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Inhibition of host- and bacteria-derived proteinases by natural anthocyanins

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Background and Objectives: Host- and bacteria-derived proteinases are considered to play critical roles in periodontitis progression. This study investigated the ability of a blackcurrant extract and its major anthocyanins (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside) to inhibit the activity of matrix metalloproteinases (MMPs), neutrophil elastase and periodontopathogen (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) proteinases.

Material and Methods: Enzyme inhibition was detected using fluorometric and colorimetric assays after incubating blackcurrant extract and its major anthocyanins (at concentrations of 6.25, 12.5, 25 and 50 μg/mL) with MMPs, elastase or bacterial proteinases, along with their specific substrates. Substrate degradation was recorded every hour for up to 4 h.

Results: The blackcurrant extract (50 μg/mL) inhibited all proteinases tested. MMP-1 and MMP-9 were significantly inhibited by pure anthocyanins at concentrations ranging from 6.25 to 50 μg/mL. Elastase activity was inhibited by cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside in the range of 6.25–50 μg/mL and by delphinidin-3-*O*-rutinoside at 50 μg/mL. *P. gingivalis*, *T. forsythia* and *T. denticola* proteinases were also significantly inhibited by pure anthocyanins. In all cases, enzyme inhibition was time-dependent.

Conclusion: Our study showed that a blackcurrant extract and its major anthocyanins were able to inhibit the activity of host- and bacteria-derived proteinases. This suggests that such natural compounds may represent promising agents for use in adjunctive treatments for periodontitis.

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Periodontitis is a complex multifactorial disorder involving gram-negative anaerobic bacteria (called periodontopathogens) and the host immune response, the combined effect of which leads to the destruction of tooth-supporting tissues and eventually to tooth loss (1). Among over 700 microbial species found in the human oral cavity, a specific bacterial group, named the 'red complex', comprising *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, has been strongly associated with clinical signs of advanced periodontal lesions, especially probing depth and bleeding on probing (2,3). These bacteria produce a large array of virulence factors, including cell-bound and secreted proteinases with a broad activity spectrum (4). Scientific evidence indicates that bacteria-derived proteinases can play multiple roles in the pathogenesis of periodontitis through the hydrolysis of plasma proteins and tissue proteins, thus contributing to neutralization of the immune defence system and to tissue destruction (5). To further support the role of bacterial proteinases as key virulence factors, it was reported that a gingipain inhibitor could prevent the progression of *P. gingivalis*-induced gingival inflammation in a rat model (6). Besides proteinases, lipopolysaccharide from these bacteria is able to trigger a host response leading to the

release of proinflammatory cytokines, chemokines, prostaglandin E_2 and matrix metalloproteinases (MMPs) (5,7). MMPs are well known to mediate connective tissue degradation during the active phases of periodontitis (8,9). These zinc-dependent proteinases are produced by the major cell types in human periodontal tissue (fibroblasts and epithelial cells), as well as by inflammatory cells, including macrophages, lymphocytes and neutrophils (8). Diseased periodontal sites exhibit higher collagenase activity than their healthy counterparts, and this has been attributed to the presence of MMP-1, MMP-8 and MMP-13 (10). The levels of gelatinases (MMP-2 and MMP-9) are also increased during periodontitis (9). The host response to periodontopathogen challenge is also associated with the release of elastase (a serine proteinase) by triggered neutrophils. Elastase is found at higher levels during periodontal inflammation and has been suggested as an indicator of disease progression (11). Moreover, it has been shown that periodontal treatment correlates with decreased elastase activity in the gingival crevicular fluid (12,13).

Research has gained insight into the beneficial properties of anthocyanins for human health. These flavonoid compounds, well known for their antioxidant activity, are found in a variety of vegetables and fruits, such as berries, red grapes and red cabbage (14). A number of therapeutic benefits have been associated with anthocyanins, including cardioprotective, neuroprotective, anti-inflammatory and anticarcinogenic properties (15). The antimicrobial property of anthocyanins has also been reported, more specifically its bactericidal effect on *Helicobacter pylori* (16).

