ORIGINAL CONTRIBUTION

ANTIOXIDANT ACTIVITY AND INHIBITION OF MATRIX METALLOPROTEINASES
BY METABOLITES OF MARITIME PINE BARK EXTRACT (PYCNOGENOL)

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Abstract—The procyanidin-rich maritime pine bark extract Pycnogenol has well-documented antioxidant and anti-inflammatory activity. After oral administration of Pycnogenol two major metabolites are formed in vivo, δ-(3,4-dihydroxyphenyl)-γ-valerolactone (M1) and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (M2). We elucidated the effects of these metabolites on matrix metalloproteinases (MMPs) and determined their antioxidant activity to understand their contribution to the effects of maritime pine bark extract. We discovered strong inhibitory effects of M1 and M2 toward the activity of MMP-1, MMP-2, and MMP-9. On a microgram-per-milliliter basis both metabolites appeared more active than Pycnogenol. The metabolites were more effective than their metabolic precursor (+)-catechin in MMP inhibition. On a cellular level, we detected highly potent prevention of MMP-9 release by both metabolites, with concentrations of 0.5 μM resulting in about 50% inhibition of MMP-9 secretion. M1 was significantly more effective in superoxide scavenging than (+)-catechin, ascorbic acid, and trolox, while M2 displayed no scavenging activity. Both metabolites exhibited antioxidant activities in a redox-linked colorimetric assay, with M1 being significantly more potent than all other compounds tested. Thus, our data contribute to the comprehension of Pycnogenol effects and provide a rational basis for its use in prophylaxis and therapy of disorders related to imbalanced or excessive MMP activity.

INTRODUCTION

Pine bark extract has been used in traditional medicine in Europe and North America for inflammatory diseases and wound healing among other uses [1,2]. Modern research with a standardized bark extract from the maritime pine, Pinus pinaster (Pycnogenol, Horphag Research Ltd., UK), indeed confirmed anti-inflammatory effects. In cell culture systems with endothelial cells or keratinocytes the maritime pine bark extract inhibited activation of the key molecule of inflammation, NF-κB, and expression of the adhesion molecules ICAM-1 and VCAM-1 [3,4]. Furthermore it was demonstrated that the release of reactive oxygen species (ROS) was inhibited by pretreatment of cells with pine bark extract before challenge with tumor necrosis factor α (TNFα) [3]. Experiments with various rodent animal models supported the observation of anti-inflammatory effects of the herbal extract. Ingestion of the maritime pine bark extract resulted in a reduction of the inflammatory response in the rodent ear endema model [5]. After topical application it prevented erythema formation after UV radiation [6]. This erythema prevention after UV challenge was also observed in humans after oral ingestion of the extract [4]. Another anti-inflammatory action of the maritime pine bark extract was noticed in asthma patients who experienced a reduction of circulating leukotriene levels and an improvement of asthma symptom scores [7].

In inflammation, ROS play a special role as signaling molecules which contribute to cell injury and degenera-
tive processes such as cartilage degradation in rheumatic diseases [8,9]. In this context, matrix degrading enzymes, matrix metalloproteinases (MMPs), significantly contribute to the pathogenesis of various chronic inflammatory diseases. MMPs constitute a family of zinc-dependent proteolytic enzymes with so far 28 identified members [10]. Interestingly, MMPs can be activated by ROS [11–14] so that both seem to contribute vitally to the inflammatory network. For the activation of MMPs by ROS various intracellular signaling pathways have been identified [15]. The maritime pine bark extract Pycnogenol has excellent radical scavenging properties [16–18] and enhances the production of antioxidative enzymes [19] which contributes to the anti-inflammatory effect of the extract.

An imbalance between MMPs and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs), is generally assumed to result in proteolytic tissue damage or remodeling in pathophysiological conditions. In patients with arthritis, upregulation of MMP-1 (collagenase 1) or MMP-9 (gelatinase B) was determined [20–22], MMP-1 also contributes to the photaging of skin induced by UV exposure [14]. In asthma MMP-9 is upregulated and found to be involved in inflammation and remodeling processes [23,24]. In pulmonary fibrosis MMP-2 (gelatinase A) plays an additional role [25]. Various MMPs contribute to cancer invasion and metastasis [26]. Based on these observations it is not surprising that the MMPs are regarded as a promising therapeutic target and the development of effective low-molecular-weight inhibitors is an important research focus of several pharmaceutical companies [27,28].

