CMLS, Cell. Mol. Life Sci. 60 (2003) 1440–1448
1420-682X/03/071440-09
DOI 10.1007/s00018-003-3119-3

CMLS Cellular and Molecular Life Sciences

Research Article

Inhibition of metalloproteinase-9 activity and gene expression by polyphenolic compounds isolated from the bark of *Tristaniopsis calobuxus* (Myrtaceae)

S. Bellosta[a], M. Dell'Agli[a], M. Canavesi[a], N. Mitro[a], M. Monetti[a], M. Crestani[a], L. Verotta[b], N. Fuzzati[c], F. Bernini[d] and E. Bosisio[a,*]

[a] Department of Pharmacological Sciences, University of Milan, via Balzaretti 9, 20133 Milan (Italy),
Fax: + 39 02 5031 8391, e-mail: enrica.bosisio@unimi.it
[b] Department of Organic and Industrial Chemistry, University of Milan, Milan (Italy)
[c] INDENA S.p.A, Milan (Italy)
[d] Department of Pharmacological and Biological Sciences and Applied Chemistry, University of Parma (Italy)

Received 28 March 2003; accepted 10 April 2003

Abstract. Excessive breakdown of extracellular matrix by metalloproteinases (MMPs) occurs in many pathological conditions, and thus inhibition of MMP activity might have therapeutic potential. The methanolic extract and the identified compounds from the bark of *Tristaniopsis calobuxus* Brongniart & Gris (Myrtaceae) were tested on the activity, production, and gene expression of MMP-9. The extract produced a concentration-dependent inhibition (50–95% at 10–50 µg/ml) of MMP-9 activity. The inhibitory activity was retained in the ethyl acetate-soluble fraction (50–95% inhibition at 10–50 µg/ml) which also reduced the release of MMP-9 by mouse peritoneal macrophages up to 80%. In the ethyl acetate-soluble fraction, two active fractions, 5A and 5B were identified. HPLC-MS and NMR analyses of these fractions indicated the presence of gallocatechin, ellagic acid, and its glycoside derivatives. Since the absolute configuration of gallocatechin was not determined, in the next experiments both (+)-gallocatechin (2R,3S) and (–)-gallocatechin (2S,3R) were tested, and (–)-epigallocatechin (2R,3R) was included for comparison. 5A and 5B inhibited MMP-9 secretion, an observation which correlated with the decrease of MMP-9 promoter activity and the downregulation of mRNA levels. All compounds decreased MMP-9 mRNA levels and secretion. Ellagic acid, (+)-gallocatechin and (–)-epigallocatechin, but not (–)-gallocatechin inhibited promoter-driven transcription. Thus configuration at C2 (*R*) of the flavanol seem to be critical for the interaction with the promoter.

Key words. Metalloproteinase-9; ellagic acid; (+)-gallocatechin; (–)-gallocatechin; (–)-epigallocatechin; gene expression; secretion; plant polyphenols; *Tristaniopsis calobuxus.*

Degradation of extracellular matrix (ECM) is an important feature in many physiological and pathological conditions. ECM can be degraded by four classes of proteolytic enzymes: cysteine proteinases, aspartic proteinases, serine proteinases and metalloproteinases (MMPs) [1]. MMPs belong to a family of structurally related, zinc-containing enzymes, and more than 20 different MMPs have been described [2]. MMPs are secreted as proenzymes, act extracellularly at physiological pH, and require activation from proenzyme precursors to attain enzymatic activity [3]. Once activated, MMPs can com-

* Corresponding author.
S. Bellosta and M. Dell'Agli contributed equally to this paper.

pletely degrade all ECM components. MMPs play an important role in ECM remodelling in physiological situations, such as fetal tissue development and postnatal tissue repair. Excessive breakdown of ECM by MMPs occurs in many pathological conditions implicated in connective tissue destruction and remodelling associated with cancer invasion and metastasis, cartilage destruction in arthritis, atherosclerotic plaque rupture and the development of aneurysms [4]. The 92-kDa gelatinase B or MMP-9 is expressed by virtually all activated macrophages and, through the degradation of the basement membrane, it facilitates macrophage extravasation. MMP-9 has been shown to be present in atherectomy materials from unstable angina [5] and abdominal aortic aneurysm [6]. In addition, tumour invasiveness is correlated with the level of MMP-9 expression [7]. MMP secretion is increased in various inflammatory, malignant, and degenerative diseases, and thus the inhibition of MMP activity might have therapeutic potential. Experimental observations indicate that lipid lowering, obtained by dietary intervention, may stabilize vulnerable atherosclerotic plaques by reducing the secretion and activity of enzymes that degrade the arterial ECM [8]. Our previous results showed that statins, a class of drugs that can achieve relatively large reductions in plasma cholesterol levels and represent an established treatment of hypercholesterolaemia [9], are able to reduce MMP-9 secretion by mouse and human macrophages, independently of their hypolipidaemic effect [10]. Besides lipid lowering, the pharmacological reduction of MMP-9 release by macrophages in vitro results in an overall reduction in collagen degradation [11].

Green tea and its polyphenolic constituents have been reported to suppress gelatin degradation mediated by MMP-2 and MMP-9 in several conditions [12, 13], to inhibit angiogenesis and tumour invasion [13–15], and human vascular smooth muscle cell proliferation [16]. A green tea extract rich in polyphenolic compounds inhibits MMP-9 activity up to 80% at 35 µg/ml [17]; the inhibition is correlated with the presence of (–)-epicatechin-3-gallate and (–)-epigallocatechin-3-gallate (IC_{50} 28 µM and 0.3 µM, respectively). Moreover, another family of plant polyphenols, flavonoids, have revealed their capacity to interact with different zinc metallopeptidases [18, 19].

In previous studies, we found that the methanolic extract of the bark of *Tristaniopsis calobuxus* Brongniart & Gris (Myrtaceae) displayed antielastase activity [20] and that ellagic acid, isolated from the extract, inhibited plasmepsin II [21], an aspartic protease specific to *Plasmodium falciparum* [22]. In the present work, we studied the effect of *T. calobuxus* extract on MMP-9 activity and secretion. Following activity-guided fractionation, several constituents were isolated and tested both on enzyme secretion and protein gene expression.

