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ARTICLE TYPE

Dietary gallic acid and anthocyanins cytotoxicity on human fibrosarcoma HT1080 cells. A study on the mode of action.

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Gallic acid and anthocyanins are abundant plant food bioactives present in many fruits and vegetables, being especially important in the composition of berries. Gallic acid has been shown to possess cytotoxic properties in several cancer cell lines and to inhibit carcinogenesis in animal models. However, its mechanism of action is not yet fully understood. The aim of this study was to elucidate whether the observed inhibitory activity of gallic acid against gelatinases corresponds to its cytotoxic activity in HT1080 cells and to determine if anthocyanins could have a similar behavior. Gallic acid and delphinidin-3-glucoside have shown selective cytotoxicity towards HT1080 cells. Further analysis in a migration and invasion assay showed anti-invasive activity of gallic acid, delphinidin and pelargonidin-3-glucosides. Zymographic analysis demonstrated the inhibitory activity of gallic acid at the level of secreted and activated gelatinases. Moreover, gallic acid inhibited MMP-2 and MMP-9 proteolytic activity with very similar potency. NMR and molecular modelling experiments confirmed the interaction of gallic acid with MMP-2, and suggested that it takes place within the catalytic center. In this work we give some new experimental data supporting the role of those compounds in the inhibition of metalloproteases as the mechanism for its cytotoxic activity against fibrosarcoma.

Abbreviations

ECM-extracellular matrix; MEM-Minimum Essential Medium; DMEM- Dulbecco's Modified Eagle's Medium; FBS-fetal bovine serum;MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS- phosphate buffer saline; DMSO- dimethyl sulfoxide; DTNB- 5,5'-dithiobis-(2-nitrobenzoic acid).

Introduction

Polyphenols are natural compounds found in plants which are the focus of increasing interest as primary chemopreventive agents. Chemoprevention is an approach used to avoid cancer formation and cancer progression.[1] Polyphenols can be classified by the number and arrangement of their carbon atoms and are commonly found conjugated to sugars and organic acids. They include: flavonoids, phenolic acids, phenolic alcohols, stilbens and lignans.[2, 3] In the flavonoid family, there is a group of natural pigments, anthocyanins, which are responsible for the red-blue colour of many fruits and vegetables. They are polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium and, most of them, are present in plants attached to sugars as mono-, di- or triglycosides, by α- or β- linkage, most frequently at C-3 of the aglycons (anthocyanidins). The effectiveness of anthocyanins in preventing or treating a range of diseases depends on their bioavailability. Our group was recently investigating the enzymatic potential of selected bacterial strains for bioconversion of selected anthocyanins to their active metabolites. It was shown that delphinidin-3-glucoside was undergoing chemical degradation to form mainly gallic acid, a phenolic acid whose role in chemoprevention is nowadays extensively investigated.[4] Besides, gallic acid is present in considerable amounts in a fruit and vegetable rich diet and, as mentioned before, can also be produced as a degradation product or metabolite from other polyphenols. Some authors have estimated its total dietary intake to be close to 1 g per day.[5]

The invasion of malignant tumors is related to several processes such as cell metastasis, proliferation and degradation of extracellular matrix (ECM) proteins. Degradation of ECM is often accompanied by over-expression of proteolytic enzyme activity, including matrix metalloproteinases (MMPs), for example MMP-2 (72 kDa) and MMP-9 (92 kDa) which are known to play a pivotal role in tumor invasion and metastasis. Their expression is increased in many malignant tumours,[6-9] among them in human fibrosarcoma.[10] It has been reported that gallic acid exerts a protective effect against cancer in many cell lines such as leukemia,[11-13] prostate cancer,[14-16] lung cancer,[17, 18] melanoma[19, 20], gastric cancer,[21] glioma,[22] osteosarcoma,[23] colon, breast, cervical and esophageal cancer.[24, 25] Frequently its anti-invasive and anti-metastatic potential is based on the inhibition of MMP-2 and MMP-9 expression and/or activities.[20] In previous studies gallic acid did not show any significant anti-invasive effect in human fibrosarcoma cell line (HT1080).[26] However, the significant effect of gallic acid in other cell lines encouraged us to check its ability to inhibit the invasion in HT1080 cells. On the

other hand, an *in vitro* assay showed that delphinidin chloride inhibited HT1080 invasion and slightly decreased MMPs activity.[27] However, there are no reports on the effect of the anthocyanidin glucosides in this type of cells.

In previous studies, the direct inhibitory activity of a series of phenol fragments including gallic acid against different metalloproteinases was reported, but no proof of their binding to the active sites of those enzymes was provided. [28, 29] In this work, a mechanism for the inhibitory activity of gallic acid against MMP-2 and MMP-9 by NMR and molecular modelling techniques is proposed.

Materials and methods

Materials.

