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Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design

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Abstract

Flavanols—a class of plant polyphenols abundant in tea leaves and grape seeds and skins—have been found to inhibit some matrix-proteases instrumental in inflammation and cancer invasion, such as leukocyte elastase (LE) and gelatinases. In order to establish the relationship between chemical structure and activity, 27 different flavonoids (antocyanidins, dihydrochalcones, dihydroflavonols, flavanolignans, flavanols, flavones, flavonols and isoflavones) and other compounds with anti-oxidant properties were evaluated for their potential in blocking LE and gelatinase activities. LE activity was measured using a chromogenic substrate: from comparison of the different levels of inhibition, it was deduced that a crucial role in inhibition might be played by a galloyl moiety or hydroxyl group at C3, three hydroxyl groups at B ring, one hydroxyl group at C4′, and a 2,3-double bond. Gelatinase activity was measured using the gelatin-zymography assay, and its inhibition showed that three hydroxyl groups at the A or B ring, or, for non-planar molecules, a galloyl moiety at C3 could be determinant. This comparative study is proposed as a basis for designing new molecules with enhanced anti-proteolytic activities, and no or reduced side-effects, for use in hindering inflammation, cancer invasion and angiogenesis.

Keywords: Polyphenols; Leukocyte elastase; Gelatinases; Inflammation; Invasion

1. Introduction

Epidemiological studies have suggested an association between the consumption of foods or beverages containing polyphenols and the prevention of some human diseases, such as chronic obstructive pulmonary and heart diseases [1,2], chronic inflammation [3], as well as reduction of risk of many types of cancer [4]. Polyphenols are widespread in nature; the two most important groups of dietary phenolics are phenolic acids and flavonoids, this latter being the largest and most studied group and responsible for the color of flowers, fruits and sometimes leaves.

One abundant source of dietary phenolics is green tea, whose compounds are mainly flavanols or catechins. In this beverage, epigallocatechin-3-gallate (EGCG) is the most prevalent secondary metabolite, followed by epigallocatechin (EGC) and epicatechingallate (ECG) [5] (Fig. 1). Recently, we reported that micromolar concentrations of EGCG inhibit in a dose-dependent, non-competitive manner the activity of gelatinase A (MMP-2) and B (MMP-9), two matrix metallo-proteinases (MMPs) instrumental in cancer invasion, metastasis and angiogenesis [6]. Moreover, at concentrations equivalent to those in the plasma of moderate green tea drinkers, EGCG inhibits invasion of tumor cells *in vitro* [7], as well as the activity of leukocyte elastase (LE) [8]. This latter is a serine-proteinase released by neutrophil leukocytes at the site of inflammation, whose activity—if not appropriately counterbalanced by tissue inhibitors—may over-disrupt the elastic architecture, as in pulmonary emphysema. LE can also activate a number of MMPs and inactivate their tissue inhibitor [9].

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Abbreviations: EC, epicatechin; ECG, epicatechingallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; IC_{50}, concentration producing 50% inhibition; IgG, immunoglobulin G; LE, leukocyte elastase; MMP, matrix metallo-proteinase; PBS, phosphate buffer saline.

