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Involvement of the modulation of cancer cell redox status in the anti-tumoral effect of phenolic compounds

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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 a gastric (AGS) and a colon (Caco-2) 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. Notably, quercetin also decreased glutathione levels in AGS cells. These results suggest that the modulation of redox homeostasis and glutathione depletion contribute to the anti-tumoral effect of quercetin.

Keywords: Phenolics, cancer, redox state.

1.0 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[1]. Indeed ROS have been implicated in signalling through modulation of the redox states of protein kinases and transcription factors[2, 3]. 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[4]. 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 glutathione system, and several anti-oxidant enzymes, such as catalase and superoxide dismutase (SOD)[5]. Growing evidence suggests that cancer cells exhibit high constitutive intrinsic ROS levels in comparison to normal cells, due in part to oncogenic transformation[6], increased metabolic activity, and mitochondrial malfunction[7]. These high constitutive levels of ROS in cancer cells seems to sustain proliferative signalling[8].

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. 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[9]. Population based studies have shown that high dietary intake of fruits and vegetables is associated to a reduced risk of several types of cancer[10], including those located in the gastrointestinal tract, where phenolics can be found in high concentrations and in direct contact with epithelium cells[11]. Being redox 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[12] and their redox potential[13]. 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[14], and interaction with signalling pathways[15] 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 superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and of *o*-semiquinones and *o*-quinones that are usually cytotoxic[16] 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 two human cancer cell lines derived from the gastrointestinal tract: a human adenocarcinoma gastric (AGS) and a colon carcinoma (Caco-2) cell lines. Also, cellular adaptation to alterations in redox homeostasis was studied through measurement of cellular anti-oxidant defences in treated cells.

2.0 Results

2.1 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 (protocatechuic and gallic acid); flavonols (quercetin and myricetin); and anthocyanins [delphinidin-3-glucoside (dp-3-gluc), malvidin-3-glucoside (mv-3-gluc) and cyanidin-3-glucoside (cy-3-gluc)]. To investigate their effect on cellular proliferation, AGS and Caco-2 cells were treated with increasing concentrations of the phenolics for 48 h (**Figs. 1A and B**).

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 the two tumor cell lines, except for quercetin for which Caco-2 were less sensitive, showing an IC50 two fold higher

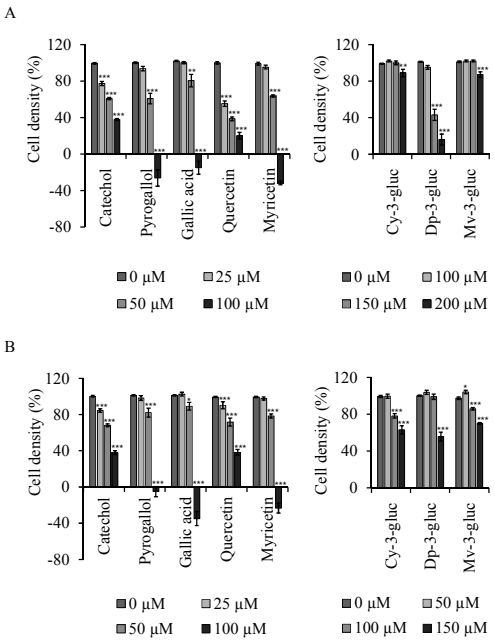


Figure 1. Effect of simple phenols, phenolic acids, flavonols and anthocyanins on (A) AGS and (B) Caco-2 cellular proliferation. Cells were treated with the indicated concentrations (or DMSO; control) for 48 h. Cell density was determined by the quantification of protein in culture through SRB assay and is expressed as percent of treated over control cells corrected for the optical density at time zero. Bars indicates means ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$ compared to vehicle control.

(69.9 ± 5.9 μM) than that observed for AGS (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. 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 both cell lines. Caco-2 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.