All reagents were obtained from Sigma Aldrich (Madrid, Spain) unless otherwise stated and were of analytical or HPLC grade where applicable. Water was purified via a Milli Q plus system (Millipore, Bedford, MA). The 3-glucosides of delphinidin, cyanidin, peonidin, pelargonidin and malvidin were obtained from Polyphenols Laboratories AS (Sandnes, Norway).

Cell culture

HT1080 (Human fibrosarcoma cell line) and 3T3 (Mouse embryonic fibroblasts cell line) were maintained as monolayer cultures in MEM (Gibco), and DMEM (Lonza) medium, supplemented with 10% heat-inactivated FBS (Lonza) and penicillin/streptomycin (100 IU/mL/100µg/mL). The growth inhibitory effect of compounds was evaluated by using a MTT assay. Briefly, cells were seeded into 96-well plates (3000 cells/well HT1080, 1500 cells/well NIH 3T3) and allowed to adhere 24 h prior to addition of the compounds. The cells were then washed twice with PBS, and treated with various concentrations of compounds (diluted in medium without FBS) for 36 h. Four hours before the end of incubation 20 µL of MTT solution (5 mg/mL) was added to each well. Formazan crystals in the wells were solubilized in 200 µL of DMSO (Panreac). Absorbance was measured at 570 nm wavelength by a spectrophotometer (Biotek). The assay was repeated in 3 independent experiment replications. The viability was calculated considering controls containing solvent control (0,1% DMSO) as 100% viable. Data represent the mean and standard deviation of three independent experiments.

Gelatin zymography

The effect of gallic acid and anthocyanins on MMP-2 expression was determined by zymography. Cells were seeded in 24-well plates at a density of $0{,}2*10^5$cells/well and cultured for 24 h. Then the cells were washed twice with PBS, and treated with non-cytotoxic concentrations of the tested compounds diluted in FBS-free medium, and incubated for 36 h. After incubation the conditioned medium was collected and the protein content of each sample was determined by the Bradford method.

Cell-conditioned medium from HT1080 containing equal amounts of total protein in each sample were subjected to substrate gel electrophoresis in non-reducing conditions. After electrophoresis the gels were washed twice with 2.5% Triton X-100 and incubated overnight in a developing buffer containing 50 mM Tris-HCl pH=7.5, 5 mM $CaCl_2$, 0.2M NaCl, 0.02% Brij-35 and 1µM $ZnCl_2$. The gels were stained with 0.1% Coomassie Blue R-250 in 40% methanol and 10% acetic acid and destained in the same solution without the Coomassie Blue dye. The bands were observed as clear zones in blue background. Densitometric analysis was performed using NIH ImageJ software (Bethesda, MD).

Matrix metalloproteinase activity inhibition

MMPs activity measurements were performed by using recombinant catalytic domains of MMP-2 and MMP-9 purchased from *Enzo Life Science International, Inc.*(Lause, Switzerland), using a colorimetric method. Proteolytic activity was measured using a thiopeptide substrate (Ac-PLG-[2-mercapto-4-methylpentanoyl]-LG—OC_2H_5) where the MMP cleavage site peptide bond was replaced by a thioester bond. [30,31] Hydrolysis of this bond by MMP produces a sulfhydryl group that reacts with DTNB to form 2-nitro-5-thiobenzoic acid, which was detected by its absorbance at 414 nm (microplate photometer Thermo Scientific Multiscan FC, Thermo Scientific, Madrid, Spain). Enzyme reactions were carried out at 37ºC in a 100 µL final volume, where the catalytic domains of the corresponding MMP were incubated in triplicate with at least seven concentrations of inhibitors. The assay buffer contained the following components: 50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35 and 1mM DTNB at pH 7.5. After addition of the substrate, the increase of absorbance was recorded in 1 min time intervals for 30 min. Data were plotted as OD versus time for each sample, in order to obtain the reaction velocity (V) in OD/min. The percentage of residual activity for each compound was calculated using the following formula: % of remaining activity = (V in the presence of inhibitor/V control) x100. An inhibitor, NNGH, was included as a prototypic control inhibitor.[32] The concentration of compound providing 50% of enzymatic inhibition (IC_{50}) was determined by semi-logarithmic dose-response plots (*Graph Pad Prism 5.0 for Windows*, Graph Pad Software Inc., San Diego, California, 2007).

Invasion assay.

To evaluate the anti-invasiveness of compounds, a fluorimetric QCM ECMatrix Cell Invasion Assay (Merck Millipore, Billerica, MA) was used, following the protocol of the manufacturer with minor modifications. The assay was performed in a 96-well invasion plate based on Boyden Chamber principle. HT1080 cells were starved overnight in FBS-free medium, followed by harvesting and placing $0.1*10^6$ of cells re-suspended in FBS-free medium (containing 0,5% BSA) in each well. The lower portion of the chamber contained medium with 10% FBS as a chemoattractant. Compounds were added to the cell suspension in triplicate. Cells were incubated in the absence (solvent control 0.1% DMSO) and presence of the compounds for 22 h. Cells able to invade through a layer of basement membrane matrix solution and cross the pores of the polycarbonate membrane were dissociated from the membrane and detected with CyQuantGR Dye. The fluorescence was read with a fluorescence plate reader at 480 nm/520 nm (Synergy Mx, BioTek).