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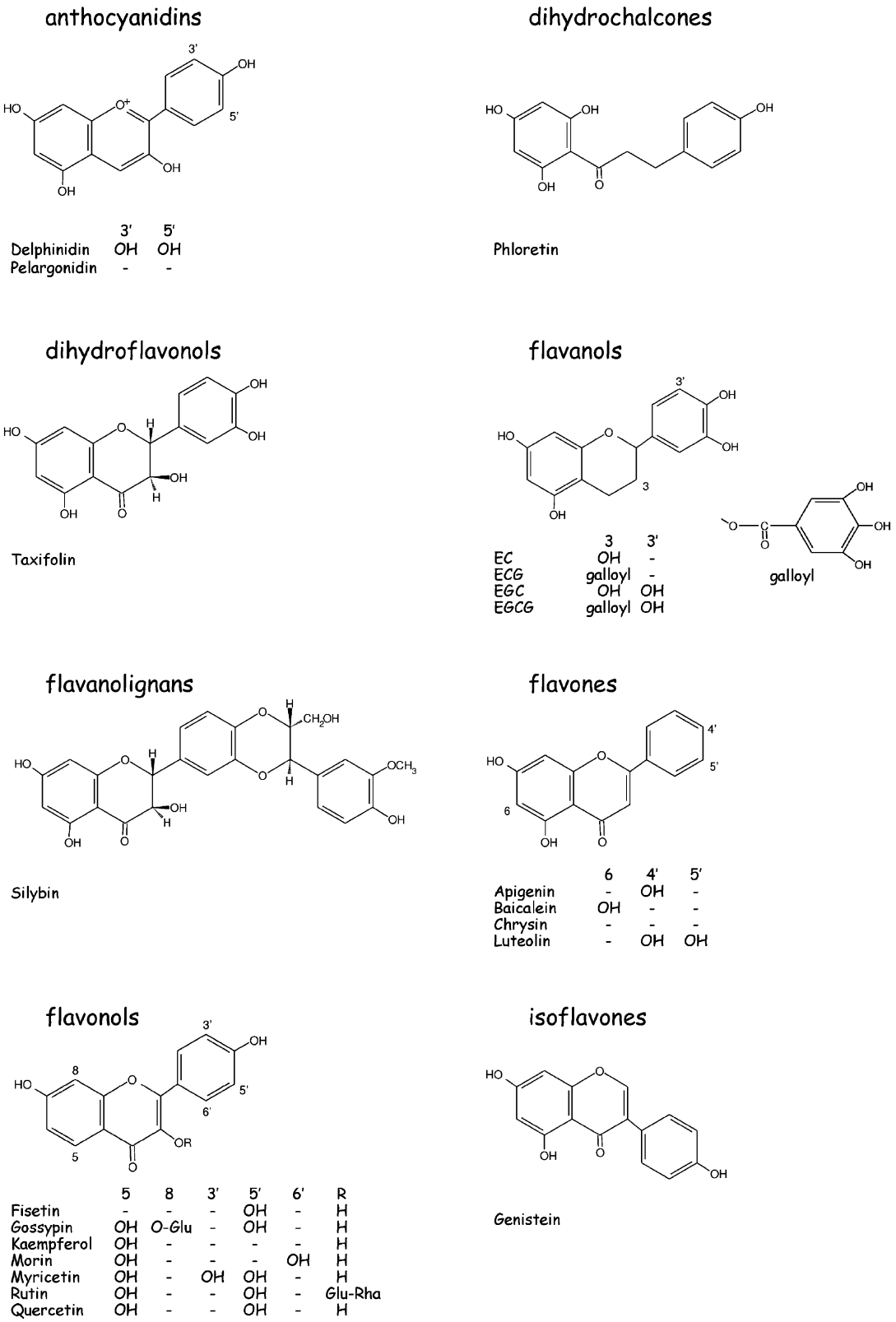

Fig. 1. Structure of the different phytofactors (grouped by class) tested in this study for anti-LE and anti-gelatinase activities.

Tumor invasion and metastasis are reduced by both natural and synthetic enzyme inhibitors—some of the latter are currently in clinical trial for cancer treatment—but they often carry unpleasant side-effects [10]. The anti-gelatinase and anti-LE properties of EGCG may offer a welcome alternative, since this catechin is a natural component of a beverage that has been consumed for centuries and is without side-effects.

The effects on proteinases are not exclusive to EGCG: also EGC and ECG exert inhibitory activity toward gelatinases A and B. However, while EGC is effective at concentrations 20–30 times higher than EGCG, ECG has been reported to block gelatinases at concentrations close to those recorded for EGCG [11]. This result fits with the suggestion that both the flavanol skeleton and the galloyl moiety are necessary for inhibition. Nevertheless, the relationship between structure of polyphenols and their targeted activity remains unclear.

In order to determine whether some particular polyphenol chemical structures may contribute to inhibitory potential, a series of vegetable secondary metabolites have been compared for their potential in blocking LE and gelatinase activities. These were polyphenols from eight different groups (antocyanidins, dihydrochalcones, dihydroflavonols, flavanolignans, flavanols, flavones, flavonols and isoflavones) (Fig. 1), and other anti-oxidant phytofactors (Fig. 2). These results are proposed as a basis for designing even more effective anti-inflammatory and anti-cancer molecules.

2. Materials and methods

2.1. Materials

Elastase from human leukocytes (EC 3.4.21.37), elastase substrate *N*-methoxysuccinil-ala-ala-pro-val *p*-nitroanilide, EGCG, ECG, EGC, EC, apigenin, baicalein, delphinidin, phloretin, gossypin, morin, pelargonidin, silybin and taxifolin were purchased from Sigma. Astaxanthin, curcumin, lycopene, luteolin, resveratrol, rosmarinic acid and D,L-α-tocopherol were obtained from Alexis Biochemicals. Chrysin, fisetin, genistein, myricetin, quercetin, and rutin were purchased from Fluka. Kaempferol was supplied by Indena. All the phenolics were purified, 95%.