Materials and methods

Chemicals

All cell culture reagents were purchased from Invitrogen (Life Technologies, Milan, Italy). The plasmid 2.2-Luc, containing the MMP-9 promoter fused to a luciferase reporter gene, was a kind gift of C. K. Glass (Department of Cellular and Molecular Medicine, School of Medicine, University of California, San Diego, CA). Restriction enzymes, luciferin, and the plasmid pGL3-promoter containing the SV40-early gene promoter were obtained from Promega (Milan, Italy). The pCMVβ-galactosidase plasmid was from Clontech (Palo Alto, CA). The Qiagen plasmid purification kit was purchased from Qiagen (Milan, Italy). The African green monkey cell line CV-1 was purchased from the American Type Culture Collection (Manassas, Va.). C57BL/6J mice were purchased from Charles River (Calco, Italy). Gallic acid, ellagic acid (EA), (–)-gallocatechin (2S,3R) (GC), Dulbecco's modified Eagle's medium and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Milan, Italy). (–)-Epigallocatechin (2R,3R) (EGC) was from Extrasynthèse (Genay, France), (+)-gallocatechin (2R,3S) was a generous gift from Prof. Delcour (Heverlee, Leuven Bioproducts, Belgium). All other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany).

Plant extraction

The extraction of the bark of *T. calobuxus* and the fractionation of the methanolic (MeOH) extract were performed as described elsewhere [23]. Briefly, the MeOH extract was fractionated into CH_2Cl_2-, ethyl acetate (EtOAc)- and water-soluble phases. The EtOAc-soluble fraction, which retained most of the biological activity of the total extract, was chromatographed on Sephadex LH-20, obtaining seven fractions. Some compounds were isolated and identified as described previously [23]: gallic acid, EA, *p*-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid. Since fraction 5 was biologically active, it was partitioned in BuOH-nPrOH-H_2O 2:1:3 to give the organic phase 5A and the aqueous phase 5B. The chemical composition of 5A and 5B was analysed by HPLC-DAD-UV-ESI-MS and NMR.

HPLC-DAD-UV-ESI-MS and NMR analysis of 5A and 5B

A Finnigan MAT (San Jose, CA) LCQ ion trap mass spectrometer equipped with a Microsoft Window NT data system and an electrospray interface (ESI) was used. The HPLC system included a Thermo Separation Product P4000 pump (San Jose, CA) and a Diode Array Thermo Separation Products UV6000LP detector. Separation was performed on a Zorbax Eclipse SB-C18 (5 µm) column (250 × 4.6 mm I.D.) from Agilent Technologies (Hewlett-Packard, Waldbronn, Germany). A step gradient of 0.01%

v/v trifluoroacetic acid in water (A) and 0.01% v/v trifluoroacetic acid in acetonitrile (B) was used according to the following profile: 0–40 min, 95–50% A, 5–50% B; 40–41 min, 50–0% A, 50–100% B; 41–44 min, isocratic 100% B. The flow rate was 1.0 ml/min. Column temperature, controlled with a column heater-cooler HP Series 1100 from Hewlett-Packard (Waldbronn, Germany), was set to 25°C. Mass spectrometer conditions were optimized to achieve maximum sensitivity. DAD detection was undertaken in the range 200–500 nm. ESI conditions: source voltage 80 kV, sheath gas flow rate 90, auxiliary gas flow 60 psi, capillary voltage 19.0 V, and capillary temperature 250°C. Full scan spectra from 150 to 1000 u in the negative ion mode were obtained (scan time 1 s). Ion trap conditions: acquisition in automatic gain control with a max-inject time of 200 ms. For the MS^n analysis, a collision energy of 35 eV was used.

^{1}H-NMR spectra were recorded on a Bruker Advance 400 MHz, in CD_3OD. The intense H_2O signal (δ 3.2 ppm) was saturated using a NOESY sequence available in the Bruker software (D1 = 5 s, mixing time = 0.3 ms, PL9 = 50 dB).

Cell culture

Mouse peritoneal macrophages were collected by peritoneal lavage with phosphate-buffered saline (PBS) from C57BL/6J mice and plated as described previously [11]. CV-1 cells were plated in 24-well plates (10^5 cells/well) the day before transfection in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum.

To generate the conditioned media, macrophages were incubated for 24 h at 37°C with DMEM, supplemented with 0.2% BSA in the absence or presence of the indicated concentrations of compounds. At the end of the incubation, the conditioned media were collected and the gelatinolytic capacity of secreted MMP-9 analysed by zymography. Cellular protein content was measured according to Lowry [24]. For quantitation of zymograms, densitometric scanning was performed using a system incorporating a video camera and a computer analysis package (NIH Image 1.52 image analysis software). Each experiment was performed at least three times with different preparations of cells. Results were normalized by cellular protein content and expressed as arbitrary optical density (OD) units. Data are presented as the mean ± SD and analysed using the Dunnett test.

SDS-PAGE zymography

MMP-9 gelatinolytic activity was evaluated as described previously [11]. Briefly, samples underwent electrophoresis on 7.5% polyacrylamide gels containing 10% SDS and gelatin (1 mg/ml). The gels were then washed in 2.5% Triton X-100 (Sigma) at room temperature and then incubated overnight at 37°C (Tris 50 mM pH 7.5 containing NaCl 150 mM, $CaCl_2$ 10 mM, $ZnCl_2$ 1 µM; activation buffer). At the end of the incubation, the gels were stained with Coomassie brilliant blue R-250 (Sigma-Aldrich). EDTA 2 mM was used as reference compound. To test the effect of the extracts on the activity of secreted MMP-9, conditioned medium was electrophoresed as described above and the tested compounds added in the activation buffer.

