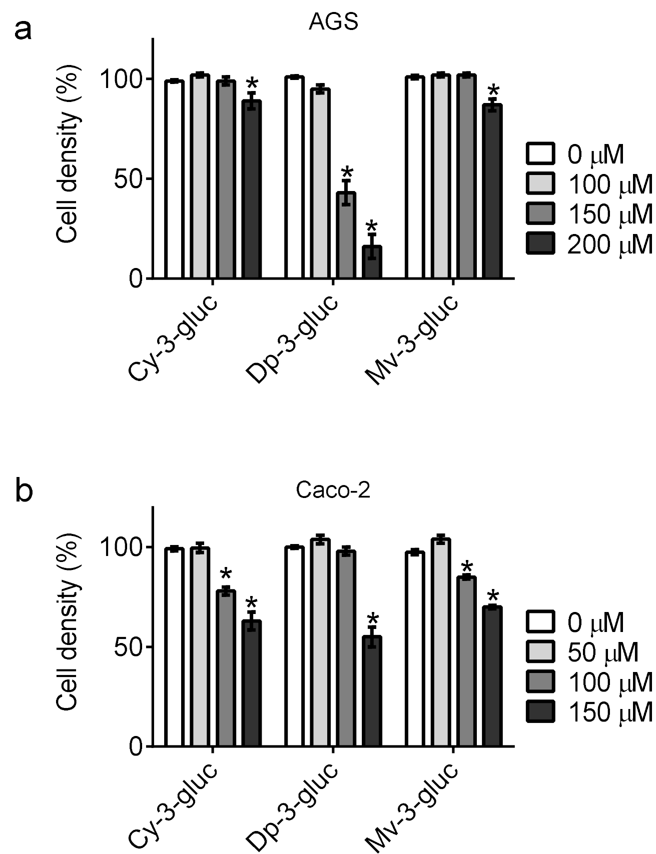


Figure 3.3 Effect of anthocyanins on (**a**) AGS and (**b**) Caco-2 cellular proliferation. Cells were treated with the indicated concentrations (or DMSO; control) for 48 h. Cell density was determined by the quantification of protein in culture through SRB assay and is expressed as percent of treated over control cells corrected for the optical density at time zero. Bars indicates means ± SEM. *$p < 0.05$ compared to vehicle control.

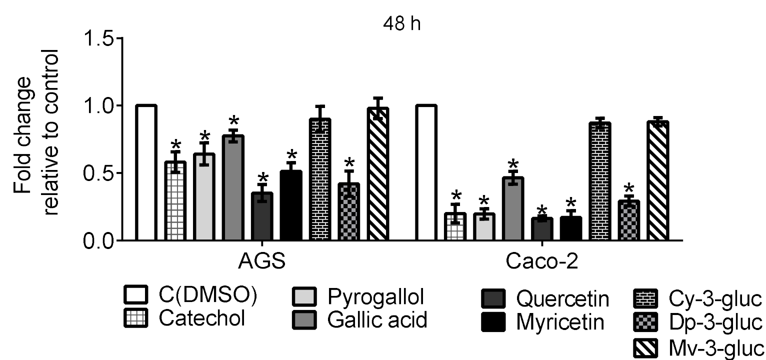


Figure 3.4 BrdU analysis of cells treated for 48 h with phenolics IC50, except for anthocyanins, for which 150 μM was selected. Bars indicates means ± SEM. *$p < 0.05$ compared to vehicle control.

Detection of H_2O_2 in culture medium with phenolics.

The anti-proliferative effect of phenolics can result from its auto-oxidation, leading to H_2O_2 production in the culture medium. Therefore, the levels of H_2O_2 were analysed in the two culture mediums (MEME and RPMI in the absence of cells) incubated with the higher concentrations used in the proliferation assay (200 μM of anthocyanins and 100 μM of the other phenolics). The results show that H_2O_2 levels were similar in the two culture media (**Figure 3.5 (a, b)**). Moreover, phenolics generated H_2O_2 in a dose-dependent manner [**(Figure S3)** for catechol, pyrogallol, gallic acid, quercetin and myricetin]. The assay was specific towards H_2O_2, as it was not detected in samples treated with catalase (**Figure S3**). In addition, H_2O_2 levels decreased over the incubation time, except in culture medium with catechol, in which H_2O_2 levels increased over time with the maximum detected after 24 h (**Figure 3.5 (a, b)**). At 1 h treatment, the compounds that generated higher levels of H_2O_2 (close to 60 μM) were pyrogallol, gallic acid and myricetin. In contrast, quercetin generated low H_2O_2 levels (close to 24 μM). Among anthocyanins, dp-3-gluc generated the highest levels of H_2O_2 (approximately 31-38 μM) while the levels detected in the case

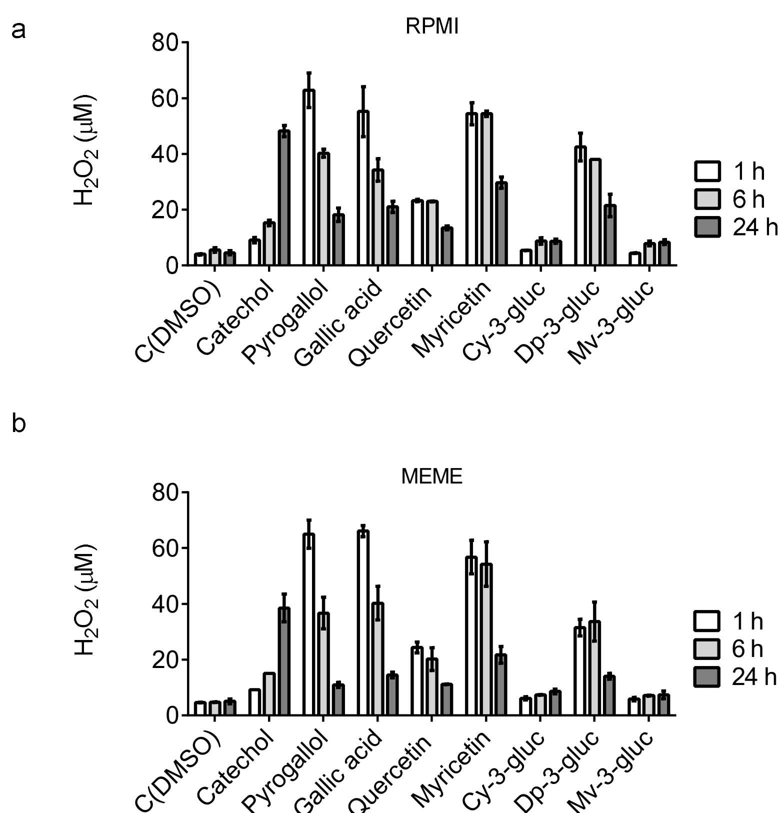


Figure 3.5 Quantification of H_2O_2 by the FOX assay in **(a)** RPMI and **(b)** MEME medium with phenolic compounds. Simple phenols, phenolic acids and flavonols were added at 100 μM and anthocyanins at 200 μM (or DMSO; control) to culture medium without cells and incubated under cell culture conditions. Bars indicate means ± SEM.

of cy-3-gluc and mv-3-gluc were similar to control. It was also confirmed the reports by other authors that H_2O_2 is not detected in the presence of cells (data not shown).

Contribution of H_2O_2 and $O_2^{\cdot-}$ to the anti-proliferative effect of phenolics.

To analyse if H_2O_2 is involved in the inhibition of AGS and Caco-2 cellular proliferation, we first tested the effect of increasing concentrations of H_2O_2 **(Figure 3.6)**. We observed a dose-dependent inhibitory effect of H_2O_2, with AGS cells being more sensitive to this oxidant. For example, in AGS cells, 25 μM of H_2O_2 decreased cell density to 71% of control, while in Caco-2 cells, 50 μM of H_2O_2 decreased cell density only to 82%. Therefore, H_2O_2 may be an intermediate in the anti-proliferative effect of phenolics in tumor cells.