Based on our knowledge of the mechanisms of host- and bacteria-mediated tissue destruction in periodontitis, targeting proteinases may be considered as a valuable strategy for periodontal treatment. Several studies have shown that MMP activity can be inhibited by low-dose doxycyclines (17) and by chemically modified nonantimicrobial tetracyclines (8), resulting in clinically relevant beneficial effects, such as reduction of the progression of periodontal attachment loss (18,19). Similarly, it was also demonstrated that these tetracycline-derived molecules were able to inhibit the proteolytic activity of *P. gingivalis* and *T. denticola* (20). In accordance with the current trend of searching for alternative therapeutic agents to prevent periodontal tissue destruction, this study investigated the ability of a blackcurrant (*Ribes nigrum*) extract and its three major anthocyanins (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside) to inhibit the activity of MMP-1 and MMP-9, neutrophil elastase and proteinases produced by *P. gingivalis, T. forsythia* and *T. denticola*.

Material and methods

Materials

All reagents used in the study were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), unless otherwise stated.

Blackcurrant (*Ribes nigrum*) extract

The blackcurrant extract, obtained from Burgundy France (Reyssouze, France), was standardized to 30% anthocyanins and contained mainly cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside.

Anthocyanins

Purified anthocyanins were obtained from PlantChem (Sandnes, Norway). Cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside (Fig. 1) were prepared in 95% ethanol and kept at 4°C, protected from light. These three anthocyanins represent 85% of the total anthocyanin composition of blackcurrant (21).

Bacteria

The bacterial strains used in this study were *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405 and *T. forsythia*

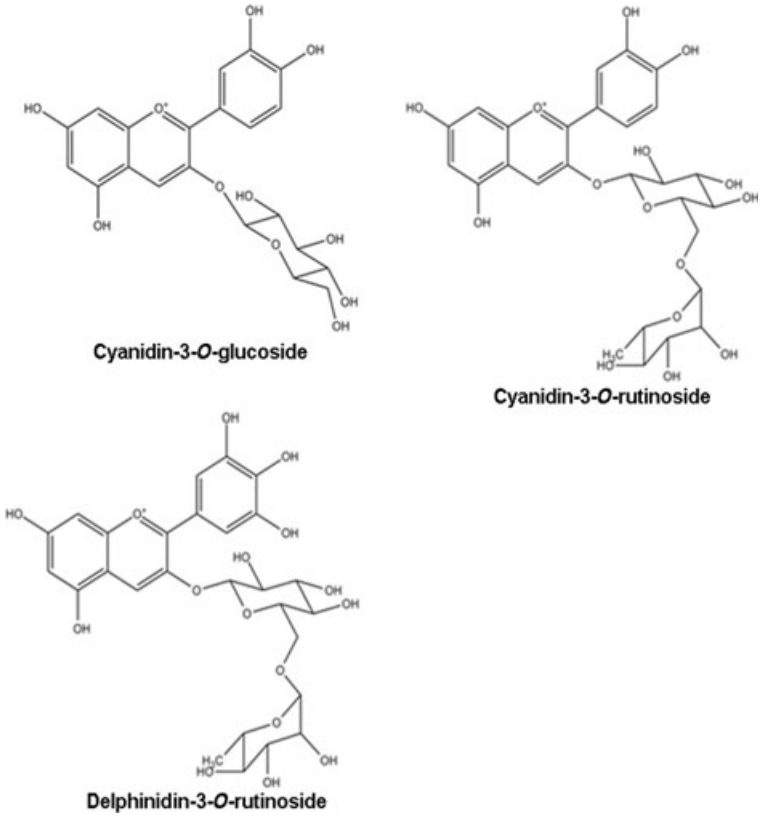

Fig. 1. Chemical structures of cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside.