The aim of our study was to determine the effects of Pycnogenol and its metabolites on the metalloproteinases that play a broad and important pathophysiological role, namely, MMP-1, MMP-2, and MMP-9. The extract is a mixture of procyanidins (procyanidinins), polyphenols, and phenolic acids that is either applied topically or administered orally [29]. The procyanidins are biopolymers comprising catechin or epicatechin monomer units in varying chain lengths. After oral intake ferulic acid and taxifolin were found in human urine [30]. These compounds are known components of the pine bark extract and ferulic acid was suggested as an excretion marker to determine bioavailability [31]. Most interestingly, two additional major metabolites (Fig. 1) were identified in human urine. These compounds, δ-(3,4-dihydroxyphenyl)-γ-valerolactone (metabolite 1, M1) and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone (metabolite 2, M2), are derived from their metabolic precursor catechin after various ring degradation and fission reactions [30]. We elucidated the effects of these metabolites on MMPs and determined their antioxidant activity to understand their contribution to the well documented anti-inflammatory action of maritime pine bark extract.

MATERIALS AND METHODS

Enzymes

MMP-1 (proenzyme, human rheumatoid synovial fibroblast), MMP 2 (proenzyme, human rheumatoid synovial fibroblast), and MMP 9 (monomer, neutrophil, granulocyte) were all obtained from Calbiochem (Schwalbach am Taunus, Germany).

Inhibitors

A spray-dried extract from maritime pine barks (Pycnogenol) was generously provided by Horphag Research Ltd. (Geneva, Switzerland). Captopril was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1, human neutrophil granulocyte) was purchased from Calbiochem. M1 and M2 were synthesized by Große Düweler [30].

Other chemicals and reagents

Dimethyl sulfoxide was purchased from Calbiochem. All other chemicals and reagents were obtained from Sigma–Aldrich if not stated otherwise.
Succinylation of collagen, elastin, and gelatin

The substrates gelatin type A (porcine skin, approx 300 bloom), collagen type I (insoluble, from bovine achilles tendon), and elastin (from bovine neck ligament) were succinylated according to [32]. Briefly, 0.200 g of each substrate was suspended in 10.0 ml of 0.05 M sodium borate buffer, pH 8.5. An equal amount of succinic anhydride was then gradually added to the solution while the pH was maintained at 8.0–8.5 by the addition of 1 M NaOH. The suspension was stirred at room temperature for 30 min. The succinated substrates were then dialyzed extensively for 12 h under gentle stirring in dialysis tubes (MWCO 12 kDa), against 0.05 M sodium borate buffer, pH 8.5, to remove unbound succinic anhydride. The final concentration of the proteins was determined by the BCA protein assay.

Activation of proenzymes

To initiate activation of MMP-1 and MMP-2, the proMMP solution was combined with 1 mM p-aminophenylmercuric acetate in 0.1 M NaOH in a 10:1 volume ratio. The mixture was incubated (Eppendorf Thermomixer, Eppendorf AG, Hamburg, Germany) at 37°C for 2 h under shaking (300 rpm). The activated MMPs were used without the removal of the organomercurial according to the manufacturer’s recommendation. The active enzymes were diluted with 0.05 M Tris–HCl (pH 7.0), containing 200 mM NaCl, 5 mM CaCl₂, and 1 µM ZnCl₂, divided in aliquots, and stored at −80°C until use [33].

Inhibition assays of MMPs

All assays were performed in 96-well flat-bottom microtiter plates with low protein binding (Microtest, BD Labware, NJ, USA). The inhibition assays of MMPs were performed with the natural inhibitor TIMP-1 (for MMP-1) and with captopril (for MMP-2 and MMP-9) as positive controls, with Pycnogenol and its metabolites. Before use, MMP-1 was diluted with 0.05 M PBS buffer (pH 7.2), with 1 mM CaCl₂, MMP-2 was diluted with 0.05 M sodium borate buffer (pH 7.0) with 10 mM CaCl₂, and MMP-9 was diluted with 0.05 M sodium borate buffer (pH 8.5). TIMP-1 was reconstituted in 0.05 M Tris–HCl with 200 mM NaCl, pH 7.0. For inhibition assays, increasing concentrations of each inhibitor were added to 666.7 μg/ml succinylated substrate and a constant concentration of MMP (83.3 ng/ml), and incubated at 37°C for 30 min. Fifty microliters of 0.03% solution of 2,4,6-trinitrobenzenesulfonic acid in 0.05 M sodium borate buffer, pH 8.5, was then added to the reaction mixture and allowed to incubate at room temperature for 20 min. Blanks were treated analogously. The optical density of each reaction was determined at 450 nm (Bio-Rad microplate reader, Benchmark, CA, USA). The efficiency of the inhibitor, expressed as IC₅₀, was determined by comparing the relative activity of enzyme in the presence and absence of the inhibitor. The remaining activities of the examined MMPs were plotted versus the logarithm of the concentration of each inhibitor, using the GraphPad prism software (GraphPad Software Inc., San Diego CA, USA). IC₅₀ was determined from the resulting regression curve. Goodness of fit to a sigmoid dose–response curve was at least 0.92.