Transient transfection assays

Transfections of CV-1 cells were performed by the calcium phosphate co-precipitation technique [25] for 16 h at 37°C. Briefly, a unique co-precipitate containing the MMP-9 promoter/luciferase or pGL3-promoter plasmid plus pCMVβ-gal was prepared and aliquoted in different wells to assure that all samples were transfected with the same amount of plasmid DNA (1.2 µg of luciferase plasmid plus 0.3 µg of β-galactosidase plasmid DNA/well). At the end of the transfection, cells were washed with PBS and incubated for 24 h in medium containing the extracts or the compounds to be tested or the vehicle (ethanol 0.1%).

Enzyme assay

Luciferase and β-galactosidase assays were performed using a luminometer (Lumat 9501; Berthold, GSG Nuclear srl, Bresso, Italy) and a microtiter plate reader (Bio-Rad, Hercules, CA) respectively, as previously described [25]. Luciferase activities were normalized versus galactosidase activities. Results are expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD values of triplicate samples. Each experiment was repeated at least twice. Statistical analyses were performed using the Student t test.

Cytotoxicity assay

Cellular toxicity caused by the extracts and the compounds was assessed both by measuring cellular protein [24] and by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) colorimetric assay [26]. Cells were treated with increasing concentrations of extracts or compounds for 24 h in DMEM supplemented with 10% heat-inactivated FBS. The medium was removed, and cells were incubated with a solution containing MTT 0.5 mg/ml in PBS at 37°C for 3 h. The MTT solution was removed and MTT formazan was extracted with isopropanol : DMSO (9 : 1, v/v; 500 µl/well) for 15 min at 37°C. Aliquots of 100 µl were read on a plate reader (Bio-Rad) at 560 nm (reference wavelength 690 nm). No sign of cytotoxicity was found. Cell proliferation was also unaffected (control cells: 51 µg protein/ plate; CV-1 cells treated with fraction 5A: 57 µg protein/ plate; CV-1 cells treated with fraction 5B: 61 µg protein/plate).

mRNA analysis by real-time quantitative RT-PCR

Murine peritoneal macrophages were treated with different compounds or extracts. Six replicates per treatment

were pooled and total RNA extracted with the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, LaJolla, CA), which includes a step of treatment with Dnase to remove contaminating genomic DNA from the samples. Pooled total RNA (1 µg) was reverse transcribed using Superscript II (Invitrogen, Milan, Italy) enzyme according to the manufacturer's instructions. The real-time PCR reactions were carried out in an iCycler iQ™ Detection System (Bio-Rad). Two-step real-time PCR reactions were performed with an SYBR Green kit (Qiagen, Milan, Italy), using the following conditions: activation step at 95°C followed by 40 cycles with 95°C denaturation step for 15 s and annealing/extension at 60°C for 60 s. After the final PCR cycle, a melting curve was performed to determine the specificity of the amplicons. Primers used were: mouse MMP-9 forward primer 5′-TGCCGTCGAAG GGATACC-3′, mouse MMP-9 reverse 5′-GACCCGAAG CGGACATTGT-3′, mouse 36B4 forward primer 5′-CG-CAACGTGGCCAGTGT-3′, mouse 36B4 reverse 5′-GGA AGGGTGTACTCAGTCTCCACAGA-3′. All the samples were tested in duplicate and relative expression of MMP-9 was calculated by normalizing the threshold cycle (C_t) of MMP-9 with the C_t of 36B4 mRNA to correct for variations in RNA loading. Dexamethasone 0.1 µM was used as reference compound. Each experiment was performed twice with different cell preparations.

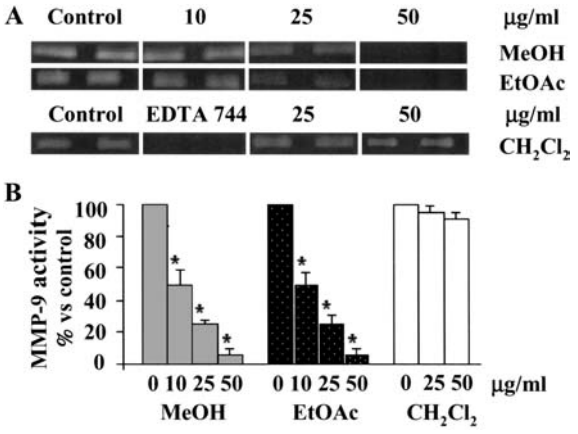


Figure 1. Effect of the *Tristaniopsis calobuxus* extracts on the activity of secreted MMP-9. (*A*) Representative gelatin zymograms showing the effect of the extracts of the bark of *T. calobuxus* on the activity of already secreted MMP-9. Mouse macrophages were incubated with medium alone for 24 h. Then the conditioned media were collected, run on gelatin gels, and the indicated compounds added into the activation buffer, as described in Materials and methods. (*B*) Data were quantified by densitometry scanning and expressed as the mean ± SD of three experiments performed in duplicate. * $p < 0.001$ vs control (untreated cells).

Results

Inhibition of MMP-9 activity and secretion by *T. calobuxus* extracts

In the first set of experiments, we tested if the methanolic extract affected the in vitro activity of MMP-9 (gelatinase B). As shown in figure 1A, the MeOH extract inhibited the activity of secreted MMP-9. The effect was concentration dependent (from 50% at 10 µg/ml up to 95% at 50 µg/ml) and statistically significant ($p < 0.001$; fig. 1B). The inhibitory activity was retained mainly in the EtOAc-soluble fraction, while the CH_2Cl_2-soluble fraction did not show an appreciable effect (fig. 1). The inhibition by the water-soluble phase was negligible (data not shown).

The MeOH extract and the EtOAc-soluble fraction (10–50 µg/ml), but not the CH_2Cl_2-soluble fraction, inhibited MMP-9 secretion by mouse macrophages in a concentration-dependent fashion and the effect started and was already statistically significant at 10 µg/ml (fig. 2). Following activity-guided fractionation, as described in Materials and methods, the inhibitory activity was associated with fractions 5A and 5B (12–50% inhibition at 1–20 µg/ml). The effect of the two fractions was comparable.

