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Sanguisorba minor extract suppresses plasmin-mediated mechanisms of cancer cell migration

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ABSTRACT

Background: Sanguisorba minor, as well as several other edible herbs and vegetables, has been used extensively in traditional medicine. The observed beneficial effects can be attributed at least in part to the direct modulation of several enzymatic activities by its polyphenolic constituents.
Methods: The ethanol extract of *Sanguisorba minor* was characterized by reversed-phase liquid chromatography, and most relevant analytes were identified by multiple stage mass spectrometry. The whole extract and the most relevant isolated constituents were tested for their ability to modulate the activity of human plasmin both toward a synthetic substrate and in human breast cancer cell culture models. Kinetic and equilibrium parameters were obtained by a concerted spectrophotometric and biosensor-based approach.
Results: Quercetin-3-glucuronide was recognized as the compound mainly responsible for the *in vitro* plasmin inhibition by *S. minor* extract, with an inhibition constant in the high nanomolar range; in detail, our approach based on bioinformatic, enzymatic and binding analyses classified the inhibition as competitive. Most interestingly, cell-based assays showed that this flavonoid was effective in suppressing plasmin-induced loss of cancer cell adhesion.
General significance: Our results show that the extract from *Sanguisorba minor* limits plasmin-mediated tumor cell motility *in vitro*, mostly due to quercetin-3-glucuronide. This glucuronated flavonoid is a promising template for rational designing of anticancer drugs to be used in the treatment of pathological states involving the unregulated activity of plasmin.

1. Introduction

Herbs have long been used as ethno-medical remedies, and recently turned out to be a valuable source of pharmaceutical agents, with drug research programs being largely aimed at discovering and designing new chemotherapeutic agents based on plant-derived compound leads. In this perspective, flavonoids [1,2], polyphenols [3,4], quassinoids [5,6], quinones [7,8], sesquiterpene lactones [9,10], together with their synthetic derivatives [11–14] represent a selection of promising bioactive natural products. Nevertheless, the comprehensive molecular mechanisms underlying the relationship between a natural polyphenol-based treatment and the decreased vulnerability to pathologies (cancer, in particular) are still being elucidated.

Among endemic herbs extensively used in the past, *Sanguisorba minor* is an edible, perennial herb with pinnate leaves and reddish-green flowers [15] widely distributed throughout Italy. Its extract (compositionally characterized by other authors [15–18]) was reported to display a number of pharmaceutical properties (antiviral [19,20], anti-ulcerogenic [21] and anti-acetylcholinesterasic [22] activities, besides some other effects common to *Sanguisorba officinalis* [23]), largely attributable to its polyphenol constituents [24–28].

In this work, in efforts to further dissect the therapeutic properties of *S. minor*, we tested the effects of its ethanol extract against human plasmin, a serine protease whose critical role in tumor cell invasion and metastasis has been clearly established [29–31]. In fact, besides catalyzing the degradation of membrane proteins such as fibronectin [32], laminin [33] and E-cadherin [34], plasmin was shown to degrade several glycoproteins of basement membranes and extracellular matrices and activate some matrix degrading metalloproteinases [35], all these events being crucial for tumor cellinvasion [36,37]. In particular, plasmin was documented to affect tumor cell adhesion by cleaving E-cadherin, an epithelial adhesion macromolecule [38] in charge of sensing intercellular contacts and controlling cell density. Extracellular proteolysis of E-cadherin yields to a 80 kDa fragment that promotes the disruption of the adherens junction and enhances cell aggressiveness [39]. Accordingly, this fragment was also suggested as a biomarker in body fluids of cancer patients [40].

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Herein, we reported that the whole *S. minor* extract significantly inhibited plasmin amidolytic activity *in vitro*, and most importantly regulated both plasmin-induced decrease of intercellular adhesion and motility in tumor cells, identifying quercetin-3-glucuronide (Q3G) as the compound mainly responsible for the observed activities. Besides, we thoroughly characterized the kinetic and equilibrium parameters of the interaction between plasmin and Q3G.

2. Materials and methods

2.1. Reagents, chemicals, and devices

Human plasmin (E.C.: 3.4.21.7), tosyl-gly-pro-lys-4-nitroanilide (chromozym-PL), CH_3OH, H_3PO_4, N-hydroxysuccinimide (NHS), N-ethyl-N-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), aprotinin and the Folin–Ciocalteau reagent were purchased from Sigma-Aldrich (Sigma-Aldrich S.r.l.—Milan, Italy). CH_3CN, NaCl, KCl, PEG-8000, Na_2HPO_4, K_2HPO_4, CH_3CH_2OH, Na_2CO_3, and gallic acid were obtained from J.T. Barker (Mallinckrodt Baker—Milan, Italy). Quercetin-3-glucuronide was obtained from Extrasynthese (Extrasynthese SAS—Lyon, France). All chemicals and solvents were of highest analytical grade available. MCF-7epithelial human breast adenocarcinoma tumor cell lines were obtained from ATCC (Rockville, MD USA). Samples of *Sanguisorba minor* were kindly provided by Agrifaber S.r.l. (Marche region, Italy). Goat polyclonal anti-human E-Cadherin antibody and rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (FL-335) antibody were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