Binding of pycnogenol and M2 to matrix proteins

The binding of Pycnogenol and M2 to matrix proteins and ovalbumin was determined according to a protocol in the Swiss Pharmacopeia (binding of polyphenols to skin powder) with slight modifications. Briefly, 10.0 ml of solution of Pycnogenol or M2 (20.0 mg/100 ml, = solution 1) was added to 100.0 mg skin powder, collagen, elastin, or ovalbumin, and shaken for 60 min at room temperature. The suspension was filtered, and 2.0 ml of the filtrate was diluted to 10.0 ml with distilled water. Two milliliters of the dilution was added to 10.0 ml distilled water, 1.0 ml Folin–Ciocalteu phenol reagent, and 10.6 % sodium carbonate solution, to obtain a final volume of 25.0 ml. After 15 min, the optical density was determined using the Bio-Rad microplate reader, set at 691 nm (= AII). Due to the good solubility of ovalbumin in water, 1.0 ml of a 0.5 M solution of zinc sulfate was added before filtration to precipitate the protein. After filtration, the assay was performed as described above. Two milliliters of solution 1 was diluted to 10.0 ml with distilled water. This dilution was treated as mentioned above and after 15 min the optical density was determined (= A1). Binding was calculated as

\[
\frac{1562.5*(AII - A1)}{\text{weighted sample (g)}*1000} = \% \text{ binding.}
\]

Isolation and culture of human monocytes

Human monocytes were isolated from blood cell suspensions pooled from different donors (Bayerisches Rotes Kreuz, Germany) by density gradient centrifugation with Ficoll–Paque [34]. The cells were cultured overnight in supplemented McCoy’s 5a modified medium (Biochrom, Berlin, Germany) at a density of 1 × 10⁶ cells/ml in a 6% CO₂ humidified atmosphere at 37°C (Hera cell incubator, Kendro, Hanau, Germany). Cell experiments were performed in Multotwell 24-well cell culture plates (polystyrene, BD Labware, NJ, USA) in a final volume of 1.0 ml/well.
Inhibition of MMP-9 release from human monocytes

Fresh medium, containing either of the two Pycnogenol metabolites M1 and M2 or hydrocortisone, was added to the wells. After 1 h, cells were treated with 10 ng/ml LPS (Salmonella minnesota Re 595) and incubated for 48 h. After incubation the number of viable cells was determined by counting living cells after staining with trypan blue. The number of living cells was equivalent after each treatment. Plates were centrifuged (Megafuge 1.0 R, Kendro Laboratory Products) and cell culture supernatants were harvested, diluted 1:25, and assayed for total MMP-9 protein concentrations by ELISA (Quantikine assay, R&D Systems, Minneapolis, USA) according to the manufacturer’s protocol.

Radical scavenging and antioxidant activity

Radical scavenging properties toward superoxide radicals. Antioxidant activity of the maritime pine bark extract Pycnogenol and its metabolites was analyzed as its ability to scavenge superoxide radicals generated by a hypoxanthine–xanthine oxidase system, using the nitroblue tetrazolium (NBT) reduction assay [35,36]. To exclude that the test compounds directly inhibit the enzyme, their respective highest concentrations were used for xanthine oxidase inhibition. Therefore, aqueous solutions of 9.9 μM EDTA, 990 μM hypoxanthine, and 33.35 mM PBS, pH 7.4, were mixed, followed by addition of 0.1 U/ml xanthine oxidase. The reaction was carried out at 25°C. Ten seconds after addition of enzyme, the formation of uric acid was measured at 290 nm in the absence and presence of the test compounds. None of the test compounds inhibited xanthine oxidase. For the NBT reduction assay, aqueous solutions of 9.9 μM EDTA, 99 μM NBT, 990 μM hypoxanthine, 33.35 mM PBS, and the test compound were mixed (25°C), followed by addition of 0.1 U/ml xanthine oxidase. Absorbance was measured at 560 nm. The change in absorbance per minute (ΔA/min) was determined in comparison to a blank sample, and the percentage of inhibition was calculated. IC_{50} was determined from regression curves; goodness of fit was at least 0.90.