2.2. Compound concentration/pH

Since some phenolics modify the buffer pH, a critical parameter in protease activity, we verified the maximum concentration that—under our conditions—left the pH unmodified for all compounds: this was found to be

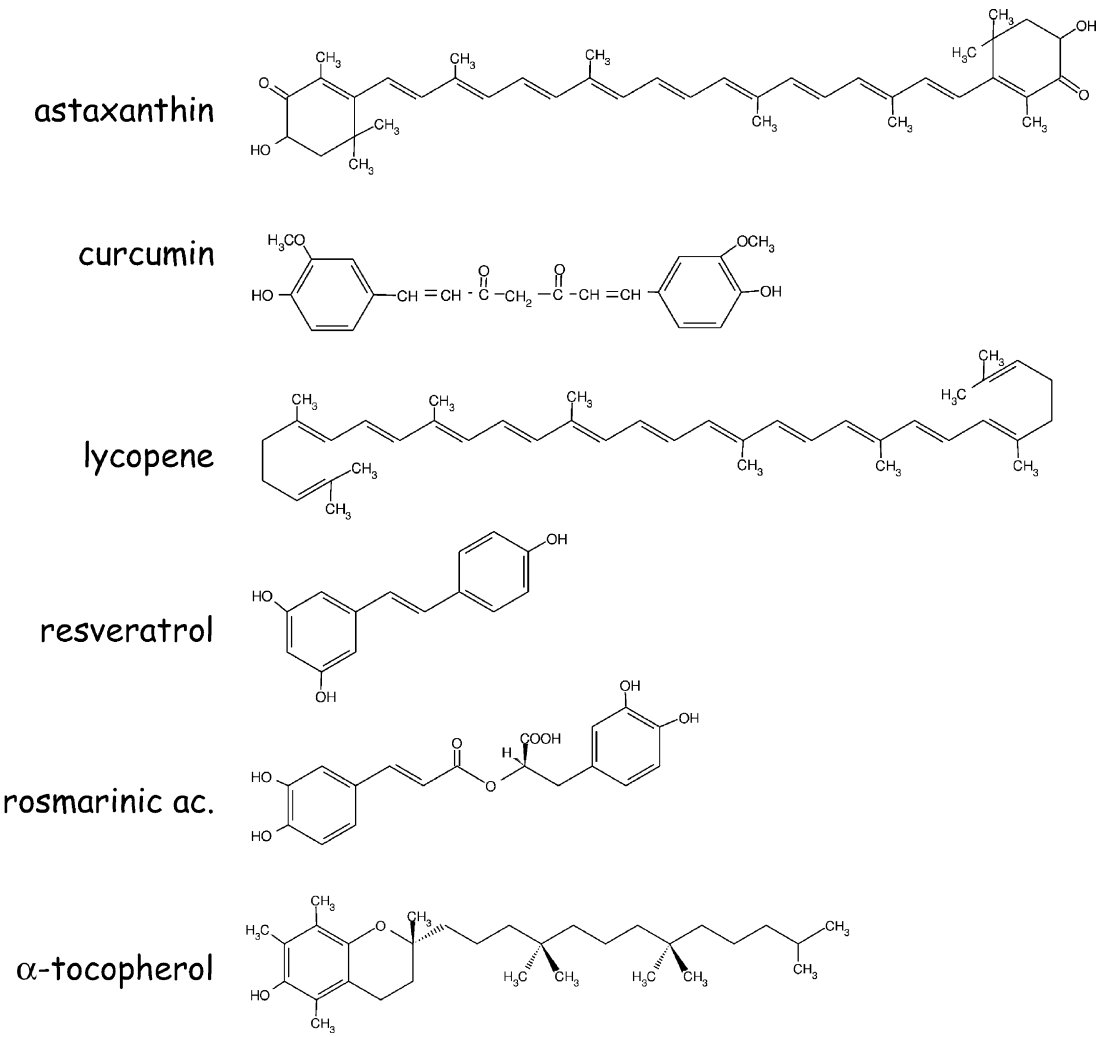

Fig. 2. Structure of the miscellaneous anti-oxidant phytofactors tested in this study for anti-LE and anti-gelatinase activities.

125 μM. This concentration was taken as the threshold for the assays (except where stated).

2.3. Substrate degradation by LE

LE was solubilized (250 mU/mL) in Hepes buffer (0.1 M Hepes, 0.5 M NaCl, 10% DMSO, pH 8.0). All the phytofactors and elastase substrate were freshly prepared 20× in ethanol. Dilutions of the compounds were pre-mixed with the enzyme in micrometers wells, and maintained 15 min at 4°. Then, 5 μL of the substrate (8 mM concentrated) were added to 100 μL final volume, and the mixture was incubated at 37°. At 20 min intervals, the intensity of the color developed by the digested substrate was measured at 405 nm using a Titertek Mutiskan (Flow Laboratories), and the control background subtracted (in triplicate experiments). The reactions developed linearly for as long as 120 min; data from 60 min were used for all plots.