Secretion experiments were also performed in CV-1 cells. This cell line is able to secrete measurable amounts of MMP-9 (by gelatin zymography). The incubation with fraction 5A and 5B for 24 h reduced the activity of MMP-9 by 37% and 49%, respectively.

Composition of fractions 5A and 5B

HPLC chromatograms of 5A and 5B are shown in figures 3 and 4. Two different wavelengths (210 and 254 nm) were used to detect all peaks in fractions 5A and 5B, since the compounds gave different responses to UV detection. MS detection allowed the compounds present in fractions 5A and 5B to be identified as gallocatechin, catechin derivatives, EA glycosides (xyloside, arabinoside and rhamnoside), and EA. 5A and 5B showed a similar composition, but in 5B, catechin derivatives and EA glycosides occurred in lower amounts. In the ^{1}H-NMR spectrum of 5A, (fig. 5), although too complex to be fully interpreted, the peaks present in specific zones of the spectrum, and indicated as reported in the legend to figure 5, supported the hypothesis derived from the MS interpretation. In both fractions, the occurrence of flavan-3-ol oligomers was proved by reaction with 5% n-butanolic HCl at 90°C, leading to a red colour.

Since the absolute configuration of GC in 5A and 5B could not be determined, in the next experiments both (+)- and (–)-GC were assayed. Their presence could not be quantified due to broad HPLC peaks.

Effect of single polyphenolics on MMP-9 secretion

EA, (+)-GC, (–)-GC, and for comparison (–)-EGC were tested on MMP-9 secretion at 30 µM (10 µg/ml). All

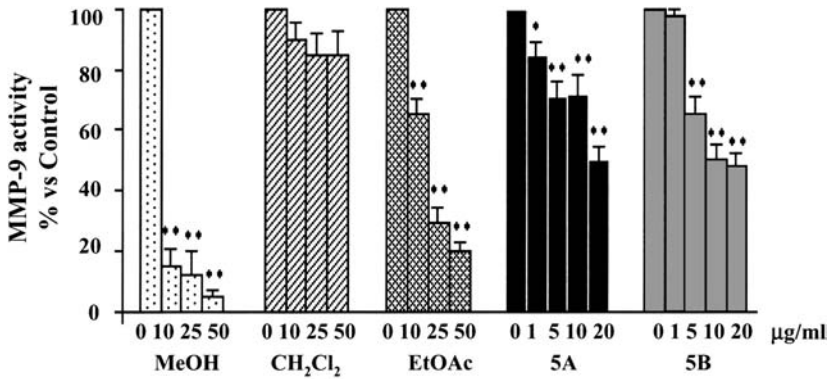


Figure 2. Effect of the *T. calobuxus* extracts on the secretion of MMP-9 by mouse macrophages. Cells were incubated for 24 h with DMEM containing increasing concentrations of the indicated extracts. Then, media were collected and MMP-9 gelatinolytic activity measured by gelatin-zymography. Data were quantified by densitometry scanning and expressed as the mean ± SD of three experiments performed in duplicate. * $p < 0.01$, ** $p < 0.001$ vs control (untreated cells).

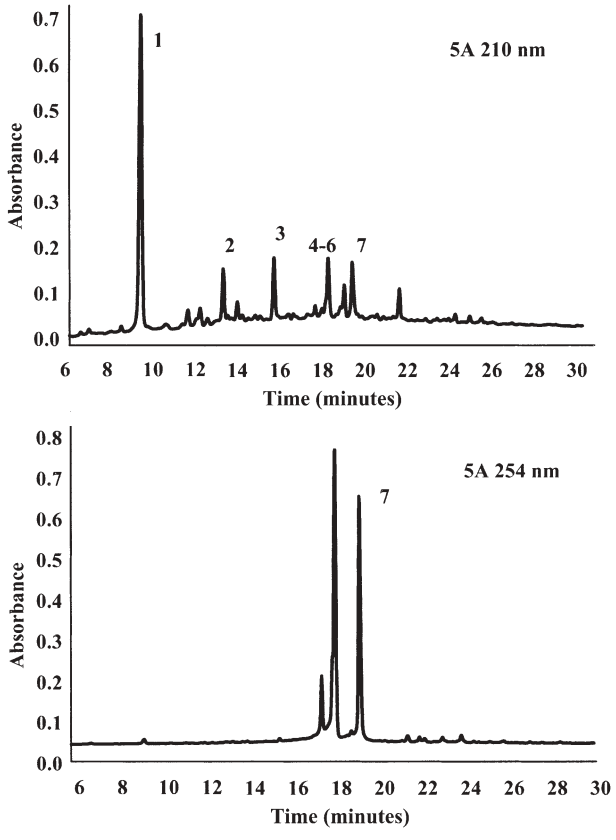


Figure 3. HPLC chromatogram of fraction 5A. The fraction was analysed as described in Materials and methods. The top panel trace was recorded at λ 210 nm; the lower trace was recorded at λ 254 nm. Peak 1: gallocatechin; peaks 2, 3: catechin derivatives; peaks 4–6: EA glycosides (xyloside, arabinoside and rhamnoside); peak 7: EA.