Ferric-reducing antioxidant potential (FRAP). Total antioxidant capacities of M1 and M2 were determined using the FRAP assay [37]. The FRAP reagent was prepared by mixing 300 mM acetate buffer, pH 3.6, and 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl_{3}-6H_{2}O solution at a 10:1:1 ratio. The reaction was carried out at 37°C. In an absorbance cell 900 μl of FRAP reagent, freshly prepared and warmed to 37°C, 90 μl distilled water, and 30 μl of either test solution or standard (ascorbic acid or FeSO_{4}) were mixed and the absorbance changes were measured at 593 nm versus the FRAP reagent with distilled water only. The final dilution of the test samples in the reaction mixture was 1/34. The reaction was monitored for 4 min using a Shimadzu UV-VIS scanning spectrophotometer equipped with a thermostated cell holder, and the 4-min readings were selected for calculations of FRAP values.

Statistical analysis

Statistically significant differences between treatment groups were determined by ANOVA calculation and subsequent Tukey or Fisher PLSD test. Significance was defined as p < .05.

RESULTS

Inhibition of the enzymatic activity of MMP-1

Inhibition of the activity of MMP-1 was determined with two different substrates, collagen and gelatin (Fig. 2). With both substrates similar results were obtained. A comparison of the maritime pine bark extract Pycnogenol and its metabolites M1 and M2 revealed that all exhibit inhibitory activity. On a microgram-per-milliliter basis both metabolites were statistically significantly more potent than the whole extract while there was no significant difference between M1 and M2. Concentrations necessary to produce 50% inhibition of MMP-1 activity were 44–57 μg/ml for Pycnogenol, while 10–23 μg/ml concentrations of the metabolites produced the same effect. Notably, the metabolic precursor of both metabo-
lites, (+)-catechin, displayed no inhibitory action on MMP activity. As a positive control the inhibitory potency of TIMP-1 was elucidated in this assay system (Table 1). As expected, TIMP-1 exhibited highly potent inhibitory action on MMP-1 activity which was more than 1000-fold superior to the metabolites of Pycnogenol. Only 60–120 ng/ml of TIMP-1 (equaling 2–4 nmol/l) was sufficient to produce 50% inhibition of MMP-1.

**Inhibition of the enzymatic activity of MMP-2**

The degradation of collagen and elastin by MMP-2 was also inhibited by Pycnogenol and its metabolites M1 and M2 (Fig. 3). While even lower concentrations of Pycnogenol were necessary to produce 50% inhibition of MMP-2 compared with MMP-1, the metabolites were similarly active inhibiting MMP-2 as MMP-1. Again, both metabolites were statistically significantly more potent than Pycnogenol on a microgram-per-milliliter basis. For comparison, the ACE inhibitor captopril was assayed as it has reported inhibitory activity against MMP-2 and MMP-9, earlier described as 72-kDa and 92-kDa gelatinases [38]. Both metabolites and Pycnogenol exhibited more potent inhibitory activity toward MMP-2 which exceeded that of captopril by a factor of about 100 (Table 1).

**Inhibition of the enzymatic activity of MMP-9**

The best inhibitory activities of the metabolites and Pycnogenol were observed against MMP-9. Only 24–27 μg/ml Pycnogenol was necessary to inhibit MMP-9 by 50%. Even significantly lower concentrations of the metabolites achieved the same effect (Fig. 4). As seen with MMP-2, the control compound captopril was about 100-fold less potent than the metabolites (Table 1).

**Binding of Pycnogenol and M2 to matrix proteins**

We determined the binding of Pycnogenol toward pulverized skin and its main matrix proteins collagen

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**Table 1. Mean Inhibitory Concentrations (IC₅₀) of the Metabolites M1 and M2 and the Respective Controls TIMP-1 and Captopril**

<table>
<thead>
<tr>
<th></th>
<th>MMP-1 (μmol/l)</th>
<th>MMP-2 (μmol/l)</th>
<th>MMP-9 (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>108 ± 14</td>
<td>104 ± 14</td>
<td>8.4 ± 1.6</td>
</tr>
<tr>
<td>Gelatin</td>
<td>45.2 ± 7.4</td>
<td>48.0 ± 7.5</td>
<td>6.9 ± 1.3</td>
</tr>
<tr>
<td>Elastin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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a The degradation of two different substrates by each MMP was determined. IC₅₀ values are the mean ± SD of six independent experiments. n.d., not determined.