NMR study

Spectra were recorded at 300 K with a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm cryogenically-cooled

TCI probe. The NMR sample contained a concentration of 5 µM MMP-2 and 100 µM gallic acid (from a 50 mM stock in H_2O), in a protein:ligand ratio of 1:20 optimal for the WaterLOGSY experiments. The NMR buffer consisted of 450 µL 10 mM Tris/HCl pH 7.4 with 50 mM NaCl, 0.02% NaN_3, 100 µM $CaCl_2$, 100 µM $ZnCl_2$ and 25 µL of D_2O. The gallic acid reference sample was prepared as above but without the incorporation of MMP-2. WaterLOGSY experiments were recorded with 8 K points, a sweep width of 9,600 Hz and a total of 1,024 scans.

Computational study.

The protein structures were obtained from the protein databank (PDB code: 1HOV for MMP-2 and 2OW1 for MMP-9). Each protein was prepared using the protein preparation wizard provided in maestro suite [33] The docking grids were settled with GLIDE software[34, 35] also from maestro package. The "add metal state" option was chosen to prepare coordinations of the catalytic zinc ion. In a first attempt, the full catalytic domain (missing the three fibronectin type II (FnII)-like inserts, as in 1HOV) was considered for docking, in order to check if the compounds were predicted to bind outside the catalytic center. Then, the grid was limited to the catalytic center of each gelatinase and docking calculations were done using XP docking, that provides a more precise sampling protocol and a more demanding scoring function [36]. After MM minimizations, using default options in MacroModel [37], the ligand (gallic acid) was prepared using LigPrep application[38]. Gallic acid was considered in its carboxylate form. Epik penalizations for metal binding state were added. For docking, the default GLIDE parameters were used, adding a penalty for non-planar conjugated π-systems.

The poses were classified according to their XP (extra precision) docking scores and studied considering geometrical criteria. Default geometrical parameters from maestro were taken for H-bonds (For a H-bond donor-H…acceptor-X, where X is the heavy atom bearing the donor atom: distance hydrogen/acceptor ≤ 2.5 Å, angle donor-H…acceptor > 120°, angle H…acceptor-X > 90°).

For the docking with a water molecule in the zinc coordination sphere, the initial structure was taken from a previous MD simulation of the MMP-2 catalytic domain [39, 40]. The protein was prepared as previously, and the water molecule was considered as taking part of the protein during the grid preparation. For the docking of two gallic acid molecules simultaneously bound to the active site of the enzyme, the following strategy was applied. The most energetically favored pose for the docking of gallic acid (one molecule) was taken to construct a new grid, including the gallic acid molecule. Using this grid, the docking was repeated. From the best pose, the rigid gallic acid molecule (included in the previous grid) was removed, whereas the other molecule was kept and considered to build the second grid. From this grid, the final binding pose was obtained, after a new docking process. In that way the "movement and flexibility" of both gallic acid molecules can be simulated.

Statistical analysis

Data are presented as means ± standard deviations (STDEV) of between three and five independent experiments performed in duplicate.

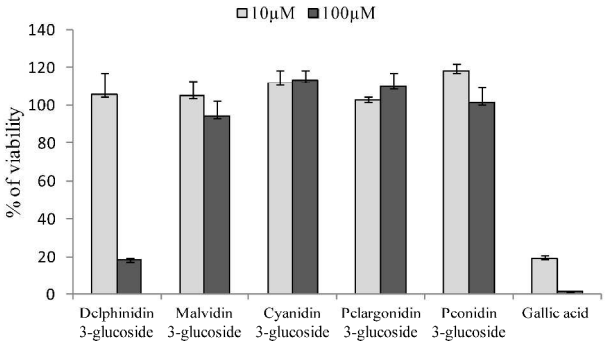

Fig.1 Effect of selected polyphenols on viability of HT1080 cells. Cells were treated with 10 and 100 µM of compounds for 36 h. The viability was measured with MTT assay. Data are the means ± STDEV of the three independent experiments performed in triplicate.

Results and discussion

Effect of anthocyanins and gallic acid on cell viability.