Extractions were carried out on an ASE 100 system, equipped with 10 μm frits and with 30 mm glass fibre filters (Dionex S.p.A.—Milan, Italy). Extracts were lyophilized on a Buchi rotary evaporator (Buchi S.r.l.—Milan, Italy). Chromatographic analyses were performed on an UV-Vis Beckman Gold HPLC (Beckman Coulter Italia - Milan, Italy), using a 5 μm, 250×4.60 mm Phenomenex Luna C-18 column equipped with a 4×3 mm Phenomenex Luna C-18 guard-column (Phenomenex S.r.l.—Bologna, Italy). The column was thermostatted in an Alltech Column Heater (Alltech Italia S.r.l.—Rome, Italy). Mass analyses were performed on a LC/MSD Trap SL (Agilent Technologies Italia—Milan, Italy) equipped with an electrospray ionization (ESI) source operating in negative ionization mode. Enzyme activities were performed on a thermostatted VarianCary 1E spectrophotometric device (Varian—PaloAlto, California). Binding studies were performed on an IAsys *plus* Biosensor (Affinity Sensor—Cambridge, UK). Carboxylate cuvettes were obtained from Neosensors (Sedgefield, UK).

2.2. Plant material

S. minor was grown by a local agrarian company (Agrifaber S.r.l.) and harvested in August 2009. The cultivation was performed according to conventional agricultural techniques, including artificial irrigation and a chemical fertilisation step before sowing [41]. No parasite contamination was reported.

2.3. Extraction procedure

Plant samples were dried at 45 °C for 72 h. Dionex ASE 100 accelerated solvent extractor equipped with 100 mL stainless extraction cells and 250-mL glass collection bottles with teflon-coated rubber caps was used for the extractions. Extraction cell was filled with 10 g of the dried sample. Extraction parameters were optimised as follows: $T = 100$ °C, pressure = 1500 psi, preheating period = 5 min, static extraction period = 5 min, solvent flash = 9.9 mL, nitrogen purge = 60 s. Ethanol was used as extracting solvent and both static extraction and solvent flash were performed twice for each sample.

The extract solution (100 mL) was collected, lyophilized with a rotary evaporator, then further dried at 40 °C for 72 h. Lyophilized samples were stored at room temperature in sealed bottles, and protected from light sources. Total phenolics were estimated according to the Folin–Ciocalteau method [42]. Variation in phenolic content ($n = 5$ replicates) always ranged between 1% and 3% relative standard deviation.

2.4. HPLC/MS analysis

Lyophilized extracts were re-suspended in 50% ethanol, treated with a 40 Hz ultrasonic bath for 4 min (insoluble particles were removed by filtration through a 0.45 μm porosity cellulose membrane filter), and finally injected (injection volume: 100 μL). The separation was performed at 26 °C using the following segmented gradient of eluent A (water adjusted to pH = 3.5 with H_3PO_4) and eluent B (CH_3CN): 0–15% of B in 30 min, 15% of B for 5 min, 15–100% of B in 10 min, 100% of B for 5 min and 100–0% of B in 10 min (flow rate = 1.0 mL/min, $t = 26$ °C). The elution was monitored at 310 nm. ESI mass spectra were carried out on Agilent Technologies LC/MSD Trap SL (G2445D SL). Multiple stage mass spectrometry (ESI-MS^n) was performed in FIA (Flow Injection Analysis) using CH_3OH as solvent (5 L/min, nebulizer pressure 15 psi), drying gas flow: 4 L/min, drying gas temperature: 325 °C. Fractions corresponding to the most representative compounds were collected, immediately freeze-dried under vacuum using a CentriVap bench-top centrifuge concentrator, and stored in sealed tubes at −20 °C.

2.5. Spectrophotometric assay of plasmin activities

To evaluate the direct effect of *S. minor* extract on plasmin activity, the apparent (substrate-dependent) equilibrium dissociation constants ($K_{d,app}$) of plasmin–inhibitor complex (either the whole extract or each isolated compound) were determined by measuring the decrease in catalytic activity of plasmin toward chromozym-PL [43] upon addition of increasing levels of inhibitor at different substrate concentrations in the range 30–150 μM. In detail, human plasmin (60 nM) was incubated at 37 °C with increasing quantities of whole extract/isolated compounds in 50 mM Na_2HPO_4, 0.1 M NaCl and 0.1% PEG 6000, pH = 7.5. After 10 min pre-incubation (enzymatic activities were not further affected at longer pre-incubation times), residual activities were measured at 410 nm by continuously monitoring the release of p-nitroaniline after the initiation of the reaction. The residual activity (a_i) was expressed as the ratio of the initial velocities of the product formation in the presence ($v_{0,i}$) and in the absence (v_0) of a given inhibitor concentration [I_i]:

$$a_i = \frac{V_{0,i}}{V_0} \tag{1}$$

The experimental dataset was constituted by a set of residual activities a_i measured at increasing inhibitor concentrations [I_i]. $K_{d,app}$ values were derived for each substrate concentration using the standard model (Eq. (2) [44]):

$$a_i = 1 - \frac{\left([E_t] - [I_i] + K_{d,app}\right) - \sqrt{\left([E_t] + [I_i] + K_{d,app}\right)^2 - 4[E_t][I_i]}}{2[E_t]} \tag{2}$$

where [E_t] is total plasmin concentration. $K_{d,app}$ is related to K_d according to the following equation (Eq. (3)):

$$K_{d,app} = K_d\left(1 + \frac{[\text{substrate}]}{K_{m,\text{substrate}}}\right) \tag{3}$$

For each substrate concentration ($m = 4$), residual activities were measured at increasing inhibitor concentrations ($n = 5$), and the resulting $n \times 2 \times m$ matrix was globally analysed by Marquardt–

Levenberg non-linear least squares fitting procedure [44] using Eqs. (2) and (3) as fitting functions.