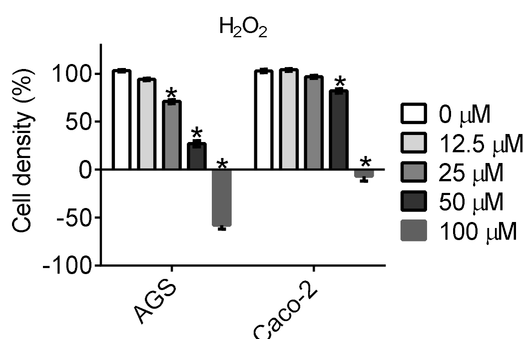


Figure 3.6 Effect of H_2O_2 on AGS and Caco-2 cellular proliferation. Cells were treated with the indicated concentrations for 48 h. Cell density was determined by the quantification of protein in culture through SRB assay and is demonstrated as percent of treated over the respective control. Bars indicate means ± SEM, *$p <$ 0.05.

This led us to assess the anti-proliferative effect of phenolics in the presence of catalase, which decomposes H_2O_2 into H_2O and O_2 (**Figure 3.7 (a, b)**). The analysis was carried out with pyrogallol, gallic acid, quercetin, myricetin and dp-3-gluc, since those were the most relevant in terms of anti-proliferative effect. Cells treated with H_2O_2 (50 μM and 100 μM in AGS and Caco-2 respectively) for 48 h were used as control. In this case, catalase reverted the anti-proliferative effect of H_2O_2. Co-incubation of pyrogallol, gallic acid, myricetin and dp-3-gluc with catalase reverted partially their anti-proliferative effect in both cell lines. Cell density in AGS cells after 48 h incubation with pyrogallol, gallic acid or myricetin at 100 μM was 6-55%, 7-60% and 9-58% in the absence and presence of

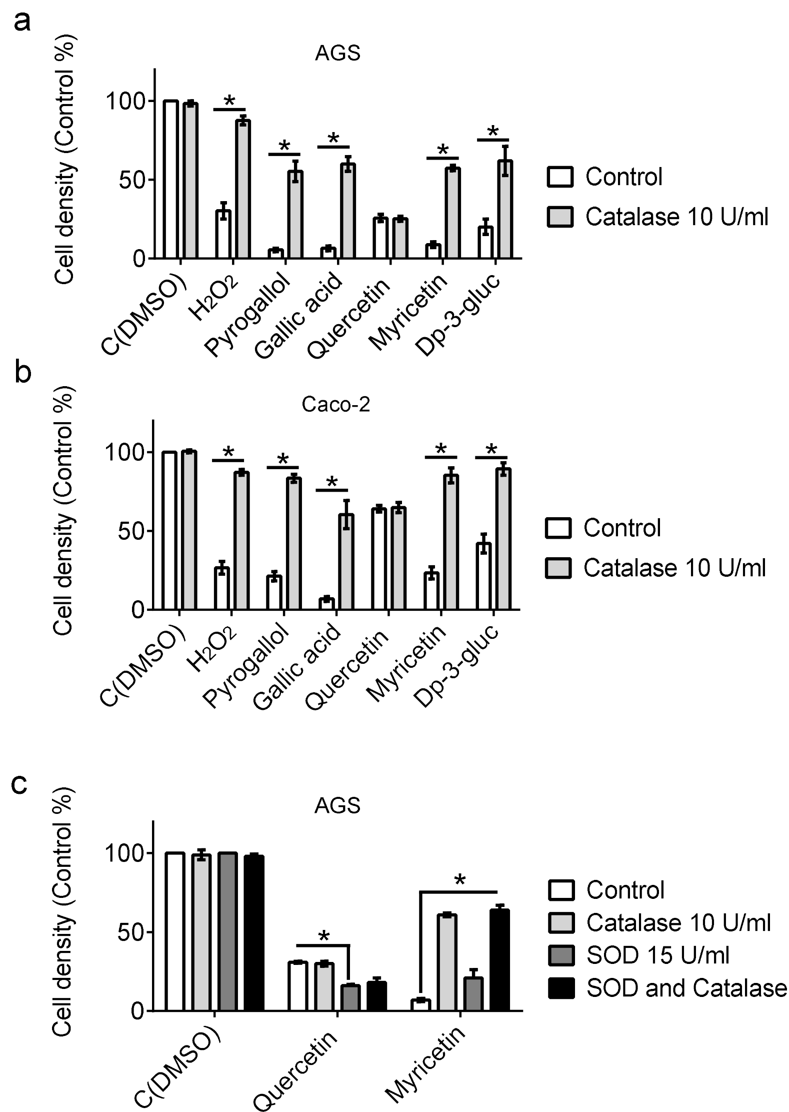


Figure 3.7 (a, b) Effect of catalase on the anti-proliferative effect of phenolics on AGS and Caco-2 cells. Cells were incubated with DMSO (control), 50 µM or 100 µM of H_2O_2, 100 µM of pyrogallol, gallic acid, quercetin and myricetin or 200 µM of Dp-3-gluc in culture medium in the absence or presence of catalase for 48 h. **(c)** Effect of SOD and/or catalase in the anti-proliferative effect of 100 µM of quercetin and myricetin in AGS cells. Cell density was determined by the quantification of protein in culture through SRB assay and is demonstrated as percent of treated over the respective control. Bars indicate means ± SEM. *$p < 0.05$ (catalase and/or SOD *vs* control).

catalase, respectively. Dp-3-gluc at 200 µM decreased cell density to 20% and 42% in AGS and Caco-2 cells respectively, but this decrease was also lower in cells co-incubated with catalase. Overall, these results indicate that H_2O_2 may be at least partially involved in the anti-proliferative effect of pyrogallol, gallic acid, myricetin and dp-3-gluc towards AGS or Caco-2 cells. In contrast, the anti-proliferative effect of quercetin was not suppressed by catalase and, therefore, is mostly independent of H_2O_2 formed in culture medium.

To analyse if $O_2^{\cdot -}$ is involved in the anti-proliferative effect of phenolics, the culture medium with quercetin or myricetin was supplemented with SOD, which reduces $O_2^{\cdot -}$ into H_2O_2, and/or catalase. The anti-proliferative effect of quercetin and myricetin was not reverted in the presence of SOD. This data indicates that the anti-proliferative effect of these compounds is not mediated by the generation $O_2^{\cdot -}$ in culture medium. In addition, the anti-proliferative effect of quercetin was modestly potentiated by the presence of SOD.

Effect of phenolic compounds in intracellular oxidation.

To analyse the impact of phenolics on intracellular oxidation, ROS levels were analysed by flow cytometry using cells stained with H_2DCF-DA, a probe sensitive to ROS, and treated with the IC50 of the following compounds: quercetin, whose anti-proliferative effect was not inhibited by catalase or SOD; myricetin, due to its structural similarities with quercetin but distinct cellular effects; and dp-3-gluc, which was the most efficient anthocyanin tested. Intracellular ROS levels were analysed 1 h and 24 h after treatment (**Figure 3.8 (a, b)**). As a positive control, cells were treated with H_2O_2 (500 µM for 10 min), which increased intracellular oxidation, as expected (data not shown). AGS cells treated with quercetin for 1 h and 24 h showed significantly lower levels of intracellular oxidation, while AGS cells treated with myricetin and dp-3-gluc only showed lower levels after 1 h (**Figure 3.8 (a)**). Interestingly, quercetin had the opposite effect in Caco-2 cells, which demonstrated transiently higher levels of intracellular ROS (only observed at 1 h) (**Figure 3.8 (b)**). Altogether, this data shows that the generation of H_2O_2 by auto-oxidation of phenolics in culture medium is not correlated to increases in intracellular ROS. Indeed, although myricetin generated high levels of H_2O_2 in culture medium, AGS cells treated with myricetin for 1 h showed intracellular ROS levels similar to control.