Dietary polyphenols are widely known as effective phytochemicals for cancer prevention. Gallic acid has been described to produce a loss of cell viability in leukemia cell lines (20% in K562 and 10% in KU812 at 10 µM) and glioma (30% in U87 and 19% U251n at 118 µM).[13, 22]

In order to investigate its cytotoxic activity towards HT1080 cells we exposed them with gallic acid (3,4,5-trihydroxybenzoic acid) for 36 h in concentrations of 10 and 100 µM (Fig. 1). As the cell viability in presence of 100 µM gallic acid was dramatically decreased, the purpose of further investigation was to determine at which concentration it is not cytotoxic.

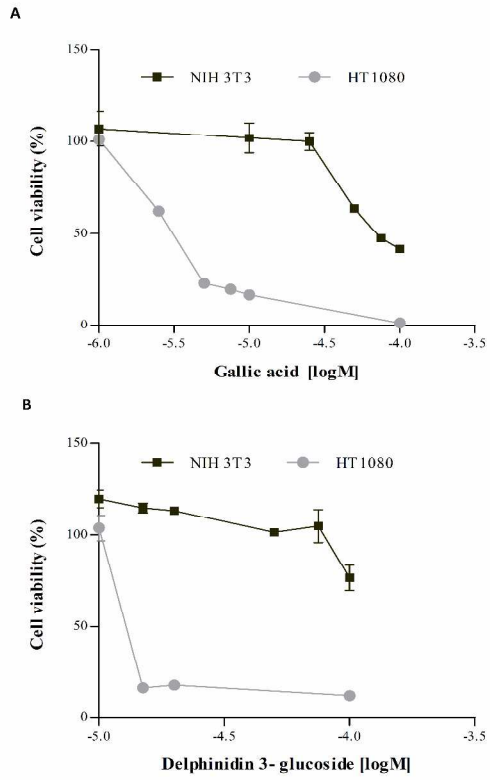

Fig.2 Effect of gallic acid (A) and delphinidin 3-glucoside (B) on cell viability of HT1080 and NIH 3T3 cells. MTT assay was used to assess the growth of cells in culture.

As showed in Figure 2A, gallic acid inhibited fibrosarcoma cells viability above 1 μM. Our results showed that gallic acid reduced HT1080 cell viability in a higher level than in reported previously in other cancer cell lines[13, 22] and the effect was dose-dependent. Moreover, gallic acid displayed 10-fold selectivity towards cancer cells. Accordingly, while it was cytotoxic up to 2.5 μM on HT1080 cells, on normal mouse fibroblasts it did not show any effect on cell viability below 25 μM.

On the other hand, anthocyanidins also have been shown to inhibit the proliferation of many human cell lines such AGS (stomach), HCT-116 (colon), MCF-7 (breast), NCI H460 (lung), and SF-268 (Central Nervous System, CNS).[41] For example, delphinidin, one of the major anthocyanidins present in red fruits and vegetables, cause a dose-dependent inhibition of cell growth of human prostate cancer cell lines such as PCa LNCaP, C4-2, 22Rv1, and PC3, without having any substantial effect on normal human prostate epithelial cells.[42] The results mentioned above and the reported activity of delphinidin chloride in HT1080 cell line,[27] prompted us to undertake studies about the role of 3-glucosides of anthocyanidins in regulating cell viability of this cancer cell line. Among the five tested anthocyanins (Fig.1), only delphinidin-3-glucoside affected HT1080 cell viability at concentrations higher than 10 μM, while displaying only 20% decrease in cell viability of normal mouse fibroblast cells at 100 μM (Fig. 2B). These results provide evidence this compound also posess selective dose-dependent cytotoxicity for fibrosarcoma cells.

Invasion assay.

Gallic acid was described to exert in vitro inhibitory effect on the invasiveness in several human cell lines such as: glioma U87 and U251n,[22] leukaemia K562 and KU812,[13] melanoma A375.S2,[20] osteosarcoma U-2 OS[23] and gastric carcinoma AGS cells.[21] Based on those results we decided to investigate whether gallic acid and anthocyanins were able to inhibit cell invasion. Inhibition was performed in HT1080 cells at non-cytotoxic concentrations, using a fluorimetric *QCM ECMatrix Cell Invasion Assay* (Millipore). Gallic acid showed reduction of cell invasiveness to 54 ± 14% of the solvent control value at 1 μM, what in comparison to a commercially approved MMP inhibitor- doxycyline hyclate (50% of control activity at 80 μM)[43] is a promising result and is in accordance with the previously reported activity in other cancer cell lines.

Similarly, there is scientific evidence that anthocyanins exert *in vitro* anti-invasive and *in vivo* anti-metastatic activities. For example, cyanidin 3-glucoside and cyanidin 3-rutinoside inhibited cell migration and invasion of lung cancer cells A549 in a dose dependent manner.[44] As mentioned before, delphinidin chloride inhibited cell invasion of fibrosarcoma cells, so it is interesting to check if the corresponding 3-glycoside displays similar activity, especially considering that anthocyanins are only found in their glycosylated form in physiological conditions.[27] From the anthocyanin compounds only delphinidin-3-glucoside and pelargonidin-3-glucoside exerted some anti-invasive effect with maximum activity with maximum activity at 10 μM for delphinidin 3-glucoside (69 ± 0.44% of solvent control) and at 100 μM for pelargonidin 3-glucoside (69 ± 14% of solvent control).