2.2 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 growth 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 (**Fig. 2A and B**). Moreover, phenolics generated H_2O_2 in a dose-dependent manner (**Fig. 2C** for

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

pyrogallol, quercetin and myricetin). The assay was specific towards H_2O_2, as it was not detected in samples treated with catalase (**Fig. 2C**). 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 the time with the maximum

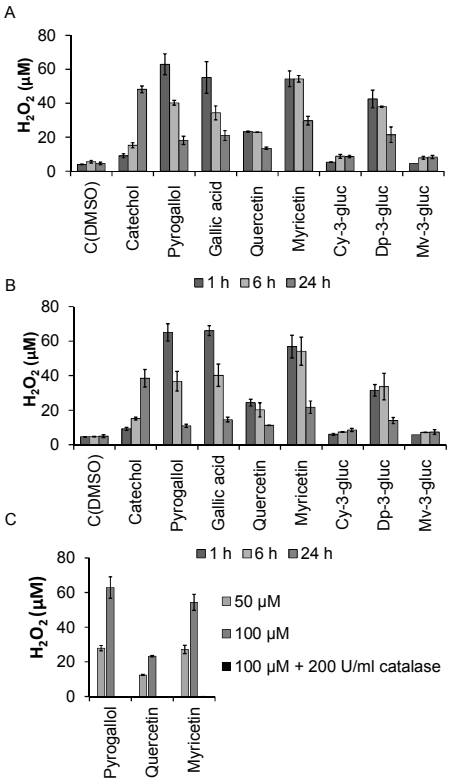


Figure 2. Quantification of H_2O_2 by the FOX assay in (A) RPMI and (B) MEME medium with phenolic compounds. Simple phenols, phenolic acids and flavonols were added at 100 µM and anthocyanins at 200 µM (or DMSO; control) to culture medium without cells and incubated under cell culture conditions. (C) Concentration dependent generation of H_2O_2 by phenolics. Compounds were added at 50 µM and 100 µM to RPMI medium for 1 h. As a control, catalase (200 U/ml) was added to RPMI incubated with 100 µM of phenolics. Bars indicate means ± SEM.

anthocyanins, dp-3-gluc generated the highest levels of H_2O_2 (approximately 31-38 µM) while the levels detected in the case of cy-3-gluc and mv-3-gluc were similar to control. It was also confirmed the reports by other authors[17] that H_2O_2 is not detected in the presence of cells (data not shown).

2.3 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 (**Fig. 3A and B**). 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. 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 (**Fig. 3C and D**). The analysis was carried out with pyrogallol, gallic acid, quercetin, myricetin and dp-3-gluc, since those were detected after 24 h (**Fig. 2A and 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. Oppositely, quercetin displayed lower H_2O_2 levels (close to 24 µM). Among

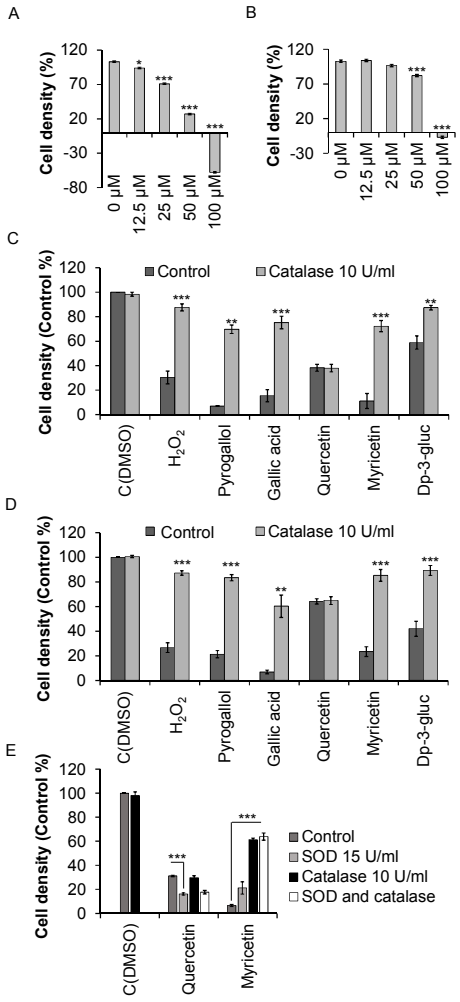
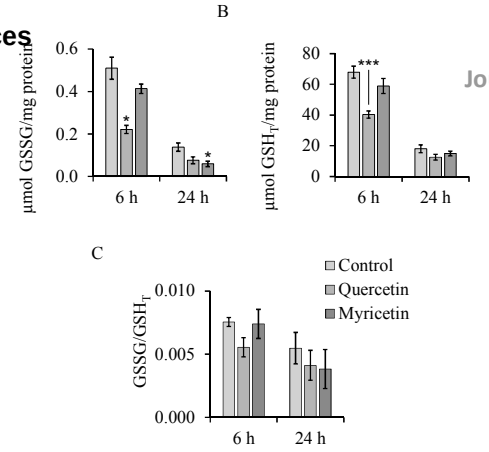