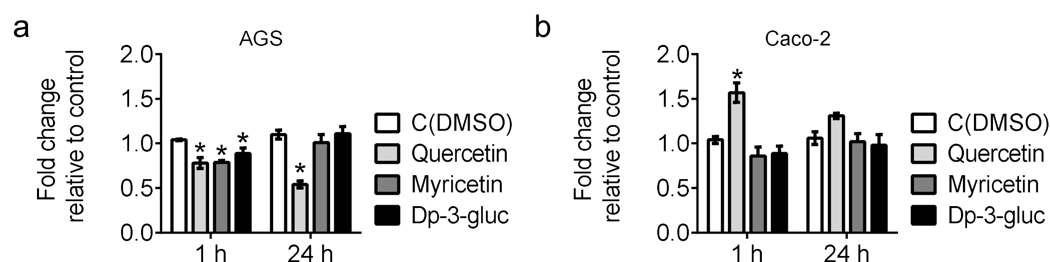


Figure 3.8 Intracellular ROS levels in **(a)** AGS treated with quercetin (30 µM), myricetin (60 µM) and Dp-3-gluc (150 µM) and in **(b)** Caco-2 cells treated with quercetin (60 µM), myricetin (60 µM) and Dp-3-gluc (150 µM). After treatment, cells were incubated with H_2DCF-DA for 30 min and PI was added before fluorescence analysis by flow cytometry. Bars indicate means ± SEM. *p < 0.05 compared with the respective time control.

To further analyse if oxidative damage was being imposed by the treatment with the phenolics, protein carbonyl levels were analysed after 1 h and 24 h of treatment. No

significant differences in protein carbonyls levels were detected between the control and treatments (**Figure 3.9 (a, b)**), supporting the absence of a pro-oxidative effect for phenolics.

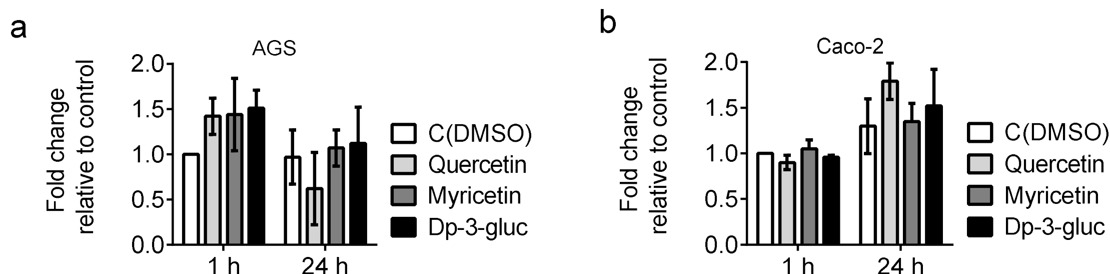


Figure 3.9 Protein carbonyl levels in **(a)** AGS and in **(b)** Caco-2 cells treated with quercetin (30 µM), myricetin (60 µM) and Dp-3-gluc (150 µM). After treatment, cells were harvested, lysed and protein carbonyl levels where analysed by slot-blot analysis as indicated in methods.

Influence of phenolic compounds on enzymatic anti-oxidant cellular defences.

Cells possess anti-oxidant defences that can be induced as an adaptation mechanism upon oxidative stress stimuli (Pelicano *et al.*, 2004). To assess the impact of phenolics on anti-oxidant defences, the activity of SOD and catalase was analysed in AGS and Caco-2 cells treated with quercetin, myricetin and dp-3-gluc. The data showed no alterations in SOD or catalase activities (**Figure 3.10 (a, b)**), indicating that phenolics did not induce an oxidative stress response leading to activation of these cellular anti-oxidant defences.

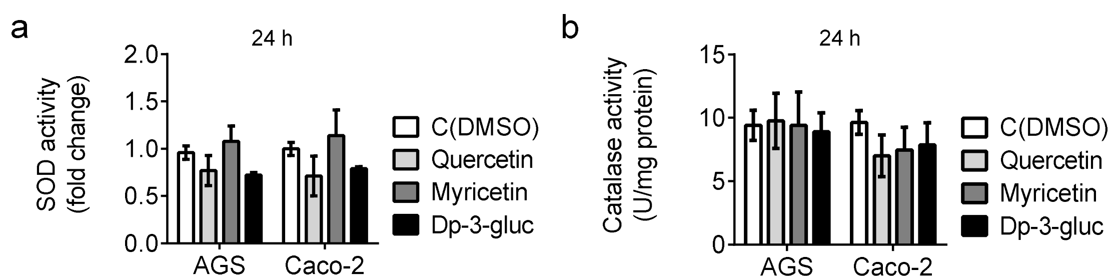


Figure 3.10 Effect of phenolic compounds on **(a)** SOD and **(b)** catalase activity. Cells were treated for 24 h with the IC50 of phenolic compounds. SOD activity was determined *in situ* after separation of protein extracts by native gel electrophoresis. Catalase activity was determined in cellular extracts by following the decomposition of H_2O_2 at 240 nm. Bars indicate means ± SEM.

Effect of quercetin and myricetin on glutathione levels in AGS cells.

Glutathione (GSH) is a tripeptide with crucial roles in redox homeostasis, protection of proteins from irreversible oxidative modification and detoxification of xenobiotics, which are extruded from cells after conjugation with GSH by glutathione *S*-transferase (GST)

(Townsend *et al.*, 2003). The ratio between glutathione disulphide (GSSG), the oxidised form of glutathione, and total glutathione levels, has been frequently used as an index of intracellular redox state. The intracellular glutathione levels were analysed in AGS cells treated with quercetin, for which the anti-proliferative effect is independent of H_2O_2 and $O_2^{\cdot -}$ generation in culture medium, and myricetin, for which the anti-proliferative effect seems to be mediated by H_2O_2 *in vitro*. After 6 h, quercetin significantly reduced GSSG levels as well as total glutathione levels in AGS cells (**Figure 3.11 (a, b))**. Myricetin had minor effects on glutathione levels, with a small decrease in GSSG levels being observed after 24 h. The differences between control and treated cells were attenuated at 24 h, probably as consequence of the low glutathione levels displayed by AGS cells after 24 h of growth. The ratio between GSSG and total glutathione levels was not affected by quercetin or myricetin (**Figure 3.11 (c)**). These results indicate that quercetin decreases glutathione levels without changing the GSSG and total glutathione ratio and suggest that glutathione depletion may contribute to its anti-proliferative effects. Indeed, the high proliferation rate of tumor cell lines has been associated with high levels of intracellular glutathione, which tend to decrease when cells reach confluence and consequently slow the growth rate (Carretero *et al.*, 1999).

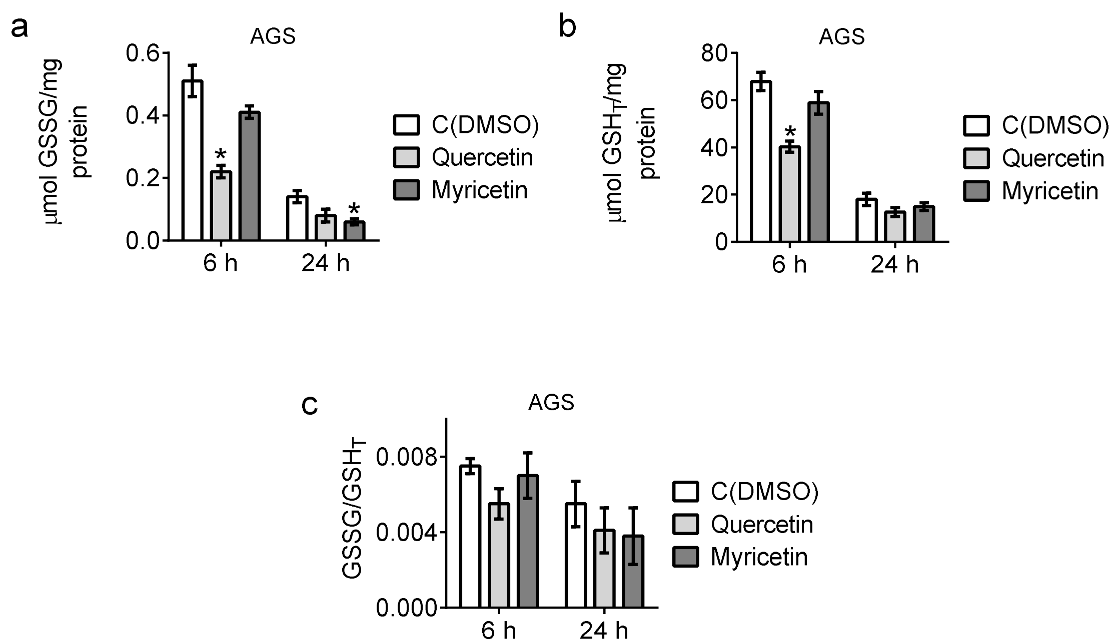


Figure 3.11 Effect of quercetin and myricetin on glutathione levels. AGS cells were treated for the indicated times with the IC50 of quercetin or myricetin. Cells were harvested and glutathione levels was determined as described in methods. **(a)** glutathione disulphide; **(b)** total glutathione; **(c)** ratio between glutathione disulphide and total glutathione levels (GSH_T). Bars indicate means ± SEM. *$p < 0.05$ compared with the respective time control.