Inhibition of secretion and activation of gelatinases.

Gelatinases have been reported to be involved in the invasive potential of tumor cells, and numerous reports show a connection between the inhibition of expression and activity of MMPs, and the prevention of cancer invasion. The activation of MMPs takes place through several mechanisms, such as activation through uPAR (the urokinase plasminogen activator receptor)[45, 46] and is controlled by tissue inhibitor metalloproteinases (TIMPs).[47] The transcription level is also regulated by various transcription factors, by the MAPK and PI3K/Akt/mTOR pathway[48-50] and by several stimulators such as growth factors, cytokines and phorbol-12-mirystate-13-acetate (PMA).[25, 51-54]

Recently, indirect role of gelatinases in cell signaling has been discovered, based on their ability to process and release bioactive molecules that target receptors involved in the regulation of cell growth, migration, inflammation and angiogenesis. Their role in facilitating tumor cell invasion and angiogenesis is based, not only on the degradation of ECM structural elements, but also on the release of cytokines, growth and angiogenic factors such as VEGF, TGF-β, bFGF among others.[55] It was previously reported that gallic acid[20, 23] and delphinidin chloride[27] possess some anti-tumor functions in human cancer cell lines through inhibition of the signalling pathways of gelatinases. Based on the results of invasion assay, we have selected gallic acid, delphinidin 3-glucoside and pelargonidin 3-glucoside to study their ability to affect the activation and secretion of MMP-2 and MMP-9 in the conditioned medium of HT1080 cells. For secretion analysis we treated fibrosarcoma cells in FBS-free medium with gallic acid (0.1-1 μM), delphinidin (0.1-10 μM) and pelargonidin 3-

glucosides (5-100 µM) for 36 h, following the analysis of gelatinolytic activity of the enzymes in conditioned medium.

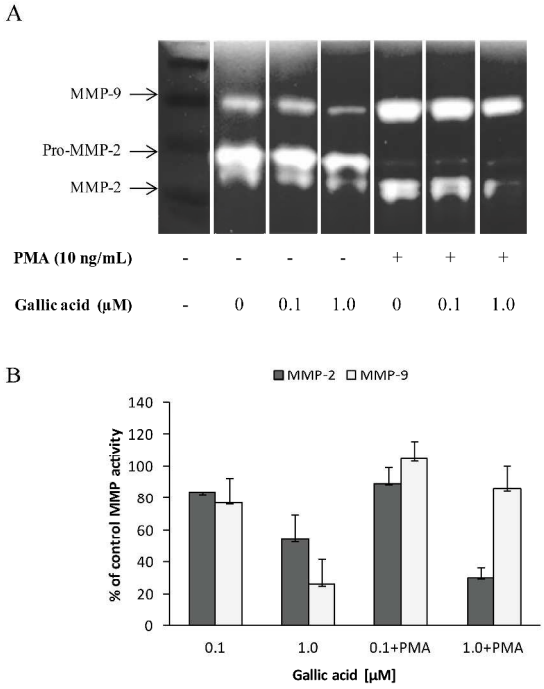

Fig. 3 Effect of gallic acid in the presence and absence of PMA on the activity of MMP-2 and MMP-9 in HT1080 conditioned medium determined by gelatine zymography (A). The densitometric analysis of gelatine digested bands done with ImageJ of data obtained from two independent experiments (B).

We treated the cells at non-toxic concentrations, and the analysis of cytotoxicity was performed in the same conditions, using FBS-free medium to ensure that the observed effect was not caused by a decrease in the viability of the cells. As presented in Figure 3, gallic acid decreased the level of secreted MMP-2 to 54% at 1µM, whereas MMP-9 decreased it to 27% at the same concentration. Similarly, in order to investigate the effect of gallic acid on the level of activated MMP-2/-9, we performed experiments in the presence of PMA (Phorbol 12-myristate 13-acetate), an activator of gelatinases. In this assay, the level of MMP-2 was decreased to 30% by 1 µM gallic acid, while MMP-9 level was 86% of the control activity. These results demonstrate that the anti-invasive activity of gallic acid can be associated to the inhibition of gelatinases. However, it is worth mentioning that in fibrosarcoma cells the activity of gallic acid was effective at much lower concentrations than in other cell lines.

By contrast, delphinidin and pelargonidin 3-glucosides did not display any significant effect on MMP-2/-9 secretion or activation. Although it is described that delphinidin slightly inhibited the activities of MMP-2/MMP-9 in fibrosarcoma cells,[27] we did not notice this effect in the glycosylated derivative.