b Mean ± mean deviation from the mean of three independent experiments.
and elastin versus binding of Pycnogenol to a non-matrix protein (ovalbumin). For comparison, we analyzed the binding of metabolite M2 to pulverized skin (Fig. 5). As expected, the procyanidin-rich maritime pine bark extract Pycnogenol binds extensively to pulverized skin. Binding to collagen is statistically significantly lower \((p \leq .01)\) while binding to elastin is significantly higher compared with that to collagen \((p \leq .001)\). A preferential binding of Pycnogenol to skin and its matrix protein components is reflected by the sharp drop in percentage binding of the compound to a nonmatrix protein, ovalbumin \((p \leq .001)\). The binding of metabolite M2 to skin powder revealed that only small amounts were bound, significantly less compared with the binding of Pycnogenol to matrix proteins \((p \leq .001)\). Thus, M2 does not have a high binding affinity toward matrix proteins. The binding is comparable to the nonselective binding of the pine bark extract to ovalbumin.

Reversal of MMP-9 inhibition in the presence of zinc

MMP activity is dependent on zinc which resides within the active site and contributes to the mechanism of proteolysis. For captopril, it has been shown that the addition of zinc ions reverses the inhibitory activity toward MMP-2 and MMP-9 \([38]\). It was concluded that the mode of the action of captopril is the chelation of \(\text{Zn}^{2+}\) at the active site of the enzymes. We determined the inhibitory action of captopril, M1, M2, and Pycnogenol toward MMP-9 (substrate: elastin) in the presence and absence of \(\text{Zn}^{2+}\) (Fig. 6). Therefore, the earlier determined IC\(_{50}\) values of the inhibitors were employed (see Table 1 and Fig. 4). Ten micromolar \(\text{Zn}^{2+}\) did not precipitate any proteins in the reaction vial. We observed complete reversal of the inhibitory activity of captopril in the presence of 10 \(\mu\text{M} \text{Zn}^{2+}\) (Fig. 6). Likewise, the inhibitory activity of M1 and M2 was reversed by \(\text{Zn}^{2+}\). There was no difference between the treatment groups and the control. In contrast, the inhibitory activity of Pycnogenol was not altered by the addition of zinc ions. The difference from the control was significant \((p \leq .001)\).

Inhibition of the release of MMP-9 from human monocytes

Incubation of human monocytes with bacterial lipopolysaccharide (LPS) (endotoxin) results in an upregulation of MMP-9 \([39]\) and subsequent release of proMMP-9 and active MMP-9 into the cell culture medium. Total secreted MMP-9 protein was quantified by ELISA after stimulation of freshly isolated human monocytes with LPS alone or together with M1, M2, or hydrocortisone. Hydrocortisone is an endogenous glucocorticoid with anti-inflammatory activity that inhibits the release of MMP-9 from mononuclear cells in vivo \([40]\). All compounds added to the culture medium along with LPS inhibited the release of MMP-9 protein in a dose-dependent manner (data not shown). M1 and M2 displayed comparative efficacy. The concentration required for approximately 50% reduction of MMP-9 release was 0.5 \(\mu\text{g/ml}\) for both metabolites (Fig. 7).

Radical scavenging and antioxidant activity of M1 and M2

Radical scavenging and anti-oxidant activity of the metabolites M1 and M2 was determined in two different assays. As controls the metabolic precursor of the metabolites, \((+)-\text{catechin}\), and the monomeric pine bark extract components taxifolin and ferulic acid were tested. Antioxidant vitamins were assayed as reference compounds. In the first assay, inhibition of superoxide-induced reduction of nitroblue tetrazolium was determined. Superoxide radicals were generated by a mixture of hypoxanthine and xanthine oxidase. None of the tested compounds had a direct inhibitory effect on xanthine oxidase at the concentrations tested, thus pretending a radical scavenging effect due to a lower production of radicals. At pH 7.4, superoxide radicals reduce nitroblue tetrazolium chloride (yellow) to formazan (purplish blue), which is insoluble in water. If radical scavenging...
compounds are present in the solution, less formazan blue is formed, thus decreasing the absorption at 560 nm. Concentrations necessary to produce 50% inhibition of superoxide production revealed that M1 was the most effective compound tested (Fig. 8). Inhibitory concentrations of M1 were statistically significantly lower compared with those of (+)-catechin, taxifolin, and vitamin C. In this assay, all tested compounds were significantly more potent \((p \leq .0001)\) in superoxide scavenging compared with soluble vitamin E (Trolox). M1 was even 12 times more potent compared with Trolox. Notably, neither M2 nor ferulic acid displayed any superoxide scavenging activities at the concentrations tested.