2.4. Zymographic analysis

Aliquots of gelatinase-containing medium conditioned by human neuroblastoma cells (for MMP-2) or HT-1080 human fibrosarcoma cells (for MMP-9) were assayed as described [12]. Without heating the samples, zymography was performed by electrophoresing 15 μL of medium in 0.1% gelatin-containing 8% polyacrylamide, in presence of SDS. After electrophoresis, the gels were washed twice for 15 min with 2.5% Triton X-100, incubated overnight at 37° in Tris buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4). For gelatinase inhibition assays, compounds were freshly solubilized in ethanol and diluted in Tris buffer used for developing the zymogram. The gel slab was then cut into slices corresponding to the lanes and then put in different tanks containing the stated concentrations of inhibitors.

The gels then were stained for 30 min with 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant Blue R-250, and destained in the same solution without dye. Clear bands on the blue background represent areas of gelatinolysis. Digestion bands were quantitated using an image analyzer system with GelDoc 2000 and Quantity One software (Bio-Rad), and the densitometric values were expressed as percentage of control bands on the same gel. Preliminary assays were carried out to determine the appropriate amount of gelatinase to be loaded, under the level giving complete digestion of the substrate.

2.5. Precipitation and dot blotting

Aliquots of gelatinase-containing medium conditioned by human neuroblastoma cells [13] were incubated 1 hr at room temperature in presence of phytofactors at indicated concentrations or 100 μM tannic acid (gallotannin). After 5 min centrifugation at 15,000 *g*, equal aliquots of supernatant were directly absorbed onto a Hybond-C Extra nitrocellulose membrane. After pre-hybridization at room temperature for 2 hr in phosphate buffer saline (PBS) with 0.1% Tween-20 (PBS-T) containing 1% non-fat dried milk (Sigma) (solution 1), the membrane was hybridized at room temperature for 1 hr in PBS-T containing 3% BSA and first antibody, anti-human MMP-2 mouse IgG (1:200) (The Binding Site). The membrane was then incubated for 1 hr at room temperature in solution 1 containing the second antibody, horseradish peroxidase-labeled anti-mouse IgG (1:2,000) (Sigma). Extensive washing in PBS-T followed each step. Antigen detection was achieved by incubating the membrane for 1 min at room temperature with 0.125 mL/cm^2 of ECL detection solution and exposure to Hyperfilm MP (both from Amersham Pharmacia Biotech) for 10 s up to 5 min.

3. Results

3.1. Inhibition of LE

To assay the inhibition exerted on human LE by the various molecules, 5 mU of purified LE were incubated with elastase substrate at increasing concentration of compound. The results are reported in Table 1, and the structure of the different chemical classes in Fig. 1:

- flavanols (or catechins): of the four molecules tested for inhibition of LE activity, only EGCG was effective in the tested range of concentration (125 μM), with an IC_{50} of 0.4 μM (Fig. 3), as already reported [8]. Conversely, no effect was registered for EC, ECG and EGC, the two latter tested up to 0.5 mM. No synergistic effects between these flavanols were observed;
- flavonols: the activity of LE was restrained with an IC_{50} between 4 and 20 μM. The highest inhibition was obtained with myricetin (IC_{50} of 4 μM) (Fig. 3) and morin (4.5 μM), followed by fisetin (16 μM) and quercetin (20 μM). In contrast, LE was inhibited at concentrations of kaempferol 1000-fold higher than those shown for other flavonols. Also the glycosides rutin and gossypin had no effect on LE;
- dihydroflavonols: taxifolin had no effect on LE;
- flavones: only baicalein was able to inhibit LE, with an IC_{50} of 25 μM (Fig. 3). The other tested flavones (apigenin, chrysin and luteolin) had no effect;
- isoflavones and flavanolignans: both genistein and silybin were completely inactive against LE;
- dihydrochalcones: phloretin did not inhibit LE;
- anthocyanidins: pelargonidin and delphinidin showed inhibition of LE comparable to that of flavonols, with IC_{50} of 3 and 12 μM, respectively;
- miscellaneous: a number of other compounds that have chemical properties common with catechins (e.g. antioxidant) were also tested (Fig. 2). None of these molecules (astaxanthin, curcumin, lycopene, resveratrol,