***In vitro* inhibitory activity of compounds on recombinant MMP-2/-9.**

As flavonoids are known to have an influence on MMPs activities at different levels, we decided to study if gallic acid is able to inhibit directly proteolytic activity of MMP-2 and MMP-9 by a colorimetric method. The recombinant catalytic/fibronectin domains of MMP-2/-9 were incubated for 45 min with gallic acid, followed by addition of 100 and 50 µM peptide substrate, respectively. The reaction was monitored at 414 nm.

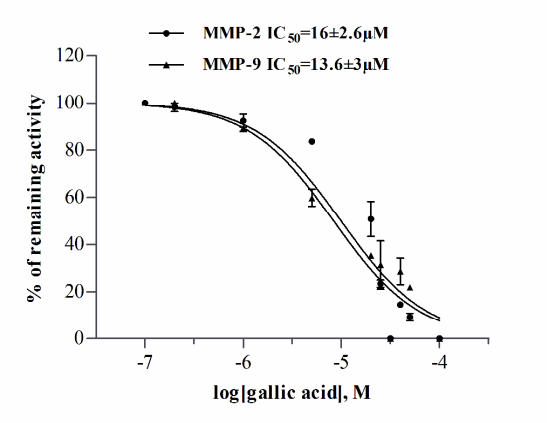

Fig. 4 Inhibition of MMP-2 and MMP-9 activity by gallic acid determined by a colorimetric method. Data are the means ± STDEV of the three independent experiments performed in triplicate.

The concentration of compound that provided 50% inhibition of enzymatic activity (IC_{50}) was determined by semi-logarithmic dose-response plots (Fig. 4). Gallic acid showed IC_{50} values of 16.0 ± 2.6 µM for MMP-2 and 13.6 ± 3.0 µM for MMP-9. This result is not in full accordance with literature, where reported activity of gallic acid for MMP-2 was much higher (IC_{50}= 2.4 ± 1.5 µM) and demonstrated selectivity towards MMP-8, 9, 14 (MMP-8/MMP-2=15, MMP-9/MMP-2=9.6, MMP-14/MMP-2= 21).[28] The observed difference could come from using proteins of different length and different substrates for analysis.

We selected also the compounds that showed anti-invasive activity in our previous experiments (delphinidin and pelargonidin 3-glucosides), to carry out tests of direct inhibition of recombinant MMP-2 and MMP-9. In the case of anthocyanins, due to the interferences of the colorimetric substrate with the anthocyanic solution colour, we decided to evaluate their effect on gelatinolytic activity by zymographic method, using catalytic/fibronectin domains of MMP-2/-9, but we did not observe any inhibition (data not shown), suggesting absence of this inhibition mechanism. Interestingly, aglycones of these compounds have been previously described to exert direct inhibition against full-length MMP-2 and MMP-9 with IC_{50} of 3 µM and 200 µM for MMP-2, and of 13 µM and 30 µM for MMP-9: (delphinidin and pelargonidin, respectively),[56] in contrast with our results for the corresponding glycosides.

NMR studies and molecular modeling.

As mentioned above, the inhibition profiles of a group of phenols, including gallic acid against MMP-2, -8, -9 and -14 was studied, in order to discover a new zinc binding group that could be derived from natural polyphenols. It was suggested that phenols with hydroxyl- and amino groups in *ortho*- and *para*-position were effective MMP inhibitors, although the mechanism of binding to MMPs was not clarified.[28] To the best of our knowledge there is no evidence of binding of gallic acid to the catalytic domain of the MMP-2, therefore we decided to explore the mode of binding of this compound by means of molecular

modelling and NMR methodologies.

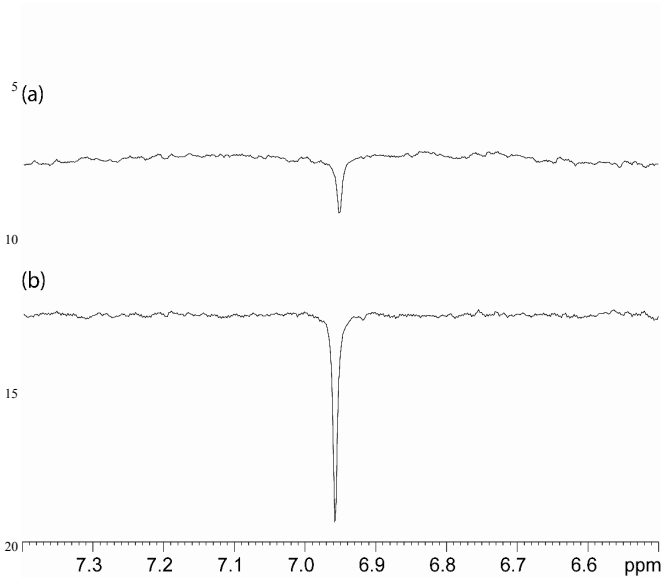

Fig. 5 WaterLOGSY-NMR experiment to determine intermolecular binding between MMP-2 (5 µM) and gallic acid (100 µM). The spectra show an expansion of the aromatic signal from gallic acid in the presence of the protein (Spectrum a), and in the absence (Spectrum b).