2.6. Biosensor studies

A detailed evaluation of binding kinetics was performed using the IAsys *plus* resonant mirror biosensor. The biosensor sensing chamber was thermostatted at 37 °C. The surface was washed and equilibrated with PBS buffer (10 mM Na_2HPO_4, 2.7 mM KCl, 138 mM NaCl, 0.05%$_{(v/v)}$ Tween 20), then standardly activated with an equimolar mixture of EDC and NHS [45]. Plasmin (0.2 mg/mL) was dissolved in 10 mM acetate buffer, pH = 5.5, and incubated over the surface for 10 min. Free carboxylic sites on the sensor surface were deactivated by injection of 1 M ethanolamine, pH 8.5. The surface was finally re-equilibrated with PBS. Quercetin-3-glucuronide was added at different concentrations, and binding kinetics were routinely followed up to equilibrium. Dissociation steps and surface regeneration were performed by addition of fresh buffer, each time assessing the baseline recovery prior to any further addition of the ligand. Raw data were fitted to both monoexponential and biexponential models, and the model validity was assessed using the *F*-test procedure [44].

2.7. Molecular docking

The most probable binding site/pose for quercetin-3-glucuronide on human plasmin were established according to predictive molecular docking analyses performed on a Pentium4/Linux Red Hat based platform using Autodock 4.0 and InsightII R2005 software (Accelrys). Quercetin-3-glucuronide three-dimensional structure (CID: 5274585) was obtained from PubChem Compound database [46]. X-ray crystal structure of human plasmin catalytic domain (PDB ID: 1DDJ [47]) was obtained from the Protein Data Bank [48]. Hydrogen atoms were added to the protein prior to any analysis. Autodock software [49] was used with a grid of 48, 48, and 48 points (in the x, y, and z directions) around the catalytic site, a grid spacing of 0.375 Å, a root-mean-square (rms) tolerance of 0.8 Å, a maximum of 2.5×10^6 energy evaluations. Other parameters were set to default values [50]. Next, InsightII was used to refine the ligand/receptor models through an energy minimization procedure, using the Discover module of the software with a consistent valence force field and a conjugate gradients algorithm to a rms derivative of 0.001 kcal/mol. Results were expressed as predicted equilibrium dissociation constants K_D derived from LUDI Score (Ludi module), Energy Estimate 3 as scoring function [51]. The output from InsightII, all modelling studies, and images were rendered with PyMOL (2010, DeLano Scientific LLC, San Carlos, CA). PyMOL was also used to calculate the predicted length of hydrogen bonds as measured between the hydrogen and its putative binding partner.

2.8. Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [52]. After individual treatments with Q3G (210 μM), quercetin (220 μM), the whole extract (100 μg/mL) and aprotinin (3 μM), MTT was added to the culture medium at a final concentration of 0.5 mg/mL and incubated for 2 h at 37 °C. The medium was replaced with 100 μL of DMSO and the optical density was measured at 550 nm after 24 and 48 h in a microtiter plate reader. At least four cultures were used for each time point.

2.9. Modulation of tumor cell adhesion

Proteolytic ectodomain fragments of E-cadherin have been proposed to promote cancer cell invasion by interfering with E-cadherin function in cells containing intact E-cadherin/catenin complexes. E-cadherin expressing MCF-7 cell line was grown in minimum essential medium (MEM), 10% FBS, antibiotics and sodium pyruvate. Confluent monolayers of MCF-7 cells were washed twice with PBS and incubated in MEM containing plasmin (66.3 nM) and aprotinin (3 μM), whole *S. minor* extract (200 μg/mL), quercetin (220 μM) or quercetin-3-glucuronide (21, 105 and 210 μM) for 24 h. Next, MCF-7 cells were lysed with PBS containing 0.1% SDS, 1% Triton X-100 and 0.5% CHAPS.

Cell membrane fractions were electrophoresed on 10% SDS-polyacrylamide gel under reducing conditions and transferred onto Immobilon-P membranes. The membranes were blocked in TBS (Tris–HCl 10 mM, NaCl 0.5 M) containing 5% bovine serum albumin overnight at 4 °C and then incubated with a monoclonal antibody recognizing the extracellular domain of human E-cadherin for 2 h at 25 °C, followed by the incubation with the peroxidase-conjugated secondary antibody for 90 min at 25 °C. Specific protein bands were visualized with an ECL detection system [53].

As a control for equal protein loading, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included (membranes were stripped and re-probed for GAPDH using a polyclonal antibody). Densitometric values were normalized to the relative GAPDH signal intensity. Ratios of band intensities were calculated within the same Western blot [54].