Table 1
Inhibitory effect of vegetable secondary metabolites on LE and gelatinase MMP-2 and MMP-9 activities, expressed as IC_{50}

Class	Name	Example sources	LE	MMP-2	MMP-9
A	Apigenin	*Chamomilla recutita*	–[a]	–	–
M	Astaxanthin	*Haematococcus pluvialis*	–	–	–
A	Baicalein	*Scutellaria* ssp.	25	2	6
A	Chrysin	*Populus* ssp., *Pinus* ssp., *Escalonnia* ssp.	–	–	–
M	Curcumin	*Curcuma domestica*	–	–	–
B	Delphinidin	*Punica granatum, Solanum tuberosum*	12	3	13
C	EC	*Camellia sinensis*	–	–	–
C	ECG	*C. sinensis*	–	95[b]	28[b]
C	EGC	*C. sinensis*	–	450	–
C	EGCG	*C. sinensis*	0.4	15	30
D	Fisetin	*Acacia* ssp.	16	8	160
E	Genistein	*Soya hispida*	–	–	–
D	Gossypin	*Gossypium indicum, Hibiscus vitifolius*	–	–	–
D	Kaempferol	*Ocimum basilicum, Citrus paradisi*	5000	–	–
A	Luteolin	*Achillea millefolium*	>300	–	–
M	Lycopene	*Lycopersicum esculentum*	–	–	–
D	Morin	*Morus alba, Chlorophora tinctoria*	4.5	25	300
D	Myricetin	*Soymidia febrifuga*	4	10	12
B	Pelargonidin	*Fragaria* ssp., *Pelargonium* ssp.	<3	200	30
F	Phloretin	*Malus domestica*	–	20	–
D	Quercetin	*Rhododendron cinnabarinum*	20	–	–
M	Resveratrol	*Vitis vinifera*	–	–	–
M	Rosmarinic ac.	*Rosmarinus officinalis*	–	–	–
D	Rutin	*Sophora japonica, Fagopyrum esculentum*	–	–	–
G	Silybin	*Silybum marianum*	–	–	–
H	Taxifolin	In many *Coniferae*	–	50	55
M	α-Tocopherol	*S. hispida, Arachis ipogea*	–	–	–

A, flavones; B, anthocyanidins; C, flavanols; D, flavonols; E, isoflavones; F, dihydrochalcones; G, flavanolignans; H, dihydroflavonols; M, miscellaneous. Example sources of each metabolite are given.

[a] Undetectable inhibition.

[b] Values from [11].

rosmarinic acid and α-tocopherol), tested up to 125 μM, was effective in inhibiting LE.

The inhibition exerted by active phenolics was dose-dependent and maintained constant slope throughout the 2 hr measurement.

3.2. Inhibition of gelatinases A and B

To determine the inhibition of MMP-2 and MMP-9, aliquots of gelatinase-containing medium were analyzed by gelatin zymography developed in presence of increasing

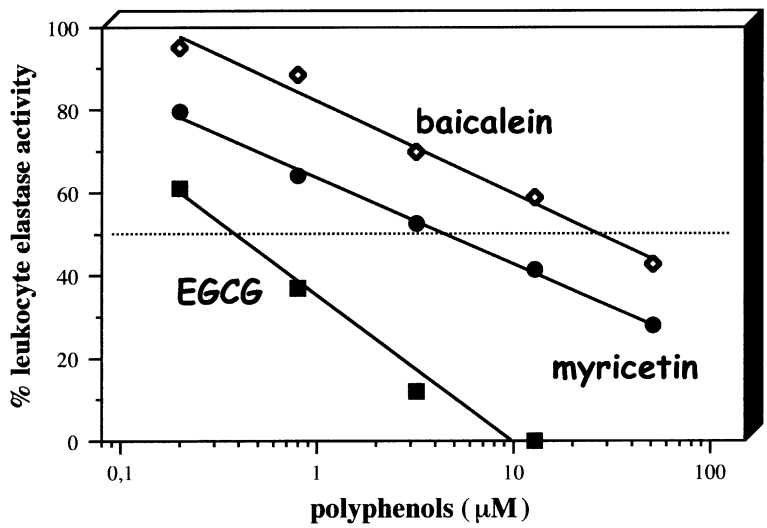

Fig. 3. Effect of polyphenols on LE activity (example of three out of the 27 compounds listed in Table 1). Leukocyte elastase (5 mU) was pre-incubated 15 min at 4° in the absence (control) or presence of increasing concentrations of the indicated polyphenols; the substrate was then added, and the mixture incubated at 37°. Data are the mean of triplicate experiments, and expressed as percent of control.