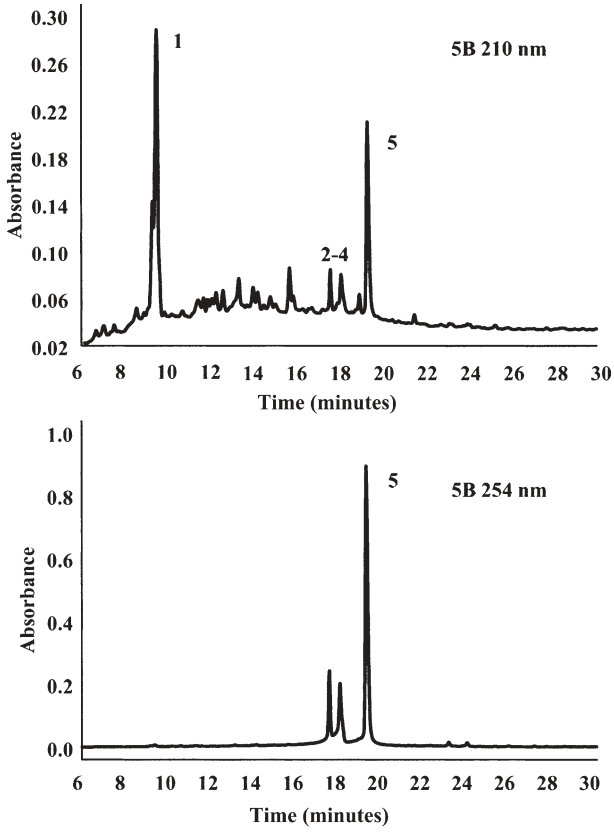


Figure 4. HPLC chromatogram of fraction 5B. The fraction was analysed as described in Materials and methods. The top trace was recorded at λ 210 nm; the lower trace was recorded at λ 254 nm. Peak 1: gallocatechin; peaks 2–4: EA glycosides (xyloside, arabinoside and rhamnoside); peak 5: EA.

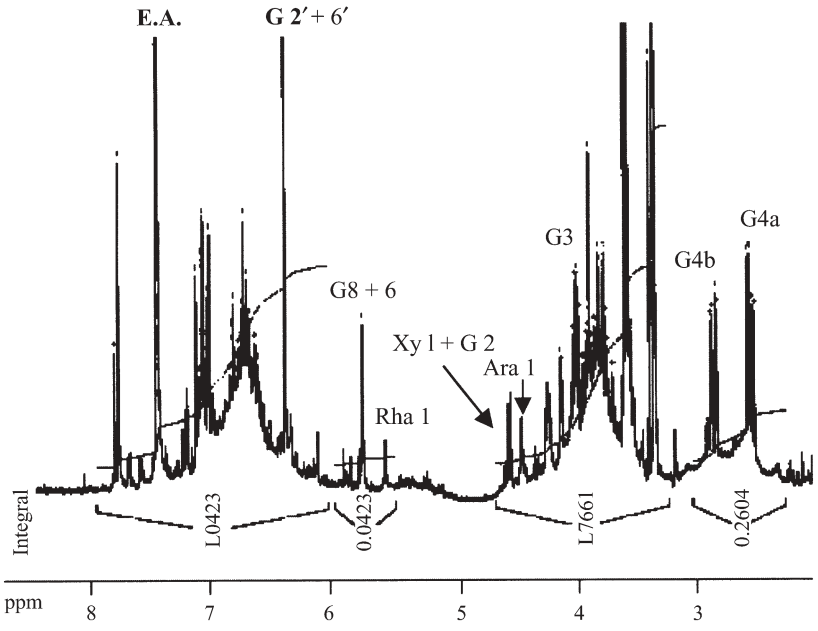


Figure 5. [1]H-NMR spectrum of fraction 5A. The fraction was analysed as described in Materials and methods. G, GC protons; Xyl, xylose protons; Rha, rhamnose protons; Ara, arabinose protons; EA, ellagic acid protons.

compounds inhibited MMP-9 secretion (table 1), (–)-EGC being less active. In contrast, gallic acid, *p*-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid did not cause a substantial effect (data not shown).

Effects on MMP-9 gene regulation

To investigate whether the effect on MMP-9 release could be linked to a modulation of protein expression at the transcription level, we tested the effect of 5A, 5B (1–20 µg/ml), and the single polyphenols (1.5–50 µM, 0.5–15 µg/ml) on MMP-9 promoter activity. CV-1 cells were used because they are easily transfectable, unlike macrophage cell lines. In preliminary experiments, we found that CV-1 cells secrete measurable amounts of MMP-9 (as assessed by gelatin zymography) and the incubation with fractions 5A and 5B for 24 h reduced the activity of MMP-9 by 37 and 49%, respectively. The range of concentrations was chosen according to the MTT assay, which indicated no sign of cytotoxicity on macrophages and CV-1 cells in those intervals. Fraction 5B displayed a potent concentration-dependent and statistically significant inhibition of MMP-9 promoter-driven transcription of the luciferase gene (from 25 up to 80% at 15 µg/ml; $p < 0.01$); 5A had a lower inhibitory effect, but still statistically significant (fig. 6A). The ability of the polyphenols to affect promoter activity was in the order (+)-GC > (–)-EGC > EA > (–)-GC, the effect of the latter not being significant (fig. 7A). To ascertain if the inhibition was specific and selective for MMP-9 promoter activity, the compounds were tested on SV40-early gene promoter-driven transcription. None of the compounds affected the activity of this promoter (fig. 6B, 7B), thus suggesting that the inhibitory effect observed on MMP-9 promoter-induced transcription is not due to a generalized suppression of gene transcription.

Table 1. Effect of polyphenols (30 µM) on MMP-9 secretion.

Compound	MMP-9 secretion % vs control
EA	61.9 ± 5.3**
(+)-GC	67.6 ± 12.5*
(–)-GC	69.2 ± 6.15**
(–)-EGC	75.9 ± 5.95**

Mouse macrophages were treated with the indicated compounds for 24 h. Secretion of MMP-9 into the conditioned medium was then measured as described in Materials and methods. *$p < 0.05$, **$p < 0.01$ vs controls.

Effect on MMP-9 mRNA levels in mouse macrophages

To study if the reduced MMP-9 promoter-driven transcription was paralleled by reduced MMP-9 mRNA production, mouse macrophages were treated with 5A and 5B at 10 µg/ml, and by the single compounds at 30 µM (10 µg/ml) for the determination of mRNA levels. The results are shown in table 2. mRNA levels were reduced to 50% of the control value by both 5A and 5B. All the tested phenols decreased mRNA levels, in the following order: (–)-EGC > (+)-GC = EA > (–)-GC.