Effect of quercetin and myricetin on cell cycle and apoptosis.

Aiming to assess if the anti-proliferative effects of phenolics were associated with changes in cell cycle or apoptotic cell death, these cellular processes were analysed in AGS cells treated with quercetin and myricetin. Cell cycle analysis revealed that quercetin, in contrast with myricetin, significantly increased the population of cells in the S and G2/M phase with a corresponding decrease in the G0/G1 phase (**Figure 3.12 (a, b)**).

In order to analyse the effect of quercetin and myricetin on cell death, AGS cells were double stained with annexinV/PI (**Figure 3.13 (a)**). Furthermore, protein levels of caspase-3 subunits were analysed by western blotting (**Figure 3.13 (b)**). Quercetin and myricetin caused a slight increase in the percentage of apoptotic cells (positive for annexin V or annexin V and PI) after 24 h. These compounds also increased the processing of procaspase 3, suggesting that both quercetin and myricetin increase apoptotic cell death.

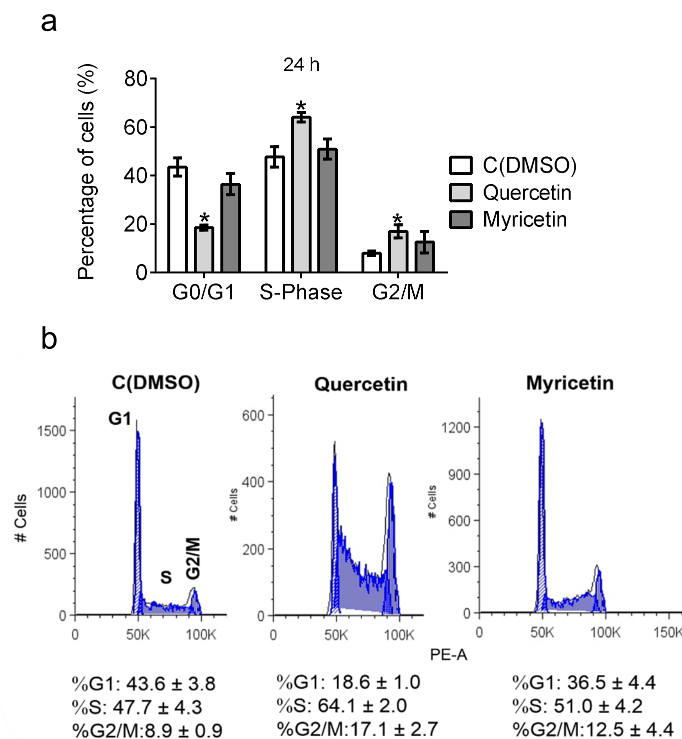


Figure 3.12 Effect of quercetin and myricetin on the cell cycle of AGS cells. Cells were treated for 24 h with the IC50 of quercetin (30 μM) or myricetin (60 μM). Cells were fixed, labelled with PI and analysed by flow cytometry. **(a)** Percentage of cells on each phase of the cell cycle. Bars indicate means ± SEM. *p < 0.05 compared with the respective control. **(b)** Representative flow cytometry histograms.

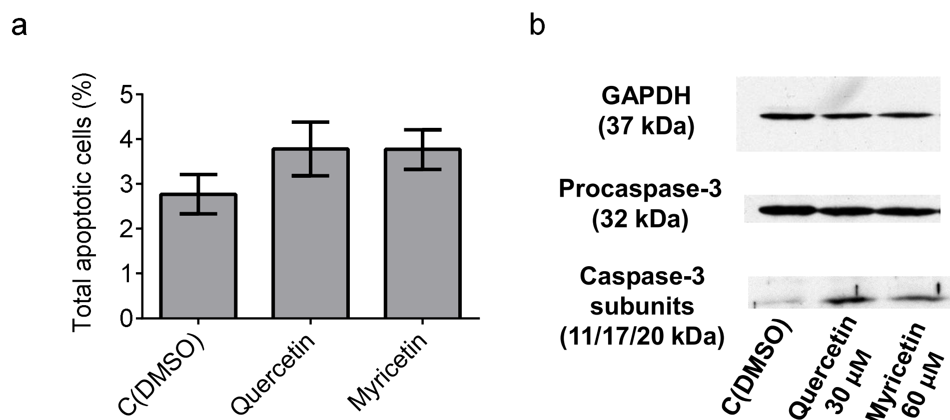


Figure 3.13 Effect of quercetin or myricetin on apoptosis in AGS cells. Cells were treated for 24 h with the IC50 of quercetin or myricetin, **(a)** double stained with annexin V/PI and analysed by flow cytometry. Values in the graph represent the percentage of cells in apoptosis. **(b)** Alternatively, protein levels of procaspase-3 and caspase-3 subunits were analysed in protein extracts through western blotting. GAPDH was used as the loading control.

Effect of quercetin and myricetin in Trx and TrxR reducing activities and Trx1/2 redox state.

The thioredoxin system and the oxidation state of thioredoxin (Trx) have a crucial role in the control of a signalling pathway involved in apoptosis. In a reduced environment, fully reduced Trx keeps ASK1 in an inactive form. Upon oxidation of Trx, ASK1 is activated with consequent induction of apoptosis mediated by p38 (Tobiume *et al.*, 2001). Therefore, it is important to analyse the state of the thioredoxin system, for which thioredoxin reductase (TrxR) has important functions for maintenance of Trx in the reduced form. In order to analyse the effect of quercetin and myricetin on the thioredoxin system, the reducing abilities of Trx and TrxR were analysed in AGS cells treated for 6 h or 24 h with the IC50 of quercetin and myricetin. Quercetin or myricetin treated cells showed a decrease in Trx reducing ability after 6 h, which was attenuated after 24 h (**Figure 3.14 (a)**). On contrary, TrxR activity was not altered by quercetin or myricetin (**Figure 3.14 (b)**).

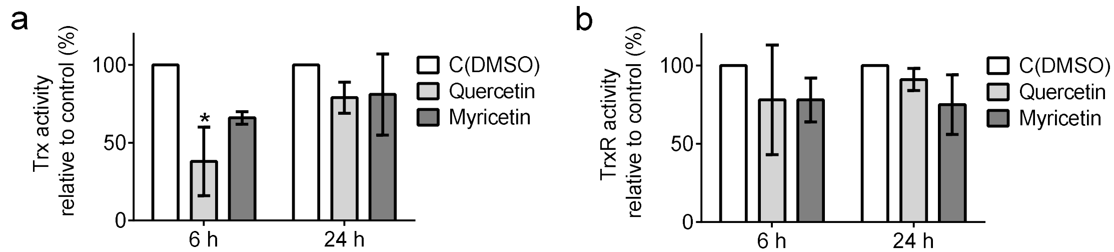


Figure 3.14 Effect of quercetin or myricetin for 6 h or 24 h on **(a)** Trx and **(b)** TrxR activities in AGS cells. Activities were determined as described in methods. Values represent mean values of percentages relative to control. Bars indicate means ± SEM. *$p < 0.05$ compared with the respective control.