All determinations were repeated in triplicate and densitometric analysis of the bands was performed using a program implemented in MatLab ver. R2010a (MathWorks Inc.—Massachusetts, U.S.A.).

2.10. Tumor cell motility

Scratch motility assay [55] was performed to evaluate the effect of Q3G on breast cancer cells migration. Briefly, MCF-7 human breast carcinoma cells were seeded at 6×10^5 cells/mL in a 6-well microtiter plate, and allowed to grow to reach confluence. The monolayer was scratched with a 10 μL-tip, washed twice with PBS buffer to remove floating cells, and eventually treated with plasmin (66.3 nM) in the absence or in the presence of Q3G (210 μM), quercetin (220 μM), the whole extract (200 μg/mL) and aprotinin (3 μM), individually. After incubation, cells migrating into the cell-depleted zone (the scratched area) were monitored at 5 randomly selected fields and cell-covered areas were calculated at 24 and 48 h using ImageJ software (http://rsb.info.nih.gov/ij/). Results were expressed as percentage wound closure with respect to control cells at 48 h.

3. Results

3.1. Chromatographic analysis

S. minor ethanol extract was characterized by reversed-phase HPLC. Chromatogram profiles obtained under the conditions described in the experimental section showed three main peaks (Fig. 1, inset A). The compound eluted at 23 min was successfully identified as quercetin-3-glucuronide, both by comparison of the retention time with a reference standard and ultimately by ion trap mass spectrometry (molecular ion $[M^-] = 476.9\ m/z$; fragment ions $[M_1] = 300.9\ m/z$, $[M_2] = 178.7\ m/z$; Fig. 1, insets B and C, respectively). Q3G accounted approximately for 10%$_{w/w}$ of total phenolic content. Purity (>98%) was assessed in accordance with a previously published method [56].

3.2. Docking analysis

Studies on the docking of Q3G to the X-ray crystal structure of human plasmin provided novel insights on both interaction strength and geometry. Q3G showed a moderately-high predicted affinity for the enzyme ($K_{D,pred} = 257$ nM), with the catalytic pocket of the protease being calculated to be the most likely to accommodate the Q3G

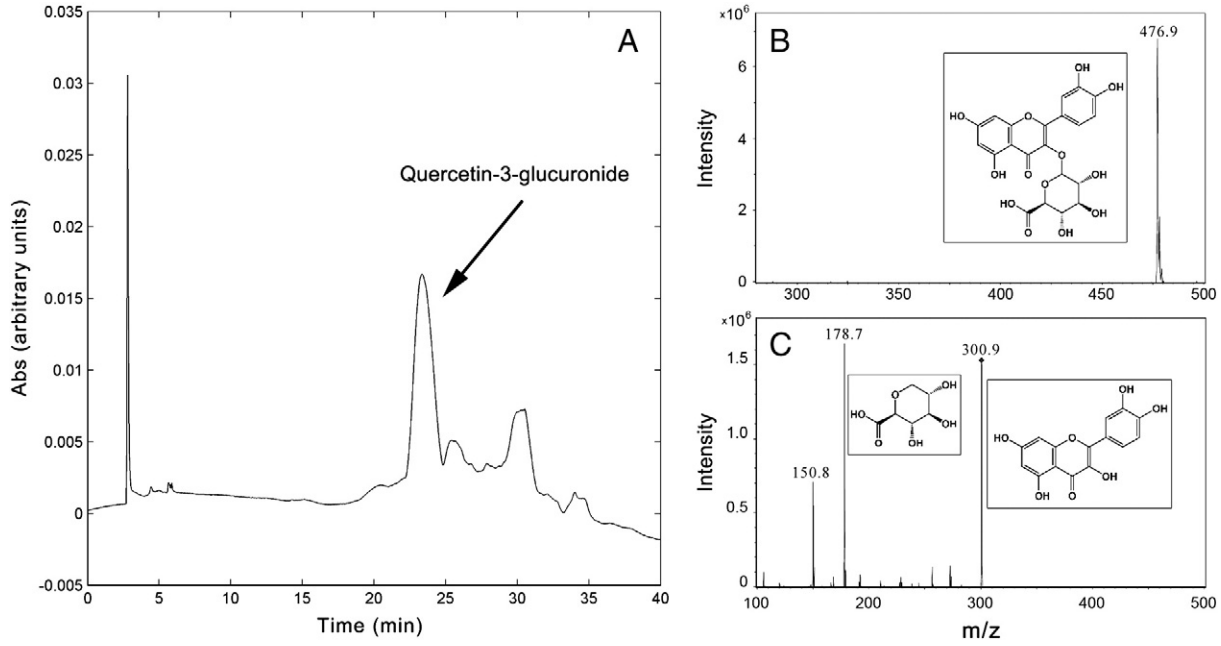

Fig. 1. Representative HPLC chromatogram of the *S. minor* extract (inset A). Molecular ion (inset B) and fragmentation pattern (inset C) of isolated quercetin-3-glucuronide obtained by the ion trap mass spectrometer (ion scan mass spectra were recorded in negative ionization mode).

molecule (Fig. 2, inset A). In particular, Q3G–plasmin complex was characterized by the formation of 4 theoretical H-bonds (mean length = 2.2 Å), respectively involving Arg^{264} ($n = 2$), Glu^{606}, Gly^{762} (see Fig. 2, inset B), and by a significant non-electrostatic energy contribution of −60 kcal/mol. Compared to previous data on quercetin/plasmin complex [44], Q3G/plasmin complex showed a different orientation, with the flavonoid skeleton being rotated of 180 degrees about its longitudinal axis, and the 3-glucuronide group pointing out to the solvent. This pose contributed to maximize the hydrophobic interactions of their binding interfaces and to stabilize the complex.