The interaction between MMP-2 (the catalytic domain missing the three FnII-like inserts) and gallic acid was investigated via WaterLOGSY-NMR experiments.[57] Positive intermolecular binding is determined by the observation of positive NMR signals from the small molecule or by a reduction of the negative signals compared to a reference WaterLOGSY acquired in the absence of the biomolecule. The latter case was observed in the MMP2-gallic acid experiment, confirming the interaction between both molecules. Figure 5 shows and expansion of the aromatic signal in the NMR spectra of gallic acid. In the presence of the protein (Spectrum a), the signal shows a less pronounced negative intensity compared to the reference experiment acquired under the same conditions, but in absence of MMP-2 (Spectrum b), therefore indicating that both molecules interact.In accordance with the colorimetric assays, the positive result showed that gallic acid binds this MMP-2 structure in a reversible manner.

As shown above gallic acid directly inhibits the proteolytic activity of MMP-2 and MMP-9 catalytic domains (IC_{50} of respectively 16 ± 2.6 µM and 13.6 ± 3.0 µM) in a colorimetric assay. In order to understand the mode of binding of gallic acid to both gelatinases, docking studies were carried out. 1HOV PDB was chosen for this study, as it is the only structure of MMP-2 in complex with a small molecule inhibitor (**I52**),[39] and we have used it in previous studies.[58] As NMR experiments were carried out with the catalytic domain of MMP-2 missing the three Fn-II like inserts, we first undertook docking experiments on the full MMP-2 catalytic domain missing those three inserts. The binding modes where gallic acid is within the catalytic center were the most energetically favorable ones under these conditions. Docking simulations were then repeated, limiting the docking grid to the catalytic center and using a more precise sampling protocol and scoring function. The results of these docking calculations are discussed here. In a first attempt to dock gallic acid, one main pose was obtained with the carboxylate anion coordinating the catalytic zinc ion (see Figure S1). This might be expected according to the many MMP carboxylate inhibitors found in the literature. However, in that pose, the zinc adopts a trigonal-based bipyramidal geometry, from which one ligand would be missing. The zinc ion is then coordinated by the three histidines (His_{120}, His_{124}, His_{130}) and the carboxylate anion. We thus repeated the docking applying two strategies to fill the zinc coordination sphere. First, we considered a water molecule, which occupied the first coordination sphere. For this we used a structure coming from a previous MD simulation of the free MMP-2 catalytic domain in explicit water.[40]

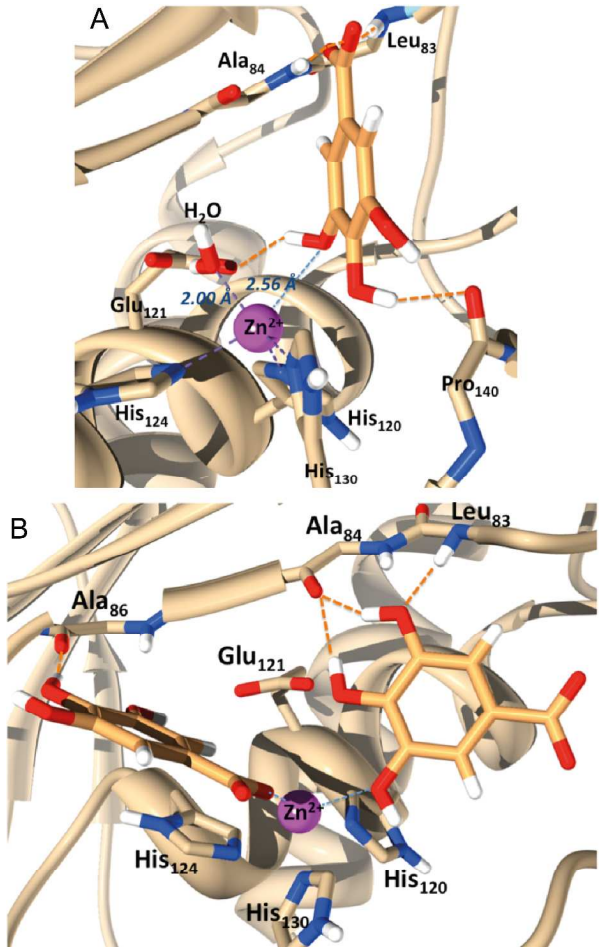

Fig.6 Representation of the most energetically favored binding mode of gallic acid in MMP-2, considering a water molecule in the zinc coordination sphere (a); and the most energetically favored binding mode of two gallic acid molecules in MMP-2 (b) (distance between the zinc ion and the two oxygens in the first coordination sphere are 2.02 Å and 2.44 Å from left to right).