Table 1. Inhibitory effect of a blackcurrant extract (50 μg/mL) on host- and bacteria-derived proteinase activities

Proteinase activity	Relative activity (%)[a]
MMP-1	35 ± 6[b]
MMP-9	46 ± 4[b]
Neutrophil elastase	39 ± 11[b]
Porphyromonas gingivalis	76 ± 2[b]
Tannerella forsythia	39 ± 4[b]
Treponema denticola	28 ± 1[b]

[a]A value of 100% was given to the activity obtained in the absence of the blackcurrant extract. Means ± standard deviation are presented.
[b]$p < 0.05$ for all inhibition values when compared with the respective level of activity in the absence of the blackcurrant extract (Student's *t*-test).

ATCC 43037. *P. gingivalis* was grown in Todd Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with haemin (10 μg/mL) and vitamin K (1 μg/mL). *T. denticola* was cultured in oral spirochete medium, as previously described (22), and *T. forsythia* was grown in Brain–Heart Infusion broth (BHI; BBL Microbiology Systems) supplemented with 5% heat-inactivated bovine serum and 0.001% *N*-acetylmuramic acid (10 μg/mL). Bacterial cultures were incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 48 h (*P. gingivalis*) or 4 d (*T. denticola* and *T. forsythia*) and then centrifuged (10,000 *g* for 10 min). Culture supernatants of *P. gingivalis* and *T. denticola* were collected, whereas the cells of *T. forsythia* were washed in 50 mM phosphate-buffered saline (pH 7.4) and suspended in the same buffer. This bacterial suspension was diluted 1 : 10 to yield an optical density at 660 nm (OD_{660}) of 2.

Determination of MMP activity

Human recombinant MMP-9 (Calbiochem, San Diego, CA, USA) was obtained in the active form, whereas human recombinant latent MMP-1 (Calbiochem) was activated at 37°C for 2 h in 10-fold TCNB buffer (50 mM Tris–HCl, 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij35, pH 7.5) containing 1 mM *p*-aminophenylmercuric acetate. Active MMPs were then diluted to a final concentration of 100 ng/mL in TCNB buffer. The assay mixtures contained 75 μL of activated MMPs at 100 ng/mL, 15 μL of fluorogenic substrate (1 mg/mL of fluorescein-conjugated DQ™ gelatin for MMP-9 and fluorescein-conjugated DQ™ type I collagen for MMP-1; both were obtained from Molecular Probes, Eugene, OR, USA), 3 μL of blackcurrant extract (at a final concentration of 50 μg/mL) or anthocyanins (final concentrations of 0, 6.25, 12.5, 25 and 50 μg/mL) and 57 μL of TNCB buffer to make the final assay volume up to 150 μL. The assay mixtures were incubated in the dark for 4 h at room temperature (for MMP-1) or at 37°C (for MMP-9). The fluorescence was measured every hour, for up to 4 h, using a fluorometer (VersaFluor™ Fluorometer System; Bio-Rad Laboratories, Hercules, CA, USA) with excitation and emission wavelengths set at 490 and 520 nm, respectively. A value of 100% was given to the activity of control assays performed in the absence of the test substances.