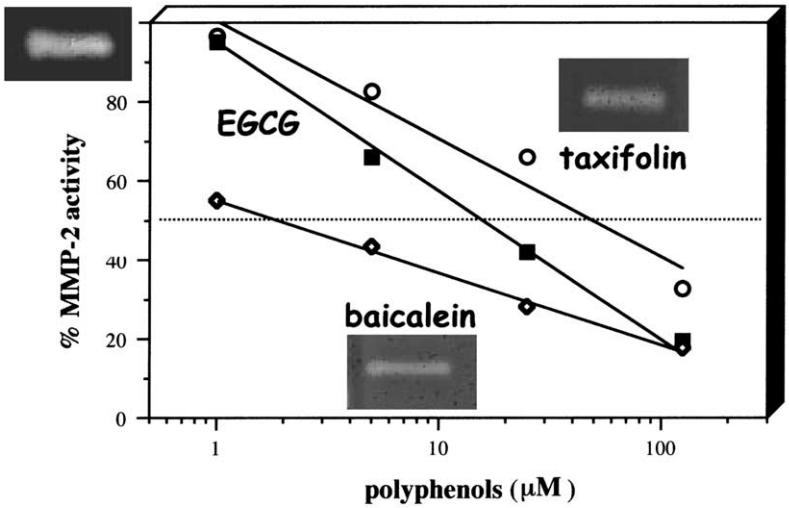

Fig. 4. Effect of polyphenols on gelatinase MMP-2 activity (example of three out of the 27 compounds listed in Table 1). Identical aliquots of MMP-2-containing medium conditioned by neuroblastoma cells were processed for zymography; each lane was then separately developed at 37° in the absence (control) or presence of increasing concentrations of the indicated polyphenols. The digestion bands were quantitated by an image analyzer system, and the data are examples of duplicate experiments, expressed as percent of control. The three inserts show example bands of digestion in absence (outside the graph) or presence of the higher concentration of baicalein and taxifolin, respectively.

concentrations of compound, and the digestion bands quantitated to determine the IC_{50}. The results are reported in Table 1:

- flavanols (or catechins): EGCG, EGC and EC inhibit the two gelatinases to a different degree. EGCG inhibits MMP-2 and MMP-9 in a dose-dependent manner with IC_{50} of 15 (Fig. 4) and 30 μM, as already reported by our group [6]. EGC has an IC_{50} approximately 20- and 30-fold higher, while EC was completely inactive;
- flavonols: the two gelatinases were inhibited by myricetin, with IC_{50} of 10 and 12 μM. Quercetin and kaempferol had little effect up to 125 μM; fisetin and morin gave an IC_{50} for MMP-2 of 8 and 25 μM, respectively, and 160 and 300 μM for MMP-9. Glycosilated compounds rutin and gossypin were not effective;
- dihydroflavonols: taxifolin inhibited MMP-2 and MMP-9 with IC_{50} of 50 and 55 μM, respectively (Fig. 4);
- flavones: baicalein was the most effective in inhibiting gelatinases, with IC_{50} of 2 μM for MMP-2 (Fig. 4) and 6 μM for MMP-9. The other flavones (apigenin, chrysin and luteolin) did not inhibit gelatinolytic activity;
- isoflavones and flavanolignans: genistein and silybin were not effective;
- dihydrochalcones: phloretin gave an IC_{50} of 20 μM for MMP-2 but showed no effect against MMP-9;
- anthocyanidins: delphinidin inhibited with an IC_{50} of 3 μM (MMP-2) and 13 μM (MMP-9). Pelargonidin was not effective against MMP-9 but gave a calculated IC_{50} of 200 μM for MMP-2;
- miscellaneous: as for LE, the other tested anti-oxidant phytofactors (Fig. 2) were shown to be completely ineffective against MMP-2 and MMP-9.

3.3. Precipitation and dot-blotting

To determine whether the gelatinase inhibition exerted by baicalein, delphinidin, EGCG, fisetin and myricetin (the most effective compounds revealed by our study) was due to enzyme precipitation by the compound, we incubated gelatinase-containing samples with the polyphenols, or with tannic acid (gallotannin) as positive control [6]. The supernatant was then analyzed by antibodies specific for MMP-2. The addition of 100 μM tannic acid completely precipitated the gelatinase antigen, while for 9-fold IC_{50} of the above molecules the enzyme remained in the supernatant.