Figure 3. (A, B) Effect of H_2O_2 on (A) AGS and (B) Caco-2 cellular proliferation. Cells were treated with the indicated concentrations for 48 h. (C, D) Effect of catalase on the anti-proliferative effect of phenolics on (C) AGS and (D) Caco-2. Cells were incubated with DMSO (control), 50 µM of H_2O_2, 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. (E) 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. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (treated vs untreated for A and B; catalase and /or SOD vs control for C-E).

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 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

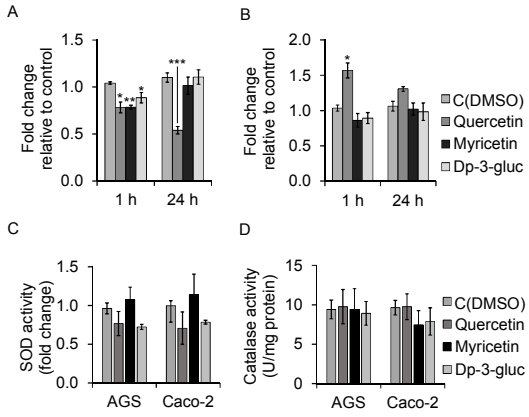


Figure 4. Intracellular ROS levels in (A) AGS treated with quercetin (30 µM), myricetin (60 µM) and Dp-3-gluc (150 µM) and in (B) Caco-2 cells treated with quercetin (60 µM), myricetin (60 µM) and Dp-3-gluc (150 µM). After treatment, cells were incubated with H_2DCF-DA for 30 min and PI was added before fluorescence analysis by flow cytometry. Bars indicate means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with the respective time control. Effect of phenolic compounds on (C) SOD and (D) catalase activity. Cells were treated for 24 h with the IC50 of phenolic compounds. SOD activity was determined in situ, as indicated in the section 2.6, 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.

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.

2.4 Effect of phenolic compounds in intracellular ROS levels

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 (**Fig. 4A and 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. Interestingly, quercetin had the opposite effect in Caco-2 cells, which demonstrated transiently higher levels of intracellular ROS (only observed at 1 h). Altogether, this data shows that the generation of H_2O_2 by auto-oxidation of phenolics in culture medium is not correlated to the increases in intracellular ROS. Indeed, although myricetin generated high levels of H_2O_2 in culture medium, AGS and Caco-2 cells treated with myricetin for 1 h and 24 h showed intracellular ROS levels similar to control.

2.5 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[5]. To assess the impact of phenolics on anti-oxidant defences, the activity of SOD, which converts superoxide radicals into H_2O_2, and catalase, which decomposes H_2O_2, 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 (**Fig. 4C and D**), indicating that phenolics did not induce an oxidative stress response leading to activation of these cellular anti-oxidant defences.