3.3. Effects on plasmin amidolytic activity

Both Q3G and the whole ethanol extract affected plasmin activity, as clearly reported in Fig. 3. In particular, in the presence of increasing levels of isolated Q3G and whole *S. minor* extract, we obtained hyperbolic inhibition isotherms, whose steepness decreased at increasing (saturating) substrate concentrations (Fig. 3, insets A, C), resulting in the apparent decrease of the inhibition potency of the compounds. In fact, under these conditions the apparent dissociation constants ($K_{D,app}$) increased linearly with substrate concentrations, unambiguously proving the competitive targeting of the catalytic pocket of plasmin by Q3G (Fig. 3, inset D)[57]. The experimentally measured equilibrium dissociation constant for the enzyme-flavonoid complex ($K_{D,Q3G} = 140 \pm 15$ nM) was in good agreement with the computationally predicted values, whereas the whole extract inhibited human plasmin activity with a $K_{D,extract} = 0.34 \pm 0.10$ mg/L.

Analytes in the fractions eluted at different retention times did not affect plasmin activity significantly.

3.4. Kinetics of binding

A more comprehensive analysis of binding kinetics of Q3G to human plasmin was performed on an IAsys *plus* biosensor. Sensing surface containing anchored protease was achieved after different

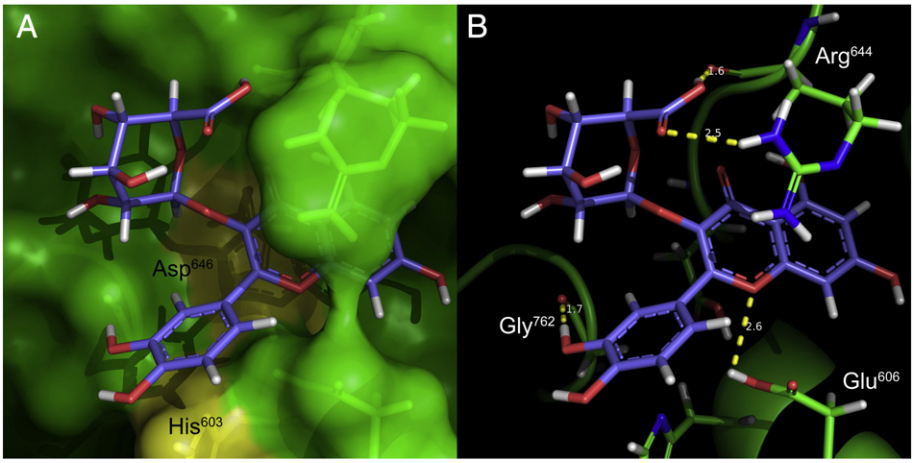

Fig. 2. Surface (inset A) and cartoon and stick (inset B) visualizations of the molecular docking of Q3G into the catalytic site of human plasmin. Theoretical h-bonds formed are represented as yellow dotted lines.