4. Discussion

We tested a series of vegetable secondary metabolites for their capacity to inhibit the enzymatic activity of LE, MMP-2 and MMP-9. The compounds were chosen for either their close similarity in chemical structure to the ''model'' molecule EGCG, or some common chemical–physical properties (e.g. anti-oxidant). Among the compounds found to be active, the inhibiting concentration for the three proteases ranges from 0.4 to over 500 μM, expressed as IC_{50} (Table 1).

Of the 27 molecules tested, seven showed inhibition of at least one of the enzymes with an $IC_{50} \leq 10$ μM: these were baicalein, delphinidin, EGCG, fisetin, miricetin, morin and pelargonidin. Some molecules from different groups of phenolics—such as flavonols, flavones, catechins and anthocyanidins—are thus able to exert inhibition of invasion-instrumental proteases at micromolar concentrations, while others show weak or no activity.

Within the same phenolic group, the presence or absence at particular locations of certain chemical groups and double bonds appears crucial to a molecule's inhibitory capacity. Most significant were the hydroxyl groups at the A and B rings, the 2,3-double bond in the flavone skeleton (Fig. 5A), and the presence of the galloyl moiety.

The most effective molecule in blocking LE activity is EGCG. The presence of six hydroxyl groups on one side of the molecule (three at the galloyl moiety and three at the B ring, Fig. 1) is probably crucial to the low IC_{50} of ECGC. In fact, molecules without the hydroxyl group in position 3′ of the B ring (ECG) or the galloyl moiety (EGC and EC) show no activity at all.

But also other molecules show good LE inhibition. Here, the presence of a hydroxyl group at C3, two hydroxyl groups at the B ring, and a 2,3-double bond lowers the IC_{50} against LE. Molecules with these three features, like myricetin and morin (Fig. 1)—but also fisetin and quercetin—inhibited LE at concentrations one order of magnitude higher than those for EGCG, but lower than many molecules lacking these features.

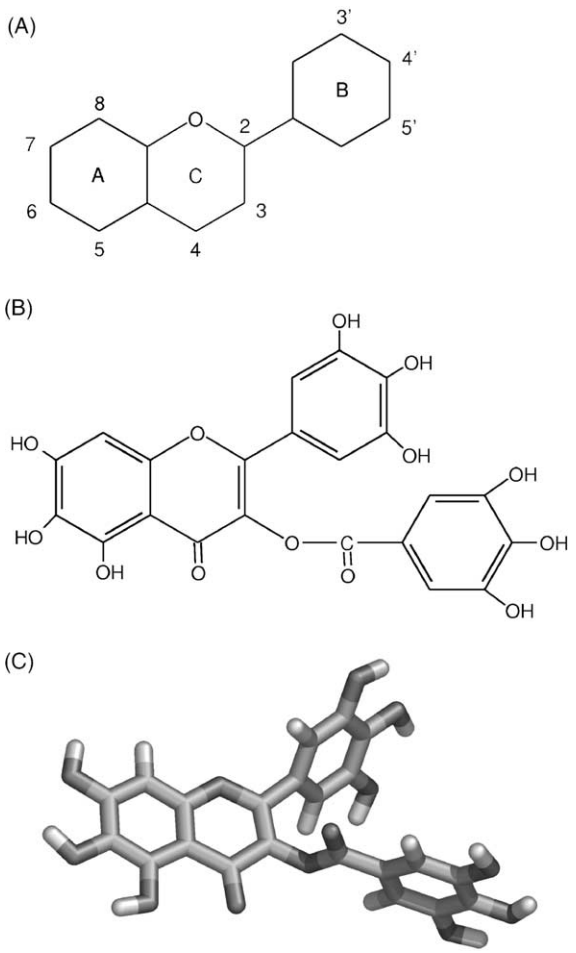

Fig. 5. (A) Structure of the flavone skeleton. (B) Example of a ''blended structure'', combining the chemical features proposed as crucial in the best anti-LE and anti-gelatinase performing phytofactors. (C) A 3-D representation of the (B) molecule.

In contrast, molecules like the dihydroflavonol taxifolin (without the 2,3-double bond), and the flavones luteolin, apigenin and chrysin (without the hydroxyl group at C3) gave IC_{50} for LE 20 times higher or out of measurement range.

The presence of hydroxyl groups in position C3 and C4′ confers to some molecules of a different class a low IC_{50}, even if the 2,3-double bond is not present. This is the case of the two anthocyanidins tested (Fig. 1)—pelargonidin and delphinidin—showing an IC_{50} of 3 and 12 μM for LE, respectively. However, this unexpected good inhibition may be attributed to the different structure of the molecules, presenting a positively charged oxygen in the C ring, which also influences the characteristics of the other rings. It is less likely that the inhibitory activity could be a property of a different structure resulting from the opening of the C ring (transformation into chalcone) [14] at the moderately alkaline pH used for detection of enzymatic activity.