In a second assay, total antioxidant capacities were determined. This redox-linked colorimetric assay

![Graph showing inhibition of MMP-9 activity with and without Zn2+](image)

**Fig. 6.** Reversal of MMP-9 inhibition in the presence of 10 \(\mu\)M zinc. Elastin was used as substrate. Concentrations of inhibitors that produced 50% inhibition of MMP-9 activity were chosen (see Table 1 and Fig. 4) and assayed in the absence and presence of 10 \(\mu\)M Zn\(^{2+}\). MMP-9 inhibition by captopril, M1, and M2 was completely reversed by zinc ions with no significant (n.s.) difference between treatment groups and control. In contrast, MMP-9 inhibition by Pycnogenol was not reversed, effects were not significantly different in the presence of Zn\(^{2+}\) (ANOVA with subsequent Tukey test). Each column represents the mean and SD of six experiments (control: 12 experiments).

![Graph showing MMP-9 secretion](image)

**Fig. 7.** Inhibition of total MMP-9 release from freshly isolated human monocytes after stimulation with 10 ng/ml LPS for 48 h. The MMP-9 release was statistically significantly inhibited by about 50% when 0.5 \(\mu\)M M1 or M2 or 1.0 \(\mu\)M hydrocortisone (HC) were added to the culture medium (ANOVA with subsequent Tukey’s test). Number of viable cells was equivalent after each treatment. Each column represents mean and standard deviation of six experiments.
employs a ferric tripyridyl-s-triazine complex (Fe$^{III}$-TPTZ) that is reduced to an intense blue ferrous form (Fe$^{II}$) in the presence of an antioxidant (reductant). To compare antioxidant efficiency, EC$_1$ (equivalent concentration) values, i.e., concentrations of antioxidant having a Fe$^{III}$-TPTZ reducing ability equivalent to that of 1 mM FeSO$_4$.7H$_2$O, were used according to the definition of Pulido et al. [41]. This parameter was calculated as the antioxidant concentration giving an increase in absorbance equivalent to the determined absorbance of a 1 mM FeSO$_4$.7H$_2$O using the corresponding regression equation. The higher the EC$_1$ value, expressed as micromolar, the lower the antioxidant activity. M1 had the lowest EC$_1$ value and, therefore, the highest reducing power in comparison to FeSO$_4$ as standard and the other antioxidants (Table 2).

### DISCUSSION

We determined effects of maritime pine bark extract (Pycnogenol) and its two major metabolites on pathophysiologically relevant matrix metalloproteinases. While there are several reports on clinical and molecular pharmacological effects of Pycnogenol [2,29] little is known about the metabolites and their possible contribution to the acknowledged anti-inflammatory action of Pycnogenol.

The Pycnogenol metabolites M1 and M2 are not genuinely present in the pine bark extract, but are generated in vivo. Both are derived from (+)-catechin which is found as a monomer in the extract and also released by cleavage of procyanidins [42,43]. After oral intake of Pycnogenol the cumulative urinary excretion of M1 exceeds the excretion of taxifolin by the factor of 6 and the excretion of ferulic acid by a factor of 4 [30]. M1 and M2 metabolites were detected in a ratio of about 2:1 in human urine [30]. Thus, highest total amounts of M1 were discovered after intake of the standardized pine bark extract Pycnogenol. M1 has also been detected in rats after oral ingestion of (-)-epicatechin gallate, but among urinary metabolites the lowest amounts of M1 were detected while other metabolites dominated [44]. In humans, M1 was identified after ingestion of green tea, again the urinary excretion of this metabolite was lower compared to other metabolite [45,46]. Several other studies reported M1 as metabolite after ingestion of tea catechins, while M2 was not mentioned.

In the present study, both pine bark metabolites M1 and M2 displayed inhibitory activity toward MMP-1
(collagenase 1), MMP-2 (gelatinase A), and MMP-9 (gelatinase B). This is the first report that ring fission metabolites of (+)-catechin have an impact on the key enzymes of inflammatory and degenerative disorders. On a microgram-per-milliliter basis both metabolites were statistically significantly more potent than the parent pine bark extract. Most notably, their metabolic precursor (+)-catechin did not inhibit MMP action within the concentration range tested. This result is in excellent agreement with the earlier reports about weak or absent inhibitory activity of catechin toward MMPs [47,48]. The potency of Pycnogenol, M1, and M2 increased from inhibition of MMP-1 to MMP-9. The lowest inhibitory concentration of 18 μM was determined for M1 toward MMP-9. This IC₅₀ value is lower than the IC₅₀ of 28 μM for the green tea-derived epicatechin gallate [48]. Both pine bark metabolites M1 and M2 are more potent in preventing proteolytic degradation of elastin by MMP-2 than epicatechin gallate [48].