The cytosolic Trx1 has five cysteines, among them three are structural and the other two form the active site (Cys32 and Cys35) (Hashemy and Holmgren, 2008), while the Trx2 has only two cysteines forming the active site (Cys90 and Cys93) (Powis *et al.*, 1997). The oxidation state of Trxs was analysed by redox western blotting in AGS cells treated for 1 h and 6 h with quercetin or myricetin (**Figure 3.15**). To analyse the redox state of the cysteines, the samples were treated with iodoacetic acid, which confers to the free cysteines a negative charge. After a reduction step (with DTT), samples were treated with iodoacetamide to add a neutral charge to the free cysteines. Then, proteins were separated in a urea-polyacrylamide gel, depending on its oxidation state. Therefore, the fully reduced form of Trx1 migrates to the gel bottom (-5 charge for Trx1 and -2 charge for Trx2). Oxidation of one cysteine within the Trxs shifts the corresponding band upwards to a higher charge, whereas the bands of the fully oxidized forms of Trx1/2 are in the top of the gel. Treatments did not result in significant changes on the redox profile of both thioredoxins. Therefore, quercetin and myricetin does not seem to affect the Trx system in AGS cells.

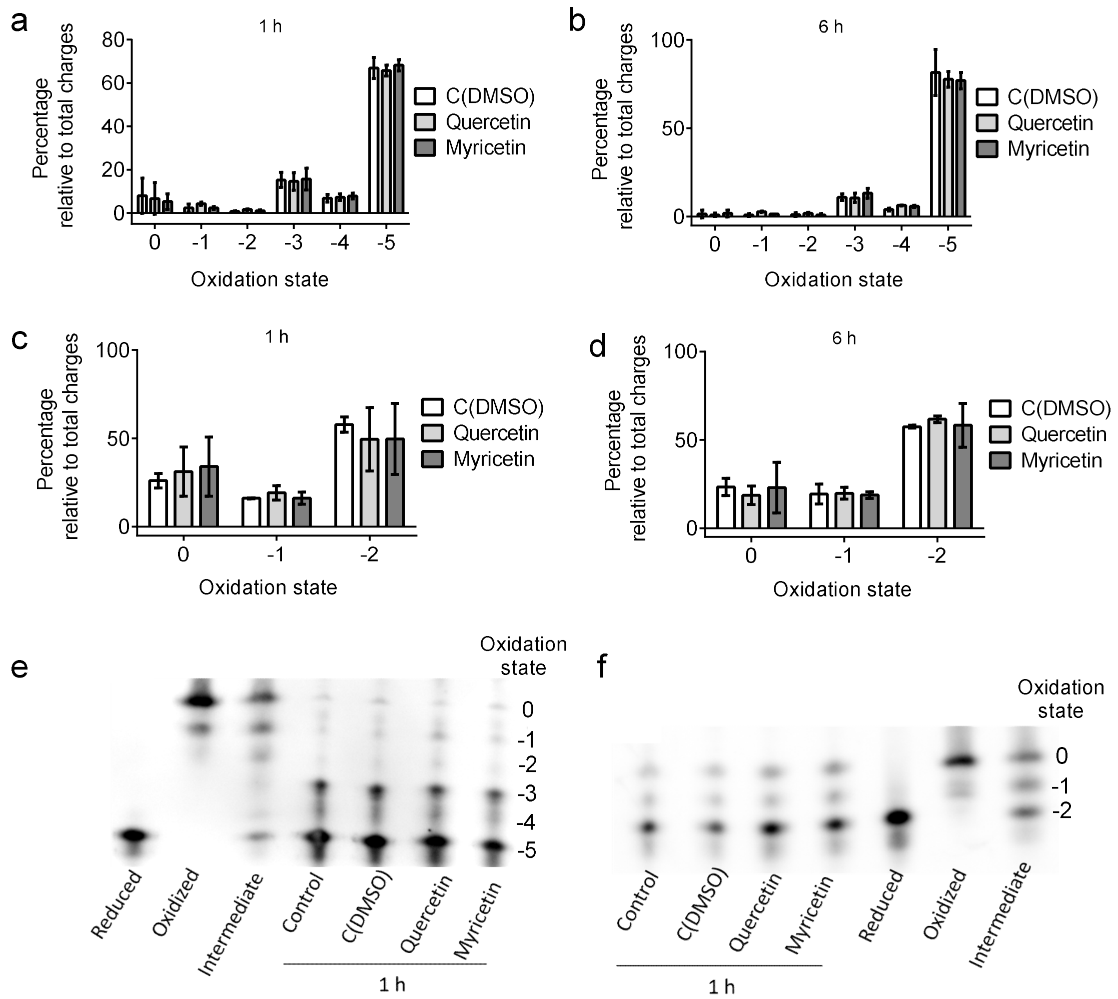


Figure 3.15 Effect of quercetin and myricetin treatment **(a and c)** for 1 h and **(b and d)** for 6 h on the oxidation state of **(a and b)** Trx1 and **(c and d)** Trx2 in AGS cells. Samples were processed as described in methods. Each bar represents the mean intensity percentage of the band corresponding to each electronic charge relative to the total intensity. **(e and f)** Representative blots of **(e)** Trx1 and **(f)** Trx2 oxidation states after 1 h of treatment. The blots include mobility standards, acquired from cellular extracts, corresponding to the protein totally reduced, oxidized and all the intermediate states.

Discussion

The cancer protective effects of plant based diets have been associated with their high content in phenolic compounds (Stevenson and Hurst, 2007). However, several studies suggest that some phenolics can auto-oxidize in culture medium producing ROS which in turn will mediate the anti-proliferative effects (Bellion *et al.*, 2009; Lee *et al.*, 2005; Weisburg *et al.*, 2004). This work focused on the study of the anti-proliferative effect of several structurally related phenolic compounds in AGS and Caco-2 cells and its correlation with ROS generation in the culture medium and changes in cellular anti-oxidant defences.

Considering a putative structure-activity relationship, the data showed that the tri-hydroxylation pattern in the B-ring of the phenolics was positively related with the anti-proliferative effect and to the generation of high levels of H_2O_2 in culture medium. Several works associate the pyrogallol moiety (tri-hydroxylation) in the B-ring to the high $O_2^{\cdot -}$ scavenging ability and consequent generation of H_2O_2 (Furuno *et al.*, 2002). A previous study performed with MCF-7 cells has demonstrated the association between tri-hydroxylation in the phenolics B-ring and a stronger anti-proliferative effect (Fernandes *et al.*, 2010). Accordingly, our results show that pyrogallol, gallic acid, myricetin and dp-3-gluc, which have a pyrogallol moiety, generated higher H_2O_2 levels in culture medium. Other studies demonstrated that the concentration of H_2O_2 generated in culture medium depend on the type of compound. For example, gallic acid, in comparison with quercetin, generated more H_2O_2 in DMEM medium and had stronger anti-proliferative activity in Caco-2 cells and in normal rat liver epithelial cells (Lee *et al.*, 2005). In agreement with our data, it was previously reported that myricetin generated more H_2O_2 than quercetin in culture medium (Yokomizo and Moriwaki, 2006). The combination of different compounds can result in improved stability and decreased ROS generation. For example, mixtures of phenolic compounds and ascorbate resulted in lower levels of H_2O_2 than the phenolics alone (Wee *et al.*, 2003).

Our results show that pyrogallol, gallic acid, myricetin and dp-3-gluc had a mild anti-proliferative effect at lower concentrations (12.5 µM and 25 µM) and cytotoxic effects at higher concentrations (100 µM) which may reflect cytotoxicity mediated by H_2O_2 generation due to auto-oxidation. In comparison to pyrogallol, phloroglucinol did not have anti-proliferative effects, indicating that the position of the three hydroxyl groups in the benzenic ring (*ortho*- position in pyrogallol; *meta*- position in phloroglucinol) is a critical feature. Quercetin and myricetin are flavonols di-hydroxylated and tri-hydroxylated in the B-ring, respectively. Although quercetin displayed the lowest IC50 in AGS, myricetin was

cytotoxic at 100 µM, indicating that myricetin is more prone to auto-oxidize in comparison with quercetin. Similarly, dp-3-gluc displayed a stronger anti-proliferative effect than its di-hydroxylated counterpart, cy-3-gluc. Mv-3-gluc had a weak anti-proliferative effect, which is in agreement with evidences that *O*-methylation in the B-ring inactivates both the anti-oxidant and pro-oxidant activities of phenolics (Cao *et al.*, 1997).