The mRNA for 36B4, which was used as an internal standard for normalization, was not affected by any of these treatments. Furthermore, we also measured the

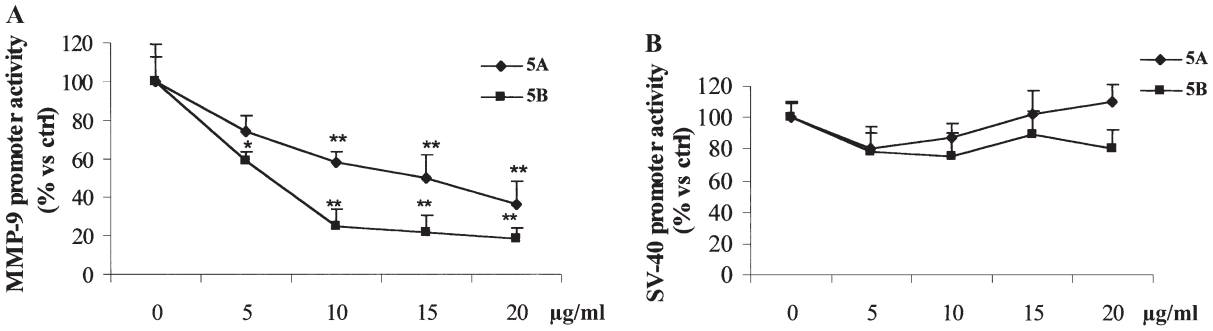


Figure 6. Effects of fraction 5A and 5B on MMP-9 (*A*) or SV40 (*B*) promoter-driven transcription. CV-1 cells were transfected with plasmids 2.2-Luc (*A*) or pGL-3 (*B*) and incubated for 24 h with the indicated concentrations of compounds. A luciferase assay was then performed as described in Materials and methods. Results are expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD values of triplicate samples. Each experiment was repeated at least twice. *$p < 0.05$, **$p < 0.01$ vs control (untreated cells).

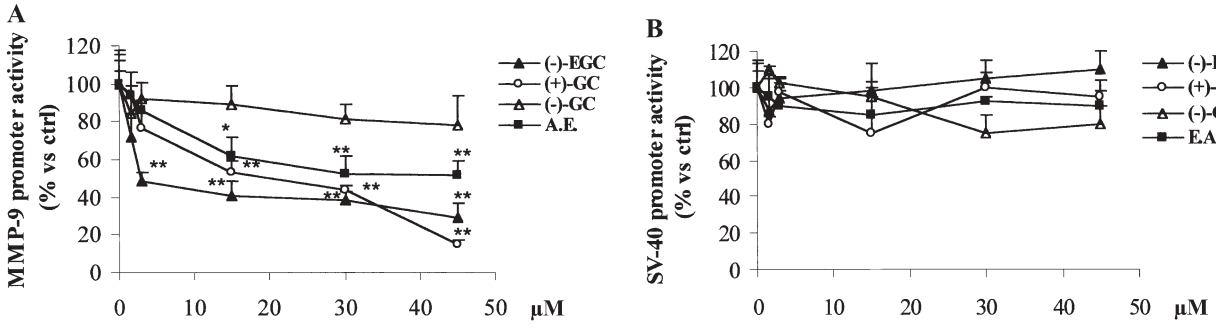


Figure 7. Effects of polyphenols on MMP-9 (*A*) or SV40 (*B*) promoter-driven transcription. CV-1 cells were transfected with plasmids 2.2-Luc (*A*) or pGL-3 (*B*) and incubated for 24 h with the indicated concentrations of compounds. A luciferase assay was then performed as described in Materials and methods. Results are expressed as the inhibition of normalized luciferase activities versus control and represent the mean ± SD values of triplicate samples. Each experiment was repeated at least twice. *$p < 0.05$, **$p < 0.01$ vs control (untreated cells).

Table 2. Effect of polyphenols on MMP-9 mRNA levels.

Compound	Concentration	mRNA levels % vs control*
5A	10 µg/ml	50.1
5B	10 µg/ml	49.1
EA	30 µM	34.9
(+)-GC	30 µM	31.9
(–)-GC	30 µM	42.8
(–)-EGC	30 µM	13.9
DEX	0.1 µM	4.9

Mouse macrophages were treated for 24 h with the indicated compounds. The levels of MMP-9 mRNA were then measured as described in Materials and methods. Dexamethasone (DEX) was used as a positive control.
* Control corresponds to cells treated with 0.1% ethanol.

level of 18S rRNA in the same samples and found that it was not changed by any of these treatments. Therefore, we conclude that the observed effects are specific for MMP-9 and are not due to global inhibition of RNA synthesis.

Discussion

In this study, we showed that the crude MeOH extract of *T. calobuxus* bark directly inhibited the gelatinolytic activity of secreted MMP-9. After fractionation, only the EtOAc-soluble fraction maintained the effect and was also able to reduce the release of MMP-9 by mouse peritoneal macrophages. The antisecretory effect was associated with both the organic (A) and aqueous phase (B) of fraction 5, obtained by chromatography of the EtOAc-soluble fraction on Sephadex LH20. The decrease in MMP-9 secretion was linked to reduced MMP-9 promoter-driven transcription which lead to a reduction in mRNA levels. The aqueous phase 5B was more effective than the organic phase 5A in inhibiting MMP-9 promoter-driven transcription. According to HPLC analysis, EA and GC were the most representative compounds in the mixture. The presence of EA glycosides was described recently in the stem bark of *Eucalyptus globulus* Labill (Myrtaceae) [27] and *Eschweilera coricea* [28].
The data obtained testing the single polyphenolic compounds did not allow assignation of the inhibitory activ-

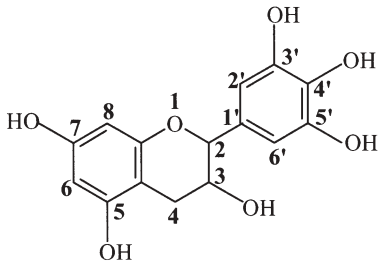
ity to a specific single constituent. If one looks at the effect on metalloproteinase promoter activity, (+)-GC was more active than EA (80 vs 40% inhibition), with no contribution given by (–)-GC (see fig. 7). Regarding the reduction of mRNA levels, EA and (+)-GC contributed to the same extent (70% reduction), while (–)-GC could also participate in the effect. Furthermore, establishing which enantiomer was present in the extract was not possible. The discrepancy between the effect of (–)-GC on promoter activity and mRNA levels could be explained by the fact that the experiments were performed with two different cell types, CV1 and murine peritoneal macrophages. CV1 was chosen to evaluate the effect on the promoter but avoiding possible interferences by other signalling pathways that could be active in macrophages. Alternatively (–)-GC which is not active on the MMP-9 promoter, could instead decrease mRNA levels through other mechanisms.