For control of the inhibitory effects of Pycnogenol and its metabolites on MMP activity, two known inhibitors were employed. TIMP-1 is a highly potent endogenous compound; the biological activity against human MMP-1 is reflected by its low IC₅₀ of 1.4–7.0 nM (manufacturer’s information) which is consistent with our own results. Captopril was chosen as positive control compound in the assays with MMP-2 and MMP-9 because it is known to inhibit both enzymes in a dose-dependent manner [38]. This inhibitory effect of captopril on metalloproteinases was suggested to be the underlying mechanism of inhibition of tumor cell invasion [49] or cardiac remodelling in end stage heart failure [50]. IC₅₀ values of captopril in in vitro assays were within the millimolar range [38,50]. Thus, the results of our own assays with captopril are in excellent agreement with previously published inhibitory concentrations, confirming the validity of our assay system. Interestingly, both M1 and M2 exceeded the inhibitory efficacy of captopril by a factor of about 100.

To elucidate whether the inhibitory activity of Pycnogenol and its metabolites toward MMPs might be (partially) due to binding to matrix proteins and thus protection of these proteins against enzymatic digestion, we determined the binding behavior of these compounds. It had been shown earlier that collagen treated with (+)-catechin became resistant to human collagenase [47]. While the pine bark extract Pycnogenol was already known to bind to proteins [51] and to inactivate certain enzymes [52] it was not clear yet whether there is a preference in binding toward collagen or elastin compared with nonmatrix proteins, such as ovalbumin. We can now provide evidence that Pycnogenol indeed binds preferentially to matrix proteins. The binding toward elastin is more pronounced than the binding toward collagen. Polyphenols tend to bind to proline-rich structures, which are highly abundant in matrix proteins, and hydrophobic bonding plays a dominant role in this interaction [51,53]. In contrast, we observed only low binding of metabolite M2 to skin powder. This is consistent with the observation that the binding affinity toward matrix proteins increases with the complexity of the polyphenols [53]. We conclude that the inhibitory activity of the pine bark extract toward proteolytic degradation of matrix proteins may be partially due to binding to and thus protecting these proteins of MMPs. This mechanism is, however, unlikely for the pine bark metabolites. M2 does not bind significantly to skin powder and it has to be assumed that it directly interacts with the catalytic action of the metalloproteinases.

To find evidence that the metabolites M1 and M2 may directly interact with the active center of MMP-9 we determined inhibitory activity in the presence of zinc ions. Zinc is located within the active site of matrix metalloproteinases. Sorbi et al. reported that the addition of zinc ions reversed the inhibition of MMP-2 and MMP-9 by captopril and concluded that this compound exhibits its action by chelation of Zn²⁺ at the active site [38]. Our experiments confirmed the reversal of MMP-9 inhibition by captopril in the presence of Zn²⁺ and revealed that both metabolites displayed analogous behavior. This means that both M1 and M2 have the ability to interact with Zn²⁺ and thus may bind directly at the active site of MMP-9. The addition of zinc ions did not alter the inhibitory activity of Pycnogenol. This is consistent with the idea that the maritime pine bark extract directly binds to and thus protects the matrix proteins. It cannot, however, be excluded that the chelating capacity of the procyandins exceeded the amount of zinc ions added to the reaction vial.

After demonstrating a direct inhibitory effect of M1 and M2 toward activated MMPs we elucidated the impact of the metabolites on LPS-induced total MMP-9 secretion from freshly isolated human monocytes. A dose-dependent reduction in MMP-9 secretion was observed when M1 or M2, was present in the incubation medium. Both metabolites exhibited equal efficacy in inhibition of MMP-9 secretion. Only 0.5 μM was required to reduce enzyme secretion by about 50% indicating the high potency of both compounds. Interestingly, the endogenous broadly acting anti-inflammatory compound hydrocortisone was less potent in reduction of MMP-9 secretion compared with M1 or M2. A reduction in MMP-9 secretion was also seen when mouse macrophages were incubated with various compounds from the bark of *Tristaniopsis calobuxus* (Myrtaceae). Thirty micromolar concentrations of ellagic acid, (+)/(-)-gallocatechin, and (-)-epigallocatechin reduced MMP-9 secretion to 62–76% of control.
Though these results cannot be compared directly with ours because we assayed total MMP-9 secretion (proMMP and activated MMP) while Bellosta et al. determined activated MMP-9 only, it indicates a higher potency of the pine bark metabolites M1 and M2. In a comparative study measuring the secretion of activated MMP-9 and its inhibition by tea polyphenols, again rather high concentrations — even of the potent epigallocatechin gallate — were required to suppress MMP-9 secretion [55]. Similar results were reported when proMMP-2 secretion from glioblastoma cells was measured. Twenty-five micromolar epigallocatechin gallate suppressed proMMP-2 secretion by about 50% [56].