In the most energetically favored docking pose (Figure 6a), a phenolic oxygen coordinates the zinc ion, forming the distorted trigonal-based bipyramid observed for hydroxamate inhibitors. Polyphenols are known Zn(II) ion chelators,[59,60] although no experimental evidence for phenol group coordinating the catalytic zinc ion of MMPs is reported in the literature. The gallic acid also established four H-bonds with Glu_{121} side-chain, and Leu_{83}, Ala_{84} and Pro_{140} backbones (1HOV numbering). It is worth noting that H-bonds involving Leu_{83} and Ala_{84} backbone can be found in several X-ray structures of inhibited MMPs, such as in 1HOV. In

a second approach, we checked if two molecules of gallic acid could simultaneously bind MMP-2 catalytic center. For that, we applied the methodology explained in Materials and methods section. In the most energetically favored pose (Figure 6b), the zinc ion is coordinated by the oxygen from a phenol of one ligand molecule, together with an oxygen from the carboxylate of the second gallic acid unit, leading to a distorted trigonal-base bipyramidal coordination of the zinc ion. Furthermore, the two gallic acid molecules participate in four H-bonds with MMP-2 backbone (NH group of Leu_{83}, CO group of Ala_{84} and CO group of Ala_{86}).

To study the binding mode of gallic acid in MMP-9, we chose 2OW1 PDB structure, in which MMP-9 is complexed to a small molecule inhibitor.[61] Gallic acid was docked in MMP-9 catalytic domain following the strategy settled for MMP-2. Analogous results were obtained, what was expected due to the similitude of the catalytic cleft of those two enzymes.

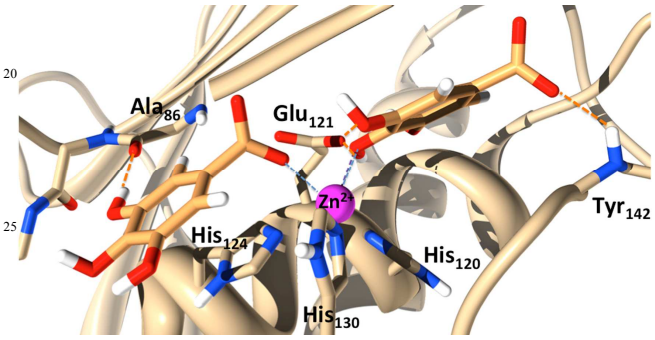

Fig. 7 Representation of the most energetically favored binding mode of gallic acid in MMP-9, considering two molecules of gallic acid (distance between the zinc ion and the two oxygens in the first coordination sphere are 2.18 Å and 2.30 Å from left to right)

In Figure 7, we report the binding mode involving two gallic acid molecules. Thus, the catalytic zinc is coordinated by an oxygen atom of the carboxylate moiety of one molecule, and a phenolic oxygen from the second one. As in MMP-2, no poses were found with the two carboxylate groups of each gallic acid coordinating the zinc, most probably due to electrostatic repulsions. One of the gallic acid units establishes three H-bonds, two with Glu_{121} side-chain and one with the NH group of Tyr_{142} backbone. The second molecule of gallic acid participates in an H-bond with Ala_{86} backbone.

Those results suggest the possible binding of gallic acid to gelatinases in a competitive manner, interacting directly within the catalytic center. Although such binding mode was not observed experimentally for MMPs, a recent study suggested the direct binding of polyphenols to the catalytic zinc ion of MMP-2.[62] In that work, another dietary polyphenol (ellagic acid) was shown to inhibit MMP-2 activity *in vitro* and *in vivo*. This process could be reversed by adding $ZnCl_2$ in a dose-dependent way, what suggests involvement of ellagic acid in the chelation of the catalytic zinc ion. Moreover, direct binding of ellagic acid to MMP-2 protein was confirmed by UV absorption.

Conclusions

In this work we give some new experimental data supporting the role of selected flavonoids in inhibition of metalloproteinases. As gelatinases are involved on different levels in promoting pathological processes such as cell invasion and angiogenesis, therefore increased consumption of natural polyphenol compounds such as gallic acid and anthocyanins, present in many dietary fruits and vegetables, could have a potential as valuable chemopreventive factor.

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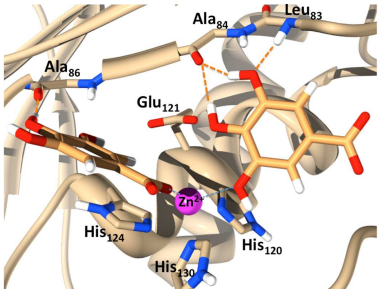

The effect of dietary gallic acid and anthocyanins on HT1080. A biochemical, computational and NMR study on the mechanisms involved.