As regards the complexity of the secretion, none of the tested compound was able to accomplish the effect achieved by the complete fractions (see table 1 vs fig. 2), indicating either synergism or the contribution of other yet unidentified constituents.

Differences in the structural features of the tested compounds are important in determining their activity. Previous studies [13, 17] showed that the presence of a galloyl group as in (–)-EGC gallate was critical for the interaction with the enzyme, and that the planarity of the molecule, as for baicalein, could be another important element in conferring a low IC_{50} against gelatinases [29]. If one considers that EA is a condensation product of two galloyl moieties with a planar conformation, such structural requisites (planarity and the presence of a galloyl group) seem also to be important for the interaction of inhibitors with the MMP-9 promoter. A 3,4,5-trihydroxyphenyl unit is also present in GCs and, in addition, since only (+)-GC and (–)-EGC were active on the promoter, a 2(*R*) configuration of the flavonol moiety seems to determine the ability to act at the transcription level (fig. 8). The stereochemical structure was shown also to affect the antitumour-promoting activity of catechins [30].

Our results provide new insights into the ability of polyphenols to act at the gene level. Tea polyphenols have been shown to inhibit androgen action by repressing the transcription of the androgen receptor gene [31] and to down- or up-regulate the expression of genes in a human lung cancer cell [32]. Moreover, red wine polyphenols were shown to possess antiproliferative effects due to their capacity to down-regulate the expression and promoter activity of the cyclin A gene, a cell cycle regulator [33, 34]. Finally, green tea polyphenols could potently enhance glucocorticoid-induced gene transcription [35].

Increased MMP activity is associated with a wide variety of pathological conditions such as arthritis, cancer, multiple sclerosis, and atherosclerosis [1, 4, 36]. The potential utility of MMP inhibitors in these diverse disease states is evident, and several MMP inhibitors have now entered clinical development [37]. However, their side-effects have greatly limited their clinical use [36].



(+)-gallocatechin: 2R, 3S

(-)-gallocatechin: 2S, 3R

(-)-epigallocatechin: 2R, 3R

Figure 8. Structure of gallocatechins.

Our data indicate that simple and natural molecules such as polyphenols, which can be readily available from dietary sources, exert an effect on MMP-9 gelatinolytic potential by reducing its release from macrophages, via the modulation of the activity of the MMP-9 promoter, in addition to their direct effect on enzyme activity. This is the first study reporting an inhibitory effect on MMP-9 gene transcription by these compounds. These results could represent a beneficial effect of these polyphenols in the therapeutic control of excessive ECM breakdown.

Acknowledgements. This study was supported in part by grant No. QLG1-1999-01007 from the European Commission to the consortium 'Macrophage Function and Stability of the Atherosclerotic Plaque (MAFAPS)' as part of the Fifth Framework Program of the European Union and by a grant from the Italian Ministry of University and Research (MIUR-FIRST).

1 Birkedal-Hansen H., Moore W. G., Bodden M. K., Windsor L. J., Birkedal-Hansen B., DeCarlo A. et al. (1993) Matrix metalloproteinases: a review. Crit. Rev. Oral Biol. Med. **4:** 197–250
2 Massova I., Kotra L. P., Fridman R. and Mobashery S. (1998) Matrix metalloproteinases: structures, evolution, and diversification. FASEB J. **12:** 1075–1095
3 Libby P. (1995) Molecular bases of the acute coronary syndromes. Circulation **91:** 2844–2850
4 Johansson N., Ahonen M. and Kahari V. M. (2000) Matrix metalloproteinases in tumor invasion. Cell. Mol. Life Sci. **57:** 5–15
5 Brown D. L., Hibbs M. S., Kearney M., Loushin C. and Isner J. M. (1995) Identification of 92-kD gelatinase in human coronary atherosclerotic lesions: association of active enzyme synthesis with unstable angina. Circulation **91:** 2125–2131
6 Newman K. M., Ogata Y., Malon A. M., Irizarry E., Gandhi R. H., Nagase H. et al. (1994) Identification of matrix metalloproteinases 3 (stromelysin-1) and 9 (gelatinase B) in abdominal aortic aneurysm. Arterioscler. Thromb. **14:** 1315–1320
7 Nordqvist A. C., Smurawa H. and Mathiesen T. (2001) Expression of matrix metalloproteinases 2 and 9 in meningiomas associated with different degrees of brain invasiveness and edema. J. Neurosurg. **95:** 839–844

8 Aikawa M., Rabkin E., Okada Y., Voglic S. J., Clinton S. K., Brinckerhoff C. E. et al. (1998) Lipid lowering by diet reduces matrix metalloproteinase activity and increases collagen content of rabbit atheroma: a potential mechanism of lesion stabilization. Circulation **97:** 2433–2444

9 Corsini A., Bellosta S., Baetta R., Fumagalli R., Paoletti R. and Bernini F. (1999) New insights into the pharmacodynamic and pharmacokinetic properties of statins. Pharmacol. Ther. **84:** 413–428

10 Bellosta S., Via D., Canavesi M., Pfister P., Fumagalli R., Paoletti R. et al. (1998) HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages. Arterioscler. Thromb. Vasc. Biol. **18:** 1671–1678