2.6 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*-transferases[18]. The ratio between glutathione disulfide (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 (**Fig. 5A and B**). Myricetin had minor effects on glutathione levels, with a small

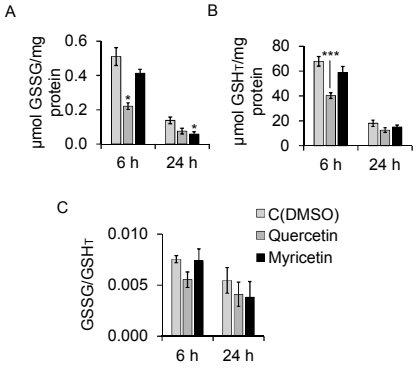


Figure 5. Effect of quercetin and myricetin on glutathione levels. AGS cells were treated for the indicated times with the IC50 of quercetin or myricetin. Cells were harvested and glutathione levels was determined as described in methods. (A) glutathione disulfide; (B) total glutathione; (C) ratio between glutathione dissulfide and total glutathione levels. Bars indicate means ± SEM. *$p < 0.05$; ***$p < 0.001$ compared with the respective time control.

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 (**Fig. 5C**). These results indicate that quercetin decreases glutathione levels without changing the GSSG/(GSH+GSSG) 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[19].

3.0 Discussion

The cancer protective effects of plant based diets have been associated to their high content in phenolic compounds[9]. However, several studies suggest that some phenolics can auto-oxidize in culture medium producing ROS which in turn will mediate the anti-proliferative effects[17, 20, 21]. 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[22]. 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[23]. 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. These compounds 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 H_2O_2 cytotoxicity. In contrast to gallic acid, protocatechuic acid did not have anti-proliferative effect. Accordingly, in other studies, gallic acid also decreased cell viability in Caco-2 cells, while protocatechuic acid did not show any effect[24]. We also found that 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. 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[12].

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 antioxidants to culture medium, such as catalase, SOD, *N*-acetyl-L-cysteine and GSH[25-27]. For instance, catalase reverted the anti-proliferative effect in Caco-2 cells and WB-F344 rat liver epithelial cells treated with gallic acid[20] and in CAL-27, a human tongue squamous carcinoma cells, treated with epigallocatechin gallate[21]. 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. This could be an indication that quercetin is being stabilized by SOD in culture medium as previously described for (-)-epigallocatechin-3-gallate (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[25]. 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 gastrointestinal tract can be

exposed to high levels of H_2O_2[28] and gut microbiota may also induce the generation of ROS[29]. Inflammation and cancer are also conditions associated with high levels of ROS. Importantly, human subjects presented high levels of H_2O_2 in saliva after chewing green tea[30].

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[31]. Also, treatment with cyanidin-3-rutinoside caused ROS accumulation in leukemic cells (HL-60) but decreased its accumulation in normal cells (PMBC)[32]. 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, which was the time point where the highest levels of H_2O_2 were detected for most compounds (except for catechol). 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. This data indicates that phenolics are not inducing oxidative stress, however, the possible modulation of signalling pathways by phenolics 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[33].

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[34, 35]. In agreement with our results, several flavonols, including myricetin and quercetin, did not change catalase and SOD activities in Caco-2 and HepG2 cells[36].

In this study, Caco-2 cells treated with quercetin for 1 h showed higher levels of intracellular ROS while in AGS cells treated for 1 h and 24 h with quercetin showed lower levels of intracellular oxidation. This difference may be associated to the higher IC50 of quercetin in Caco-2 cells comparing with AGS cells. Several studies implicate moderate ROS levels on proliferative signalling[37] and, therefore, decreasing ROS levels results in a down-regulation of proliferative signalling pathways[8]. On the other hand, increasing even more ROS levels can cause cell cycle arrest or, above a critical threshold, result in the activation of apoptosis[38]. Assuming that higher concentrations of phenolics are more prone to have pro-oxidant effects, quercetin maybe inducing apoptosis in Caco-2 cells, whose quercetin IC50 is higher, but it may block ROS-mediated proliferative signalling in AGS cells, in which quercetin IC50 is lower.

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[39] and of 40 μM in AGS cells[40]. Myricetin was also reported to have anti-tumoral activity in several tumor cell lines, including HCT116[41], HepG2[42], PC-3[43], among others. Generally the concentrations used to inhibit tumor cell proliferation are much higher for myricetin when compared with quercetin.