The dependency on phenolics auto-oxidation products and consequent generation of ROS in culture medium for the anti-proliferative effect of phenolics *in vitro* has been demonstrated in numerous works. *In vitro*, the biological effects of several phenolics is reversed after addition of anti-oxidants to culture medium, such as catalase, SOD, N-acetyl-L-cysteine (NAC) and GSH (Atsumi *et al.*, 2006; Erlank *et al.*, 2011; Hou *et al.*, 2005). For instance, catalase reverted the anti-proliferative effect in human colon tumor Caco-2 and rat liver epithelial WB-F344 cells treated with gallic acid (Lee *et al.*, 2005) and in CAL-27, a human tongue squamous carcinoma cells, treated with (-)-epigallocatechin-3-gallate (EGCG) (Weisburg *et al.*, 2004). Our results indicate that the anti-proliferative effect of quercetin is independent of the generation of H_2O_2 and $O_2^{\cdot-}$ in culture medium. In addition, it was observed a moderate potentiation of the anti-proliferative effect of quercetin in AGS cells in the presence of SOD. This could be an indication that quercetin is being stabilized by SOD in culture medium as previously described for EGCG. The addition of SOD to culture medium stabilized EGCG, reverting the inhibition of EGFR and HER-2/*neu* phosphorylation in KYSE 150 cells and OE19 cells respectively, but increasing EGCG-mediated growth inhibition (Hou *et al.*, 2005). The presence of SOD may increase H_2O_2 in culture medium leading to an additive effect of quercetin and H_2O_2 in growth inhibition. In this work we show that the generation of H_2O_2 by SOD did not potentiate quercetin effects since the moderate increase of the anti-proliferative effect of quercetin in the presence of SOD was not reverted by the presence of catalase.

The extrapolation of extracellular oxidation of phenolic compounds in culture medium *in vitro* to the *in vivo* must be applied with caution. Phenolics oxidation will certainly be limited *in vivo* in comparison to *in vitro* conditions. However, in several physiological conditions, we cannot exclude totally the occurrence of phenolics oxidation in extracellular environment. *In vivo*, the upper GI tract can be exposed to high levels of H_2O_2 (Long *et al.*, 1999) and gut microbiota may also induce the generation of ROS (Neish and Jones, 2014). Inflammation and cancer are also conditions associated with high levels of ROS. Also of note, human subjects presented high levels of H_2O_2 in saliva after chewing green tea (Lambert *et al.*, 2007).

Literature concerning the influence of phenolics compounds in intracellular ROS levels is highly variable, depending on several factors. For instance, in the presence of a peroxyl generator, delphinidin and cyanidin had a pro-oxidant effect in the doxorubicin-resistant

cell line LoVo/ADR, while in Caco-2 cells, a more stable tumoral cell line, they had an anti-oxidant effect (Cvorovic *et al.*, 2010). Also, treatment with cyanidin-3-rutinoside caused ROS accumulation in leukemic cells (HL-60) but decreased its accumulation in normal cells (PMBC) (Feng *et al.*, 2007). This evidence suggests that, when using moderate concentrations of phenolics, the effect on intracellular ROS levels depends on the cell type, treatment time and experimental conditions (if there is an inducing stress condition). Most of the studies evaluate intracellular ROS levels in cells treated with phenolics and subjected to stress factors without considering the possible role of extracellular generation of ROS. To our knowledge, this is the first work that correlates the extracellular (in culture medium) generation of ROS by phenolic compounds and the impact on intracellular levels of ROS and on the cellular response through oxidative stress defences. Our results show that the levels of H_2O_2 detected in culture medium with phenolics were not associated to higher levels of intracellular ROS after treatment for 1 h (except for quercetin in Caco-2 cells), which was the time point where the highest levels of H_2O_2 were detected for most compounds (except for catechol). On the contrary, in AGS cells, quercetin, myricetin and dp-3-gluc decreased fluorescence of H_2DCF after 1 h and quercetin decreased after 24 h. In addition, although the levels of H_2O_2 in culture medium with myricetin were high, the AGS cells treated with myricetin did not show glutathione oxidation.

In relation to the modulation of catalase and SOD activities, the effects of phenolics on tumor cells are scarce. The induction of cellular anti-oxidant defences by phenolics is often observed in situations of a pre-existing stress (Hernandez-Ortega *et al.*, 2012; Vidyashankar *et al.*, 2013). In agreement with our results, several flavonols, including myricetin and quercetin, did not change catalase and SOD activities in Caco-2 and HepG2 cells (Aherne and O'Brien, 1999) .

The anti-proliferative effect of these compounds depends on their concentration, time of treatment and also on the cell type. Our results are in agreement with previous studies showing an IC50 for quercetin of 50 µM in Caco-2 cells (van Erk *et al.*, 2005) and of 40 µM in AGS cells (Wang *et al.*, 2011). Myricetin was also reported to have anti-tumoral activity in several tumor cell lines, including HCT116 (Shiomi *et al.*, 2013), HepG2 (Zhang *et al.*, 2013), PC-3 (Xu *et al.*, 2013), among others. Generally the concentrations used to inhibit tumor cell proliferation are much higher for myricetin when compared with quercetin.

In this study, quercetin and myricetin induced apoptosis, indicated by the increase in the levels of caspase-3 subunits. In addition, quercetin effect was also associated to a cell cycle arrest in S and G2/M phase. Together this data may indicate that cells underwent cell cycle arrest and were trying to adapt to quercetin treatment, while myricetin treatment was more aggressive. It is likely that cells adapt through glutathione mediated extrusion of

quercetin or quercetin metabolites. Apart from extracellular oxidation, quercetin can be oxidized inside cells (Awad *et al.*, 2002; Walle *et al.*, 2003) forming *o*-semiquinones, *o*-quinones and ROS. Those quercetin metabolites can be further methylated and conjugated with glutathione (Galati *et al.*, 2001; Spencer *et al.*, 2003) and extruded from cells, depleting intracellular glutathione levels without causing glutathione disulphide formation (Ishikawa, 1992). The glutathione quercetin adduct was identified in the human plasma in one work (Lee *et al.*, 2012) indicating that cellular oxidation may be possible *in vivo*. In agreement with our data, several reports show the depletion of intracellular glutathione by several phenolic compounds (Odenthal *et al.*, 2012), including quercetin in human leukemia cell lines (Ramos and Aller, 2008). However, several data under different experimental conditions also showed that quercetin and myricetin could increase intracellular levels of reduced glutathione in human breast cancer MCF-7 cells (Rodgers and Grant, 1998). There are also indications that phenolic compounds can modulate transporters involved in the extrusion of glutathione, such as MRP1/2, in HeLa cell lines (Leslie *et al.*, 2001) and modulate the activity of enzymes involved in the synthesis and metabolism of glutathione in intestinal and breast cancer cell lines (Odenthal *et al.*, 2012; Rodgers and Grant, 1998). The modulation of those enzymes depends on the cell type, phenolics structure, concentration, treatment time and experimental design (cells in exponential growth or in confluence; tumoral or non-tumoral cells subjected to stress conditions or under normal conditions).