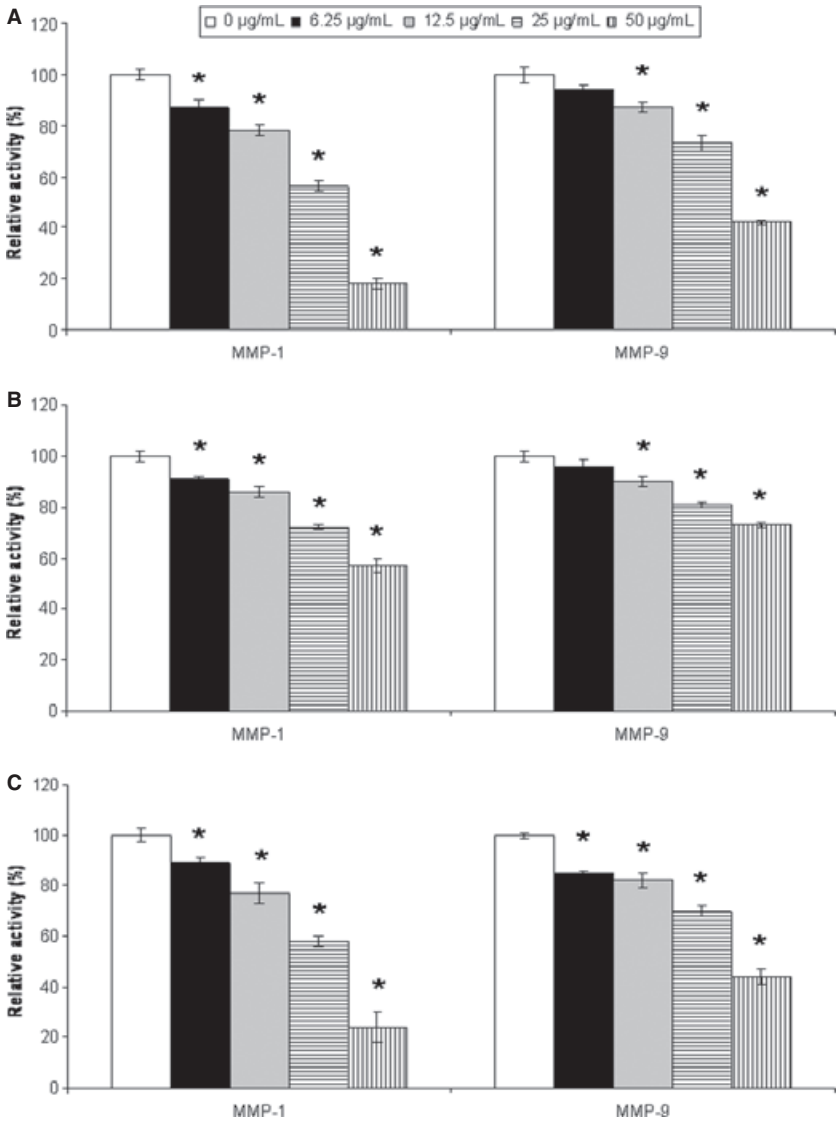

Fig. 2. Inhibitory effect of anthocyanins on MMP activity. A value of 100% was assigned to degradation observed after incubation (for 4 h) of substrate with either MMP-1 or MMP-9 in the absence of anthocyanins. Bars marked with an asterisk (*) indicate significant inhibition of enzyme activity when compared with the untreated control ($p < 0.05$). (A) Cyanidin-3-*O*-glucoside. (B) Cyanidin-3-*O*-rutinoside. (C) Delphinidin-3-*O*-rutinoside.