Conversely, kaempferol—a flavonol with the three structural features mentioned above—gave an IC_{50} for LE two orders of magnitude higher than that of other active flavonols. The unexpected lower inhibitory effectiveness obtained for this compound can be partially explained by its high sensitivity to oxidation, which causes decomposition during the assay [15].

Moreover, differences in just the absence or presence in the molecule of ''small'' chemical groups may also confer different biological properties. In particular, the presence in the flavonoid skeleton of an hydroxyl group at C3 (Fig. 5A) confers effectiveness in countering LE, while its absence confers anti-proliferative activity. In fact, while the flavones luteolin and apigenin (without hydroxyl group at C3) were highly effective in inhibiting cell proliferation (IC_{50} of 9 and 12 μM, respectively) [16], they instead were ineffective (out of measurement range) against LE.

Conversely, the structural features characterizing the most effective molecules against gelatinases MMP-2 and MMP-9 differ from those needed to inhibit LE. One requirement was found to be the presence of at least three hydroxyl groups at the A or B ring (Fig. 5A). In fact, the strongest inhibition of MMP-2 was obtained with baicalein (IC_{50} of 2 μM) which has three hydroxyl groups at the A ring, followed by delphinidin and myricetin, which have two hydroxyl groups at the A ring and three at the B ring. The exception was fisetin (8 μM), which has only one hydroxyl group at the A ring and two at the B ring (Fig. 5A). As previously shown for EGCG, dot-blot of supernatants excluded the possibility that gelatinase inhibition was due to precipitation of the protease induced by binding to the numerous hydroxyl groups, as in the case of tannic acid [6].

In the compounds tested, the ''planarity'' of the molecule was found to be another important element in conferring a low IC_{50} against gelatinases. EGCG proved to be the most active catechin in countering MMP-2 and MMP-9.

Its non-planar structure contains three hydroxyl groups at the B ring and a galloyl moiety at C3 (Fig. 5A). ECG (non-planar) showed an inhibition 6-fold lower [11] and has only two hydroxyl groups, but like EGCG has a residue of gallic acid at C3. EGC (non-planar), despite having three hydroxyl groups at the B ring, gave a very low inhibition. Thus, while three hydroxyl groups present at the A or B ring of planar compounds (such as baicalein) confer a low IC_{50} for gelatinases, they are not sufficient for non-planar compounds, which also need a galloyl moiety in position C3 to be effective. This moiety could also increase the potential of flavones and flavonols.

In contrast, additional glycosidic groups present on the flavone skeleton of phytofactors (as in rutin = quercetin 3-rutinoside) may affect the steric conformation and lead to a weak phenolic-enzyme interaction, and thus to a weaker inhibition of the proteolytic activity. Glycosidic compounds were indeed ineffective against both LE and gelatinases (see rutin and gossypin), but also have been reported by other research groups to be unable to inhibit oxidation [15].

Other authors have demonstrated that polyphenols may vary significantly in their activities, depending on slight variations in their structural features [15–17]. In particular, it has been shown that only flavonoids with a hydroxyl group at the C3 and C4′ and the 2,3-double bond (Fig. 5A) show high antioxidant activity. With the same structural features, molecules like quercetin, morin, fisetin and myricetin are among the most effective against LE. However, the lack of synergistic effects among inhibitory and non-inhibitory flavanols indicated that the anti-oxidant activity and protease inhibition are not necessarily interdependent properties. This is confirmed by the ineffectiveness of some other strong anti-oxidant molecules—such as astaxantin, curcumin, lycopene, resveratrol, rosmarinic acid and α-tocopherol [18–23]—in inhibiting LE and gelatinases.

These data may be used as the basis for proposal of new molecules, such as the example shown in Fig. 5B and C, which combines chemical structures that may enhance both LE and gelatinase inhibition: a galloyl moiety at C3, three hydroxyl groups at the B ring (>LE), and three hydroxyl groups at the A ring fused with a pyrone ring (>gelatinases).

Given the key role of matrix-proteases on inflammatory, angiogenic, invasive and metastatic processes, the synthesis of new compounds with improved anti-LE and anti-gelatinolytic activities represents a valid approach in preventing and combating severe pathologies—from chronic obstructive pulmonary disease (which may lead to emphysema) to cancer—where proteolytic enzymes are instrumental to progression. The ''model'' EGCG phytofactor passes, at least partially, from the intestinal tract to the blood [24], and is without reported side effects; any synthetically modified derivatives should maintain these clinical advantages.

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