In mammalian cells, Trx and GSH systems can act as a backup system for each other (Du *et al.*, 2012). For that reason, the disruption of both systems is considered as an anticancer strategy. The TrxR1 knockout cancer cells are highly susceptible to GSH depletion induced by buthionine sulphoximine (BSO), a compound that inhibits γ-glutamylcysteine synthetase (Mandal *et al.*, 2010). There are few data on the effect of quercetin and myricetin on the Trx system in cancer cells. Quercetin and myricetin and their oxidized products were identified as inhibitors and substrates of mammalian TrxR (Lu *et al.*, 2006). Nitrogen saturation or SOD attenuated the inhibition effect while xanthine/xanthine oxidase system amplified the inhibitory effect of the flavonols on TrxR, demonstrating the dependency on the redox environment. The inhibition of TrxR induced by quercetin and myricetin and the decrease in the reduced form of Trx was further confirmed in the human lung adenocarcinoma epithelial A549 cell line (Lu *et al.*, 2006). In addition, the quinone species of quercetin and myricetin can confer $O_2^{\cdot -}$-producing NADPH oxidase activity to mammalian TrxR (Cenas *et al.*, 2004). In this work, quercetin and myricetin treatment did not affect the reducing ability of TrxR and the oxidation state of Trx1 and Trx2 in AGS cells. Other studies with non-tumoral cells showed that quercetin treatment decreases the expression of TXNIP, a negative regulator of Trx, decreasing the

inflammation process in non-alcoholic fatty liver disease in rats (Wang *et al.*, 2013b), and increases the activity of TrxR in normal human keratinocytes (Sugahara *et al.*, 2010).

The data in the present work indicates that quercetin and myricetin do not induce oxidative stress in AGS cells. However, the possible modulation of signalling pathways through ROS generation cannot be totally excluded, since low levels of ROS in cells may be rapidly transformed into cellular signalling through oxidation of cysteines in proteins crucial for cellular signalling (Miki and Funato, 2012). In summary, this work emphasizes the importance of analysing possible artefacts in *in vitro* studies to limit the interference of ROS generated in culture medium in the interpretation of data regarding the anti-tumoral properties of phenolics. Our results show a correlation between the structure of these compounds and the generation of H_2O_2 in the growth medium, with phenolics with a tri-hydroxylation pattern on the B-ring being more prone to exert pro-oxidant effects. Importantly, the inhibition of AGS and Caco-2 cells proliferation by most compounds was partially dependent on H_2O_2 production in the medium but the anti-proliferative effect of quercetin was completely independent of H_2O_2 and $O_2^{\cdot-}$ generation. In contrast with the other phenolics tested, quercetin anti-tumoral effect was independent of changes in the activity of SOD or catalase and of the Trx system but associated with glutathione depletion in AGS cells.

Supplemental data

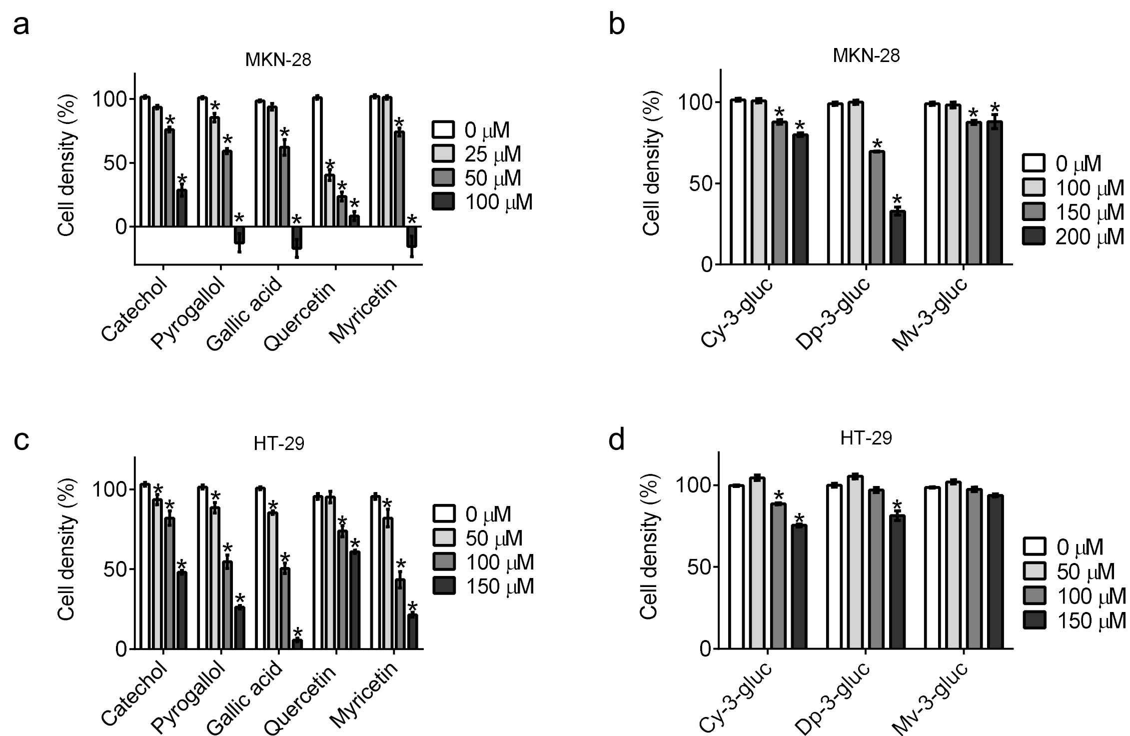


Figure S1 Effect of phenolic compounds on **(a, b)** MKN-28 and **(c, d)** HT-29 cellular proliferation. Cells were treated with the indicated concentrations (or DMSO; control) for 48 h. Cell density was determined by the quantification of protein in culture through SRB assay and is expressed as percent of treated over control cells corrected for the optical density at time zero. Bars indicates means ± SEM. **p* < 0.05 compared to vehicle control.

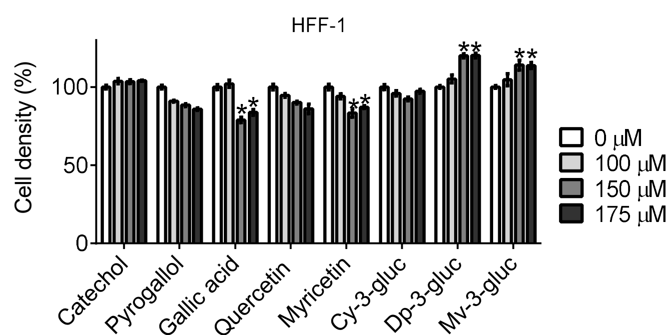


Figure S2 Effect of phenolic compounds on cellular proliferation of human foreskin fibroblasts HFF-1 cells. Cells were treated with the indicated concentrations (or DMSO; control) for 48 h. Cell density was determined by the quantification of protein in culture through SRB assay and is expressed as percent of treated over control cells corrected for the optical density at time zero. Bars indicates means ± SEM. **p* < 0.05 compared to vehicle control.

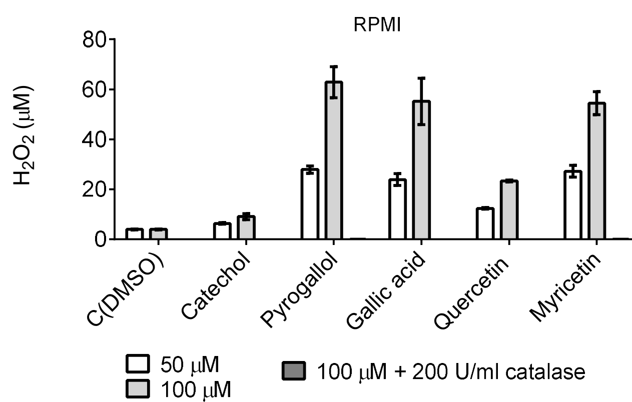


Figure S3 Dose-dependent generation of H_2O_2 in RPMI with phenolic compounds. Simple phenols, phenolic acids and flavonols were added at to culture medium without cells and incubated under cell culture conditions for 1 h. As a control, catalase (200 U/ml) was added to the medium with 100 µM of the phenolics. Bars indicate means ± SEM.

Chapter 4.