11 Bellosta S., Canavesi M., Favari E., Cominacini L., Gaviraghi G., Fumagalli R. et al. (2001) Lacidipine [correction of Lalsoacidipine] modulates the secretion of matrix metalloproteinase-9 by human macrophages. J. Pharmacol. Exp. Ther. **296:** 736–743

12 Maeda-Yamamoto M., Kawahara H., Tahara N., Tsuji K., Hara Y. and Isemura M. (1999) Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells. J. Agric. Food Chem. **47:** 2350–2354

13 Garbisa S., Sartor L., Biggin S., Salvato B., Benelli R. and Albini A. (2001) Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. Cancer **91:** 822–832

14 Benelli R., Vene R., Bisacchi D., Garbisa S. and Albini A. (2002) Anti-invasive effects of green tea polyphenol epigallocatechin-3-gallate (EGCG), a natural inhibitor of metallo and serine proteases. Biol. Chem. **383:** 101–105

15 Jung Y. D. and Ellis L. M. (2001) Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. Int. J. Exp. Pathol. **82:** 309–316

16 Locher R., Emmanuele L., Suter P. M., Vetter W. and Barton M. (2002) Green tea polyphenols inhibit human vascular smooth muscle cell proliferation stimulated by native low-density lipoprotein. Eur. J. Pharmacol. **434:** 1–7

17 Demeule M., Brossard M., Page M., Gingras D. and Beliveau R. (2000) Matrix metalloproteinase inhibition by green tea catechins. Biochim. Biophys. Acta **1478:** 51–60

18 Bormann H. and Melzig M. F. (2000) Inhibition of metallopeptidases by flavonoids and related compounds. Pharmazie **55:** 129–132

19 Parellada J., Suarez G. and Guinea M. (1998) Inhibition of zinc metallopeptidases by flavonoids and related phenolic compounds: structure-activity relationships. J. Enzyme Inhib. **13:** 347–359

20 Bosisio E., Mascetti D. and Cabalion P. (2000) Screening of plants from New Caledonia and Vanuatu for inhibitory activity of xanthine oxidase and elastase. Pharm. Biol. **38:** 18–24

21 Dell'Agli M., Parapini S., Basilico N., Verotta L., Taramelli D., Berry C. et al. (2003) In vitro studies on the mechanism of action of two compounds with antiplasmodial activity: ellagic acid and 3,4,5-trimethoxyphenyl(6′-O-galloyl)-beta-D-glucopyranoside. Planta Med. **69:** 162–164

22 Le Bonniec S., Deregnaucourt C., Redeker V., Banerjee R., Grellier P., Goldberg D. E. et al. (1999) Plasmepsin II, an acidic hemoglobinase from the *Plasmodium falciparum* food vacuole, is active at neutral pH on the host erythrocyte membrane skeleton. J. Biol. Chem. **274:** 14218–14223

23 Verotta L., Dell'Agli M., Giolito A., Guerrini M., Cabalion P. and Bosisio E. (2001) In vitro antiplasmodial activity of extracts of *Tristaniopsis* species and identification of the active constituents: ellagic acid and 3,4,5-trimethoxyphenyl-(6′-O-galloyl)-O-beta-D-glucopyranoside. J. Nat. Prod. **64:** 603–607

24 Lowry D. H., Rosenbrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265–275

25 Crestani M., Stroup D. and Chiang J. Y. (1995) Hormonal regulation of the cholesterol 7 alpha-hydroxylase gene (CYP7). J. Lipid. Res. **36:** 2419–2432

26 Denizot F. and Lang R. (1986) Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods **89:** 271–277

27 Kim J. P., Lee I. K., Yun B. S., Chung S. H., Shim G. S., Koshino H. et al. (2001) Ellagic acid rhamnosides from the stem bark of *Eucalyptus globulus*. Phytochemistry **57:** 587–591

28 Yang S. W., Zhou B. N., Wisse J. H., Evans R., Werff H. van der, Miller J. S. et al. (1998) Three new ellagic acid derivatives from the bark of *Eschweilera coriacea* from the Surineme rainforest. J. Nat. Prod. **61:** 901–906

29 Sartor L., Pezzato E., Dell'Aica I., Caniato R., Biggin S. and Garbisa S. (2002) Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design. Biochem. Pharmacol. **64:** 229–237

30 Gao H., Kuroyanagi M., Wu L., Kawahara N., Yasuno T. and Nakamura Y. (2002) Antitumor-promoting constituents from *Dioscorea bulbifera* L. in JB6 mouse epidermal cells. Biol. Pharm. Bull. **25:** 1241–1243

31 Ren F., Zhang S., Mitchell S. H., Butler R. and Young C. Y. (2000) Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. Oncogene **19:** 1924–1932

32 Okabe S., Fujimoto N., Sueoka N., Suganuma M. and Fujiki H. (2001) Modulation of gene expression by (–)-epigallocatechin gallate in PC-9 cells using a cDNA expression array. Biol. Pharm. Bull. **24:** 883–886

33 Iijima K., Yoshizumi M., Hashimoto M., Kim S., Eto M., Ako J. et al. (2000) Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin A gene. Circulation **101:** 805–811

34 Iijima K., Yoshizumi M. and Ouchi Y. (2002) Effect of red wine polyphenols on vascular smooth muscle cell function – molecular mechanism of the 'French paradox'. Mech. Ageing Dev. **123:** 1033–1039

35 Abe I., Umehara K., Morita R., Nemoto K., Degawa M. and Noguchi H. (2001) Green tea polyphenols as potent enhancers of glucocorticoid-induced mouse mammary tumor virus gene expression. Biochem. Biophys. Res. Commun. **281:** 122–125

36 Kahari V. M. and Saarialho-Kere U. (1999) Matrix metalloproteinases and their inhibitors in tumour growth and invasion. Ann. Med. **31:** 34–45

37 Whittaker M., Floyd C. D., Brown P. and Gearing A. J. (1999) Design and therapeutic application of matrix metalloproteinase inhibitors. Chem. Rev. **99:** 2735–2776