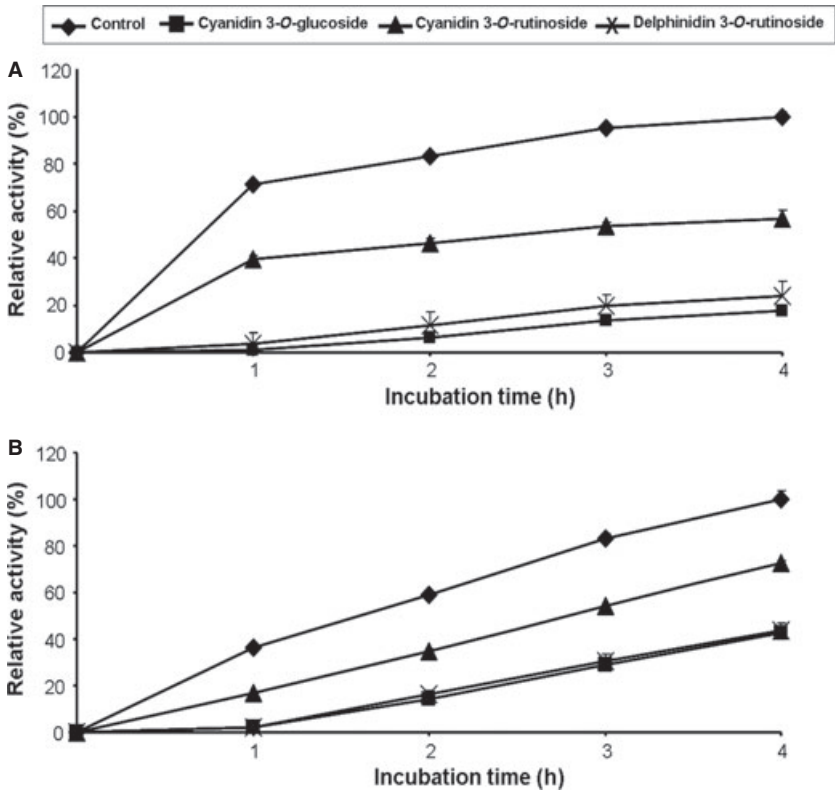

Fig. 3. Time-dependent inhibition of MMP activity by anthocyanins (50 μg/mL). (A) MMP-1. (B) MMP-9.

Determination of elastase activity

Human neutrophil elastase (Calbiochem) was used to verify the inhibitory effect of anthocyanins. The assay mixtures contained 2 μL of elastase (50 μg/mL), 25 μL of elastase colorimetric substrate I (4 mM; Calbiochem) and 73 μL of reaction buffer (100 mM Tris–HCl, 500 mM NaCl, pH 7.5), with or without blackcurrant extract (at a final concentration of 50 μg/mL) or anthocyanins (at final concentrations of 0, 6.25, 12.5, 25 and 50 μg/mL). Incubation was performed in a 96-well plate at 37°C for up to 4 h. Hydrolysis of the chromogenic substrate was determined by measuring the absorbance at 415 nm in a microplate reader (Bio-Rad Laboratories). A value of 100% was given to the activity of control assays performed in the absence of the test substances.

Determination of bacterial proteinase activity

Blackcurrant extract or anthocyanins (3 μL) were added to a mixture containing 7.5 μL of *P. gingivalis* supernatant, *T. denticola* supernatant or *T. forsythia* cells, 15 μL of the fluorogenic substrate at 100 μg/mL, and 124.5 μL of TCNB buffer, to obtain final concentrations of 0, 6.25, 12.5, 25 and 50 μg/mL. Fluorescein-conjugated type I collagen, incubated at room temperature, was used to measure the collagenase activity of *P. gingivalis*. Fluorescein-conjugated gelatin, incubated at 37°C, was used to measure the gelatinase activity of *T. denticola* and *T. forsythia*. Incubation was carried out in the dark, and fluorescence was measured every hour for up to 4 h using a fluorometer, as described earlier, under 'Determination of MMP activity'. A value of 100% was given to the activity of control assays performed in the absence of the test substances.

Statistical analysis

Each experiment was performed in triplicate and repeated three times. Data were expressed as mean ± standard deviation. Statistical analysis was performed using the Student's *t*-test and analysis of variance with the Dunnett *post-hoc* multiple comparison test. The confidence level was set at $p \leq 0.05$.

Results

In our search for natural products with beneficial properties for oral health, we evaluated the ability of a blackcurrant crude extract to inhibit the activity of host- and bacteria-derived proteolytic enzymes. The results presented in Table 1 show that this crude fraction significantly inhibited ($p < 0.05$) all proteolytic activity tested. The activities of the host enzymes MMP-1, neutrophil elastase and MMP-9 were reduced to 35 ± 6%, 39 ± 11% and 46 ± 4%, respectively, of the control activity. Regarding bacterial enzymes, the activity levels dropped to as low as 28 ± 1% of the control activity.