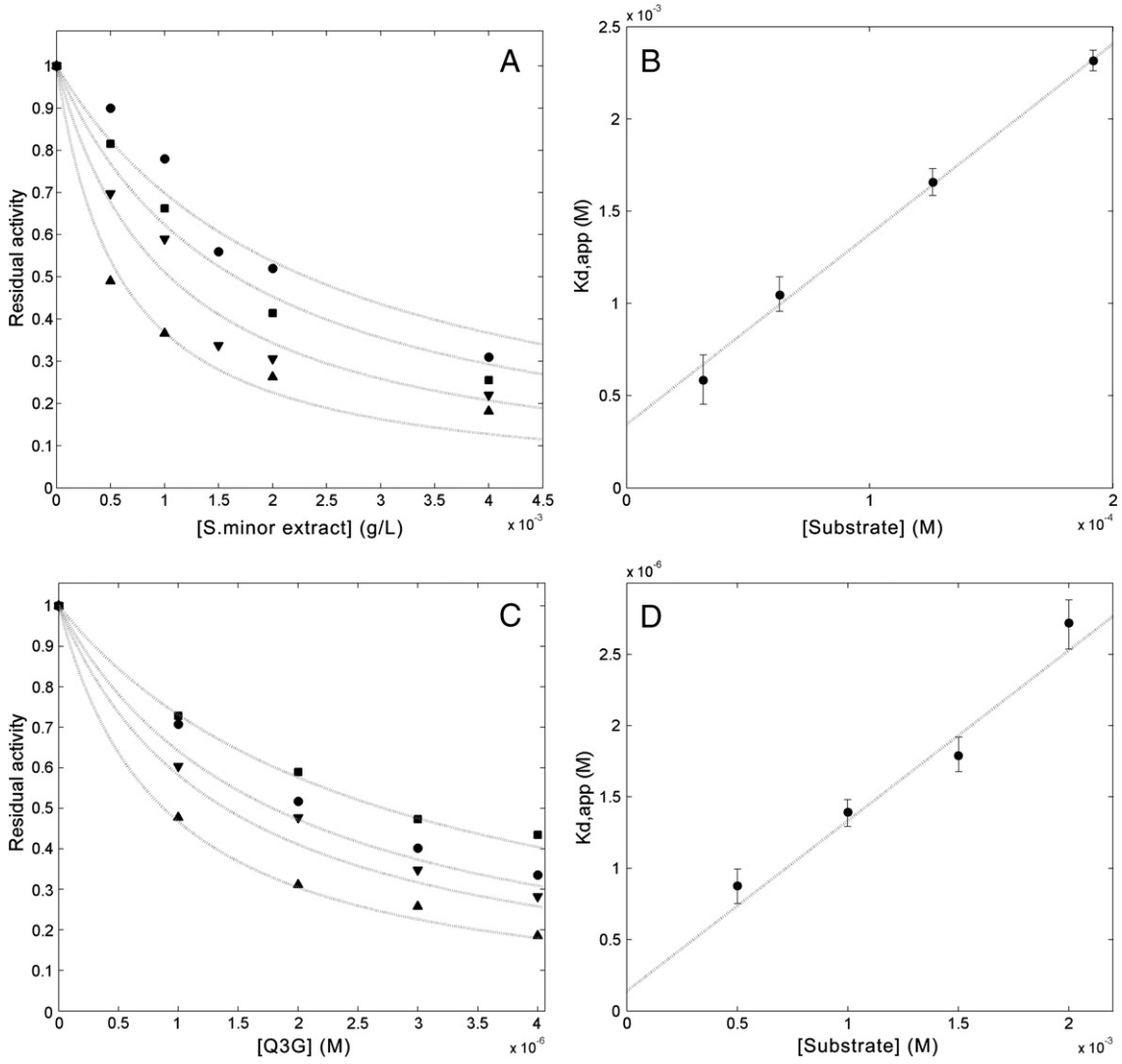

Fig. 3. Inhibition of human plasmin amidolytic activity. Plasmin residual activities versus modulator concentration measured at four different levels of plasmin substrate 30 μM (▲), 90 μM (▼), 120 μM (●), 150 μM (■) (insets A and C, respectively for the whole *S. minor* extract and isolated Q3G). Linear dependence of apparent equilibrium dissociation constants resulting from global fit of raw data upon increasing substrate levels (insets B and D, respectively for the whole *S. minor* extract and isolated Q3G).

experiments based upon the optimization of both the enzyme concentration and immobilization buffer composition. Readout of about 900 arcsec was obtained upon the immobilization of human plasmin, and the corresponding amount of the capturing agent was calculated. These conditions resulted in the coupling of a 'Langmuir' partial monolayer for a 75 kDa protein (surface concentration = 1.6 ng/mm^2). Then, Q3G was added at increasing concentrations in the range 0.036–6.64 μM, and kinetics of association were followed up to equilibrium (Fig. 4, inset A). Whenever needed, surface regeneration rate was increased without affecting its stability by CH_3COONa washes at pH 5.5, the affinity of the interaction being significantly diminished at lower pH conditions. The longevity of the bio-layer was also tested: surface stability and enzyme activity were fully retained upon at least 50 binding events. Raw data of time courses measured at several ligand concentrations were globally-analysed as previously reported [58]. This experimental approach confirmed the high affinity interaction between soluble Q3G and anchored plasmin ($K_D = 175.2 \pm 22.8$ nM), with both fast association ($k_{ass} = 17692 \pm 262\ M^{-1}\ s^{-1}$) and slow dissociation ($k_{diss} = 3.1 \pm 0.4\ ms^{-1}$) events significantly contributing to the stability of the complex. Monophasic time courses were always reported upon the addition of soluble Q3G (the validity of monophasic model to fit time courses was assessed by a standard *F*-test procedure, with the biphasic model being statistically non-significant at 95% statistical confidence). The binding response at equilibrium was calculated for each time course with a resulting fully comparable equilibrium dissociation constant ($K_{D^*} = 199.2 \pm 21.8$ nM). Moreover, the hyperbolic nature of the saturation plot (Fig. 4, inset B), proved the non-cooperative binding of Q3G to human plasmin.

3.5. Modulation of tumor cell adhesion

The cleavage of E-cadherin in MCF-7 cells was performed upon treatment with human plasmin. In order to evaluate the inhibitory effect exerted by Q3G on the proteolytic cleavage of E-cadherin, we exposed cells to increasing amounts of the flavonoid and a fixed amount of plasmin in the culture medium. Clearly, the addition of plasmin to the medium led to a decrease in the level of the 120 kDa E-cadherin (Fig. 5, lane 3) with respect to non-treated cells (lane 1), whereas co-treatment with aprotinin (a potent plasmin inhibitor, used as positive control) fully suppressed proteolysis of E-cadherin (lane 2). The treatment with Q3G significantly reduced the cleavage of E-cadherin by plasmin in a concentration dependent manner (Fig. 5, lanes 6–8) to a similar extent. Comparable concentration of quercetin and the whole extract showed lower effects (lanes 4 and 5, respectively).