Apart from extracellular oxidation, quercetin can be oxidized inside cells[44, 45] forming *o*-semiquinones, *o*-quinones and ROS. Those quercetin metabolites can be further methylated and conjugated with glutathione[46, 47] and extruded from cells, depleting intracellular glutathione levels without causing glutathione disulfide formation[48]. The glutathione quercetin adduct was identified in the human plasma in one work[49] 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[50], including quercetin in human leukemia cell lines[51]. However, several data under different experimental conditions also showed that quercetin and myricetin could increase intracellular levels of reduced glutathione in MCF-7 human breast cancer cells[52]. There are also indications that phenolic compounds can modulate transporters involved in the extrusion of glutathione, such as MRP1/2, in HeLa cell lines[53] and modulate the activity of enzymes involved in the synthesis and metabolism of glutathione in intestinal and breast cancer cell lines[50, 52]. The modulation of those enzymes depends on the cell type, phenolics structure, concentration, treatment time and experimental design (cells in exponential growth *vs* cells in confluence; tumoral or non-tumoral cells subjected to stress conditions or under normal conditions).

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 superoxide generation. In contrast with the other phenolics tested, quercetin modulated the intracellular oxidation status by mechanisms independent of changes in the activity of SOD or catalase but associated with glutathione depletion in AGS cells. The overall results suggest that the modulation of redox homeostasis and glutathione depletion contribute to the anti-tumoral effects of quercetin.

4.0 Experimental

4.1 Phenolic compounds

Quercetin, myricetin, pyrogallol, gallic acid, protocatechuic 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 dimethyl sulfoxide (DMSO; Fluka, Madrid, Spain) at a stock concentration of 200 mM and aliquots were stored at -20ºC.

4.2 Cell lines and growth conditions

A human colorectal carcinoma cell line, Caco-2 cells [HTB-37 from American Type Culture Collection (ATCC)], was 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. A gastric adenocarcinoma cell line, AGS cells (CRL-1739 from ATCC), was 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% CO2. For all experiments, 24 h before treatment, AGS and Caco-2 cells were seeded at a cell density of 2.42 x 10^4 cells/cm^2 and 4.84 x 10^4 cells/cm^2 respectively. All cell culture medium components were obtained from Sigma-Aldrich (Madrid, Spain).

4.3 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)[54]. The treatment conditions were as follows: Caco-2 and AGS were seeded in 96 well plates (100 µl/well) and after 24 h cells were treated with increasing concentrations of phenolics (or DMSO as control). In the case of co-incubations with enzymatic antioxidants, 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.

4.4 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[55]. 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.

4.5 Intracellular ROS levels

The oxidant-sensitive probe 2´, 7´-dichlorodihydrofluorescein (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) at the respective IC50 concentration. 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.

4.6 SOD and catalase activities

Cells were seeded at 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[56]. SOD activity was determined in situ after native gel (10% acrylamide) electrophoresis using 20-30 µg of protein, as previously described[57]. Bands intensities were measured using QuantityOne software with local background subtraction. Catalase activity was measured spectrophotometrically by following the decomposition of H_2O_2, as previously described[58].

4.7 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 the other data were compared by Student´s *t*-test.

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Abbreviations: cyanidin-3-glucoside (Cy-3-gluc); delphinidin-3-glucoside (Dp-3-gluc); dimethyl sulfoxide (DMSO); ferrous oxidation in xylenol orange (FOX); fetal bovine serum (FBS); glutathione dissulfide (GSSG); half maximal inhibitory concentration (IC50); hydrogen peroxide (H_2O_2); malvidin-3-glucoside (Mv-3-gluc); Minimum Essential Medium Eagle (MEME); nicotinamide adenine dinucleotide phosphate (NADPH); phosphate buffered saline (PBS); propidium iodide (PI); reactive oxygen species (ROS); reduced glutathione (GSH); Roswell Park Memorial Institute medium (RPMI); sulforhodamine B (SRB); superoxide ($O_2^{\cdot-}$); superoxide dismutase (SOD); 2′, 7′-dichlorodihydrofluorescein (H_2DCF-DA).

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