Conclusions and Future Perspectives

Phenolic compounds have been regarded as potent anti-oxidants, and this property has been considered important for the health beneficial effect of phenolics. However, the anti-oxidant evidence arises mainly from chemical *in vitro* studies and is not always in agreement with *in vitro* studies using biological models. In addition, several epidemiological studies fail to find any positive association between a diet rich in phenolics and health improvement (Janjua *et al.*, 2009) and even some clinical trials has been interrupted because of the clear damaging effect that the tested anti-oxidant compounds were having in the treated subjects ("The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group," 1994).

For their anti-oxidant potential and according to the oxidative stress theory of aging, it is believed that phenolic compounds can increase lifespan of cells. Indeed, this was observed in several works. We found that the effect of the phenolic compounds (myricetin, pyrogallol and pholoroglucinol) on lifespan may be independent of their intrinsic anti-oxidant properties. Otherwise, compounds would have increased lifespan of all yeast cells tested. So the effects of several phenolics on lifespan may be mediated by other mechanisms, such as through the translocation of the FOXO transcription factor DAF-16 to the nucleus in *C. elegans* treated with quercetin (Kampkotter *et al.*, 2007a). It is also relevant that cells seem to benefit more from phenolics when they are under oxidative stress conditions. In this work we found that H_2O_2-induced oxidative stress, intracellular oxidation and protein carbonylation was decreased in yeast cells pre-treated with myricetin and pyrogallol, and myricetin increased the lifespan of the *sod2*Δ yeast mutant in contrast to the lack of effect in parental and *sod1*Δ mutant cells. A number of works show this contrasting effect, in which phenolics only have anti-oxidant effects in cells exposed to oxidative stress (see Introduction).
Several phenolics have been associated to a decrease in aging at the cellular level, such as in yeast or in mammalian cells (Roh *et al.*, 2015) and retard age-related declines, such as cognitive function in rats and humans (for more information check the recent review (Murphy *et al.*, 2014)). However, most of the works in animals are performed in already aged subjects and do not evaluate the effect of permanent exposure to phenolics in the lifespan, but in age related diseases.
The activation of cellular anti-oxidant defences has been considered as one of the mechanism by which phenolics act as anti-oxidants. However, the studies only demonstrate associations between phenolics treatments and expression/activities of catalase or SOD. However, multiple works, including ours, show lack of effect. In the present work, the tested phenolics failed to alter SOD and catalase activities in the yeast

model and also on tumor cell lines. Therefore, the biological effects of phenolics seem to be independent of the activities of SOD and catalase. However, the available scientific data points to the induction of Nrf2 translocation to the nucleus by phenolic compounds. Therefore, phenolics are able to induce a cellular adaptive response. The transcription factor Nrf2 is one of the main regulators of γ-glutamyl cysteine synthetase gene, which encodes for the enzyme that catalyses the limiting step in the glutathione synthesis *de novo*, and therefore is important for glutathione homeostasis. There are indications that phenolics alter intracellular GSH levels, and in our work we demonstrate that this is not associated with a disruption of the cellular redox environment. Several indications also demonstrate that GSH can be conjugated with phenolics as a cellular detoxification mechanism. In our work we found that the anti-tumoral effect of quercetin was associated to a depletion of intracellular glutathione. In yeast cells in basal conditions, myricetin and pyrogallol caused depletion of intracellular GSH. The mechanism by which the Nrf2 is activated by several phenolics may be associated with GSH depletion. The activation of cellular defence mechanisms may increase the resistance of cells towards oxidative stress, as we observed in yeast cells under oxidative stress. Consistently, myricetin and pyrogallol pre-treatment attenuated the H_2O_2 induced glutathione depletion in yeast cells.

In our work, quercetin effect was independent of H_2O_2 generation in culture medium, in contrast to myricetin. Myricetin anti-tumoral effect *in vitro* seems to be mediated mainly by ROS, since it was abolished by both catalase and SOD. Also, myricetin decreased GSSG levels after 24 h. The anti-tumoral effect was associated with decrease in intracellular levels of glutathione without affecting intracellular redox environment. Quercetin and myricetin did not affect cellular catalase and SOD activities and the thioredoxin system. Therefore, the anti-tumoral effect of quercetin and myricetin was independent of an induction of anti-oxidant defences in tumor cells and glutathione depletion was the main cellular response to these compounds.

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Appendix

List of abbreviations

ABAP - 2,2´-azobis(2-amidinopropane) dihydrochloride

ABTS - 2,2`-azinobis(3-ethylbenzothiazoline)-6-sulfonate

AMPK-α - 5´AMP-activated protein kinase

AP-1 - activator protein 1

ARE – anti-oxidant responsive element

ASK1 - apoptosis signal-regulated kinase-1

ATP – adenosine triphosphate

BrdU - 5-bromo-2'-deoxyuridine

CA – caffeic acid

C-EBP-β - CCAAT/enhancer binding protein-β

CK2 - casein kinase 2

CLS – chronological lifespan

CREB - cAMP response element-binding protein

Cy-3-gluc – cyanidin-3-glucoside

DCF – dichlorofluorescein

DHE – dihydroethidium

DHR123 – dihydrorhodamine

DMSO - Dimethyl sulfoxide

DNA - deoxyribonucleic acid

Dp-3-gluc – delphinidin-3-glucoside

DPPH - 2,2-diphenyl-1-picrylhydrazyl

DUSP3 - dual-specific phosphatase 3

EGCG - (-)-epigallocatechin-3-gallate

EGF(R) – epidermal growth factor (receptor)

Erk - extracellular signal-regulated kinases

FBS – fetal bovine serum

FOX - ferrous oxidation xylenol

FRAP - ferric reducing anti-oxidant power

GCG - (+)-gallocatechin-3-gallate

GI - gastrointestinal

GPx – glutathione peroxidase

Grx – glutaredoxin

GSH – reduced glutathione

GSK-3β - glycogen synthase kinase-3β

GSSG – oxidized glutathione

GST - glutathione *S*-transferase

H_2DCF - 2´,7´-dichlorodihydrofluorescein

H_2DCF-DA - 2´,7´-dichlorodihydrofluorescein diacetate

hnRNPA1 - heterogeneous nuclear ribonucleoproteins 1

HRPO – horseradish peroxidase

JNK - c-Jun N-terminal kinases

Keap1 - Kelch-like ECH-associated protein-1

MAPK - mitogen activated protein kinase

MCL1 - myeloid leukemia cell differentiation protein 1

MDM2 - E3 ubiquitin protein ligase

MEME - Minimum Essential Medium Eagle

MKP-1 - mitogen-activated protein kinase phosphatase-1

MPP - 1-methyl-4-phenylpyridinium

Msr - methionine sulfoxide reductase

mTOR - mammalian target of rapamycin

Mv-3-gluc – malvidin-3-glucoside

NAC - n-acetyl cysteine

NADPH - nicotinamide adenine dinucleotide phosphate

NF-κB - nuclear factor kappa B

Nrf2 - Nuclear factor erythroid 2-Related Factor

ORAC - oxygen radical absorbance capacity

PBS - phosphate-buffered saline

PEITC - phenethyl isothiocyanate

PIG3 - p53-inducible gene 3

PIP2 - phosphatidylinositol 4,5 bisphosphate

PIP3 - phosphatidylinositol 3,4,5 triphosphate

PI3K - phosphoinositide 3-kinase

Prx – peroxiredoxin

PKA - cAMP-dependent protein kinase

PKC – protein kinases C

PKG – cGMP-dependent protein kinase

PTEN - phosphatase and tensin homology

PTP – protein tyrosine phosphatases

Ref-1 – reducing factor 1

ROS – reactive oxygen species

RPMI - roswell park memorial institute medium

SHP2 - Src homology 2 domain containing protein tyrosine phosphatase

SOD – superoxide dismutase

SRB - sulforhodamine B

STAT3 - signal transducer and activator of transcription 3

TNF-α - tumor necrosis factor-α

TNPO1 - nuclear import receptor transportin-1

TRAIL - TNF-related apoptosis-inducing ligand

TXNIP - Thioredoxin Interacting Protein

8-OHdG - 8-hydroxydeoxy guanosine