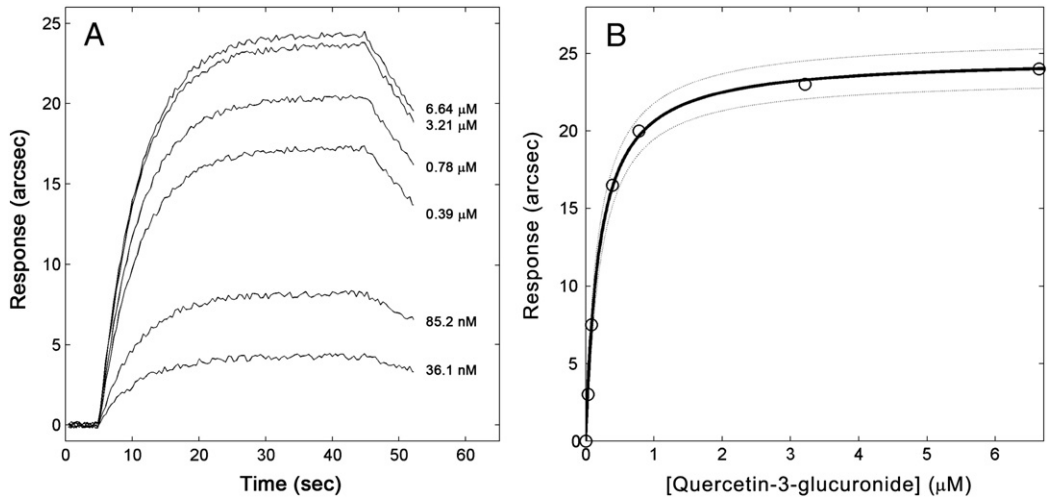

Fig. 4. Overlay of association and dissociation kinetics of soluble Q3G to surface-bound human plasmin (inset A); dependence of the extent of binding from ligand concentration (inset B). Nonlinear fit (solid line) and 95% confidence-bound (dashed lines) are reported. Each experimental point was the average of five replicates.

3.6. Inhibition of cancer cell motility

The effect of Q3G on the plasmin-induced migration of MCF-7 cells was tested according to a standard scratch motility assay. Upon creation of a gap on the confluent cell monolayer, the number of migrating cells within the cell-depleted zone was calculated from five randomly selected fields *per* sample and represented as percentage wound closure with respect to fully healed wound (control cells at 48 h). Control cells exhibited a progressive wound closure after treatment for 24 and 48 h, respectively (Fig. 6), whereas plasmin treatment notably increased the rate of cell motility, resulting in the complete wound closure yet at 24 h. On the contrary, Q3G-treated cells significantly retarded wound closure at any time point, displaying a minor narrowing of wound margins. Q3G displayed higher

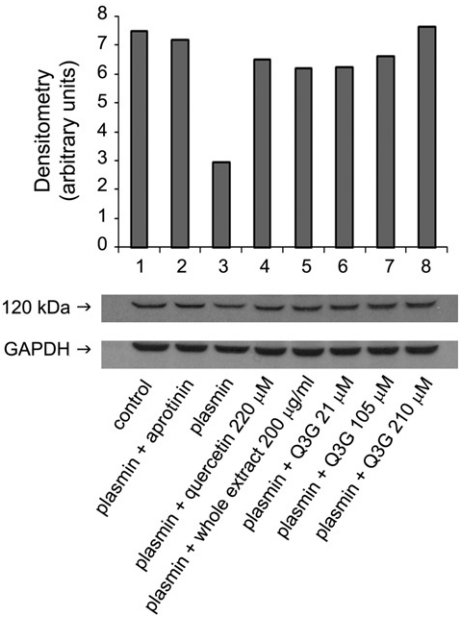

Fig. 5. Suppression of plasmin-mediated release of E-cadherin. A representative western blot of E-cadherin is presented and the densitometric analysis from three separate blots (upper inset) provided for E-cadherin 120 kDa cell membrane fragment. Cells were treated with plasmin (66.3 nM) in the absence (lane 3) or in the presence of Q3G (lane 6: 21 μM; lane 7: 105 μM; lane 8: 210 μM), quercetin (lane 4: 220 μM), or the whole *S. minor* extract (lane 5: 200 μg/mL). Untreated loading control is labelled 1. Positive control (cells treated with plasmin + aprotinin 3 μM) is reported in lane 2. GADPH was used to check protein loading (lower panel).