To elucidate whether the mechanism of the inhibition of MMP-9 secretion by M1 and M2 might be related to the radical scavenging properties of these molecules, we tested possible antioxidant properties of these molecules. ROS play an important role as signaling molecules as many cellular pathways are subjected to redox regulation [57]. Bacterial LPS can activate the redox-sensitive transcription factor NF-κB via ROS and might thus induce pro-inflammatory cytokine production [58,59]. MMPs are known to be activated by ROS [11–14] via various intracellular signaling pathways [15]. Thus, ROS most probably played a central role in the LPS-induced secretion of MMP-9 from monocytes. We indeed found that metabolite M1 inhibits superoxide-induced reduction of NBT. Most notably, M1 was statistically significantly more potent compared with all other compounds tested. Thus, we identified a novel comparatively potent radical scavenging property of a pine bark metabolite. The pine bark extract Pycnogenol itself is a highly potent antioxidant [2,29]. Surprisingly, within the concentration range tested, neither M2 nor ferulic acid displayed any radical scavenging activity. It can be assumed that the catechol structure (3,4-dihydroxyphenyl) is essential in this assay. Neither M2 nor ferulic acid has this structural feature. This is consistent with earlier observations that the radical scavenging ability increases with a catechol structure [60]. Previously, IC_{50} values of 14 and 8.7 μM were reported for vitamin C and (+)-catechin, respectively, in the NBT reduction assay [61]. Thus, (+)-catechin is a more potent scavenger compared with vitamin C. In our assay we did not observe a statistically significant difference between these two compounds, (+)-catechin appearing only a little less active than vitamin C. Inhibitory concentrations for reduction of superoxide level were reported as 1.73 μM for taxifolin and 1.61 μM for (+)-catechin [62]. Though we observed generally higher values we also found that catechin is a better scavenger than taxifolin. However, although we identified M1 as a novel radical scavenging compound, this cannot explain the inhibitory effects on LPS-induced MMP-9 secretion from human monocytes. The concentrations of M1 required for effective radical scavenging exceed those required for inhibition of MMP-9 secretion by a factor of 50. Also, M1 and M2 displayed equal efficacy in attenuating MMP-9 release while M2 was not active in the NBT reduction assay within the range of concentrations tested. Thus, the radical scavenging properties of M1 most probably do not contribute significantly to the inhibition of LPS-induced MMP-9 release from monocytes.

In the second assay, we determined the total antioxidant activity of the metabolites and found that both are active. Consistent with our results from the first assay we saw that M1 was the most potent of the compounds tested. We found the following order of reducing power: M1 > (+) catechin > vitamin C > M2 > ferulic acid > taxifolin. M1 was statistically significantly more potent than all other compounds tested. In a recent work using the same method a similar order of catechin > vitamin C ≈ ferulic acid was established [41]. Due to the fact that M1 and M2 displayed very different antioxidant activities while they were equally potent in inhibition of MMP-9 release we conclude that this property does not provide a satisfying mechanistic explanation for the observed effect.

To summarize, we characterized molecular pharmacological effects of two major metabolites of the standardized maritime pine bark extract Pycnogenol. We discovered strong inhibitory effects of M1 and M2 toward the activity of MMP-1, MMP-2, and MMP-9. On a cellular level, we detected highly potent inhibition of MMP-9 release by both metabolites and identified radical scavenging activity of M1 and general antioxidant activity of both metabolites. However, the inhibition of MMP-9 secretion appeared to be regulated by a redox-independent pathway yet to be identified. Interestingly, on a microgram-per-milliliter basis both metabolites were more active than the pine bark extract in MMP inhibition. The metabolites were more potent than their metabolic precursor (+)-catechin in MMP inhibition and M1 was a better scavenger than catechin. Our data contribute to the comprehension of the well-documented effects of Pycnogenol and provide a rational basis for the use of standardized maritime pine bark extract in prophylaxis and therapy of multiple disorders related to imbalanced or excessive MMP activity.

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REFERENCES