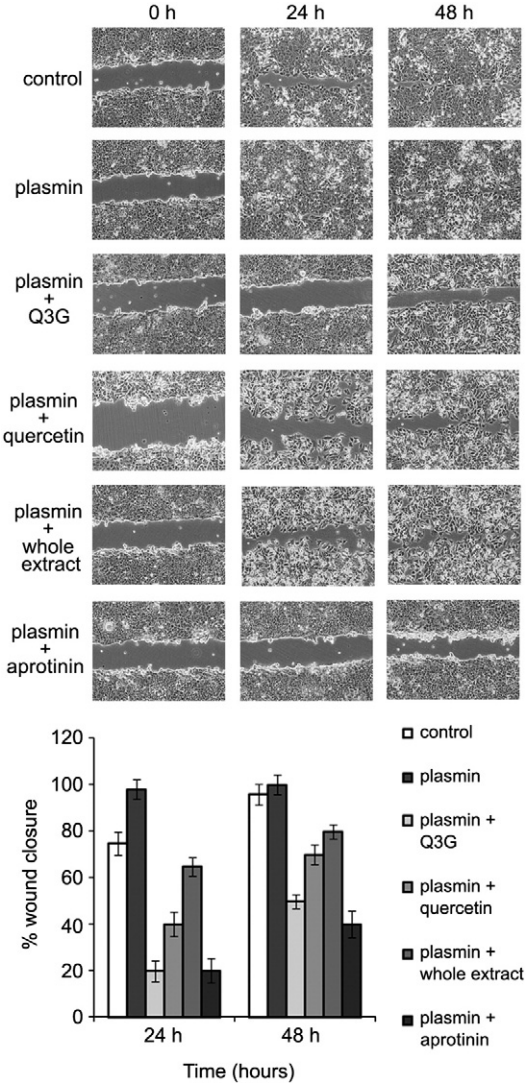

Fig. 6. Effect of Q3G, quercetin and whole extract on MCF-7 cell migration. A confluent monolayer was scratched and wound closure was monitored using a microscope equipped with a camera after 24 and 48 h in the absence or in the presence of plasmin (66.3 nM) and of isolated Q3G (210 μM), quercetin (220 μM), whole extract (200 μg/mL), or aprotinin (3 μM). Each experiment was repeated at least five times. Results were expressed as percentage wound closure (*$P<0.05$ compared with control).

efficacy with respect both to quercetin and to the whole extract. Interestingly, the effect exerted by Q3G was still significant compared to aprotinin, particularly at 24 h.

4. Discussion

Natural products are ideal templates for drug design and engineering, as they evolved to serve specific purposes in biological systems such as modulating functions of target enzymes [59–61]. With this regard, flavonoids (and polyphenols in general) have been proposed to be potentially central in cancer prevention and treatment, as these secondary metabolites are abundant in plants traditionally associated with decreased cancer rates [62,63], and a number of plausible mechanisms by which the intake of vegetables and fruits may inhibit carcinogenesis have been offered. In general, these mechanisms involve anti-estrogenic [64] and anti-proliferative activities [65,66], induction of apoptosis [67,68], protection from oxidation [69], induction of detoxification enzymes [70], regulation of the host immune system [71], changes in cellular signalling [72], or direct enzyme targeting [73,74].

Concerning these latter, fibrinolytic enzymes, plasmin in particular, were shown to promote cancer cells growth and the invasiveness [75], suggesting that their unregulated activities could enhance metastatic potential of cancer cells [76]. This hypothesis was supported by various experimental models, reporting the inhibition of both cancer invasion and metastasis upon plasmin inactivation [20]. Interestingly, over-activated plasminogen–plasmin pathway was reported in lung cancer from early stages of disease [77], supporting the potential efficacy of plasmin(ogen) inhibitors for the treatment of lung cancer. Based on these evidences, the effect of an ethanol extract from *Sanguisorba minor* obtained under sub-critical extractive conditions on human plasmin activity was here evaluated and dissected. Quercetin-3-glucuronide, a glucuronated flavonol approximately accounting for 10% of total phenolic content of the extract, was identified as the major contributor to the observed anti-plasmin properties. The interaction between Q3G and plasmin was quantitatively studied and the 1:1 reversible complex was characterized by an equilibrium dissociation constant in the high nanomolar range, displaying a 3-fold higher stability in terms of K_D values with respect to parent quercetin. Consistently with previous studies on site-directed ligands of plasmin (structurally resembling quercetin)[44], Q3G acted as a competitive inhibitor, by targeting the protease active site. These results were supported by data from *in silico* docking experiments, reporting a favourable positioning of Q3G into the catalytic site of plasmin.

Similar to *in vitro* results on the isolated enzyme, both plasmin-induced loss of cell adhesion and increase in cell motility were relevantly suppressed by non-cytotoxic levels of Q3G in MCF-7 cancer cells (cell viability data not shown), more efficiently than both the whole extract and quercetin, and most importantly, comparably to aprotinin, a potent inhibitor of plasmin reported to suppress cancer cell invasiveness [30]. Hence Q3G represents a noteworthy dietary regulator of intercellular adhesion and cell motility, although significant effects were reported only upon treatment with higher levels of the flavonol ($[Q3G] \gg K_D$): this is attributable to the general ability of flavonoids to interact with a number of cell components (redox enzymes, in particular), as extensively documented [78–80]. Nevertheless, the physiological importance of these findings can be positively claimed, as several evidences recently demonstrated that glucuronide and sulphate metabolites of quercetin (one of the most abundant flavonoid in human diet) accumulate in plasma [81], and here we demonstrated that the metabolic conversion could enhance (at least some of) its biological effects.

Globally, the relevant properties displayed by this flavonoid from *S. minor* represent a stimulating starting point both for the design of strategies for cancer prevention/co-treatment through diet customization, and for the development of new pharmaceutical agents that may be used in the treatment of pathological states characterized by unbalanced plasmin activity, possibly due to a deficiency in α2-antiplasmin [82], its major systemic inhibitor.

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