The antiproteinase properties of blackcurrant were further investigated by assessing the individual inhibitory effects of its major anthocyanins, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside. As these substances were prepared in ethanol, we first showed that ethanol, the final concentration of which was always ≤ 2% in our assays, had no inhibitory effect. Cyanidin-3-*O*-glucoside, at all concentrations tested, caused a significant reduction of type I collagen degradation by MMP-1 (Fig. 2A). More specifically, 50 μg/mL of cyanidin-3-*O*-glucoside inhibited MMP-1 activity by 82 ± 2%. Cyanidin-3-*O*-glucoside also significantly inhibited MMP-9 activity when used at 12.5, 25 and 50 μg/mL, with a reduction of 13 ± 2%, 27 ± 3% and 58 ± 1% observed, respectively (Fig. 2A). Cyanidin-3-*O*-rutinoside, at all concentrations tested, significantly inhibited MMP-1 activity (Fig. 2B). The highest concentration (50 μg/mL) of cyanidin-3-*O*-rutinoside inhibited MMP-9 activity by 43 ± 3%. MMP-9 activity was also significantly decreased by 12.5 μg/mL (10 ± 2% inhibition), 25 μg/mL (19 ± 1% inhibition) and 50 μg/mL (27 ± 1% inhibition) cyanidin-3-*O*-rutinoside (Fig. 2B).

Delphinidin-3-*O*-rutinoside inhibited MMP-1 by 11 ± 2%, 23 ± 4%, 42 ± 2% and 76 ± 6%, at 6.25, 12.5, 25 and 50 μg/mL, respectively (Fig. 2C). Similarly, MMP-9 activity was also significantly reduced (by 15 ± 1%) even at the lowest concentration tested (6.25 μg/mL) (Fig. 2C). The inhibitory effect of anthocyanins (50 μg/mL) on MMP-1 and MMP-9 activities was time-dependent (Fig. 3).

Elastase activity was significantly inhibited by anthocyanins, mainly cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, which were effective at all concentrations tested (Fig. 4A). At 50 μg/mL, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside yielded a reduction in activity of approximately 30%. Delphinidin-3-*O*-rutinoside significantly inhibited elastase activity at 12.5, 25 and 50 μg/mL, resulting in a reduction of 8 ± 4%, 8 ± 3% and 18 ± 3%, respectively (Fig. 4A). A time-dependent inhibitory effect of anthocyanins (50 μg/mL) on elastase was also observed (Fig. 4B).

Anthocyanins also affected the degradation of gelatin and type I collagen by bacterial proteinases. Cyanidin-3-*O*-glucoside significantly inhibited *P. gingivalis* collagenolytic activity at all concentrations tested. Of note, an inhibition of 96 ± 1% was obtained at 50 μg/mL (Fig. 5A). *T. forsythia* and *T. denticola* proteinase activities were also significantly inhibited at all concentrations tested (Fig. 5A). The activity of *T. forsythia* appeared to be more susceptible to cyanidin-3-*O*-glucoside. Cyanidin-3-*O*-rutinoside was able to efficiently reduce protein degradation by *P. gingivalis* and *T. forsythia* at all concentrations tested. Inhibition ranged from 14 ± 2% to 56 ± 4% for *P. gingivalis* and from 18 ± 3% to 50 ± 1% for *T. forsythia* (Fig. 5B). A significant reduction of *T. denticola* proteolytic activity was obtained with 25 and 50 μg/mL of cyanidin-3-*O*-rutinoside, which yielded inhibition of 12 ± 1% and 25 ± 1%, respectively (Fig. 5B). Similarly, delphinidin-3-*O*-rutinoside caused a significant inhibition of *P. gingivalis* and *T. forsythia* proteolytic activities at all concentrations, whereas reduction of *T. denticola* proteolytic activity could only be achieved at 25 and 50 μg/mL (Fig. 5C). Reduction in activity values ranged from 50 ± 2% to 96 ± 1% for *P. gingivalis*, from 18 ± 1% to 50 ± 3% for *T. forsythia* and from 13 ± 2% to 31 ± 1% for *T. denticola* (Fig. 5C). As observed for MMP and elastase activity, the effect of pure anthocyanins (50 μg/mL) on bacterial proteinases was also time-dependent (Fig. 6).

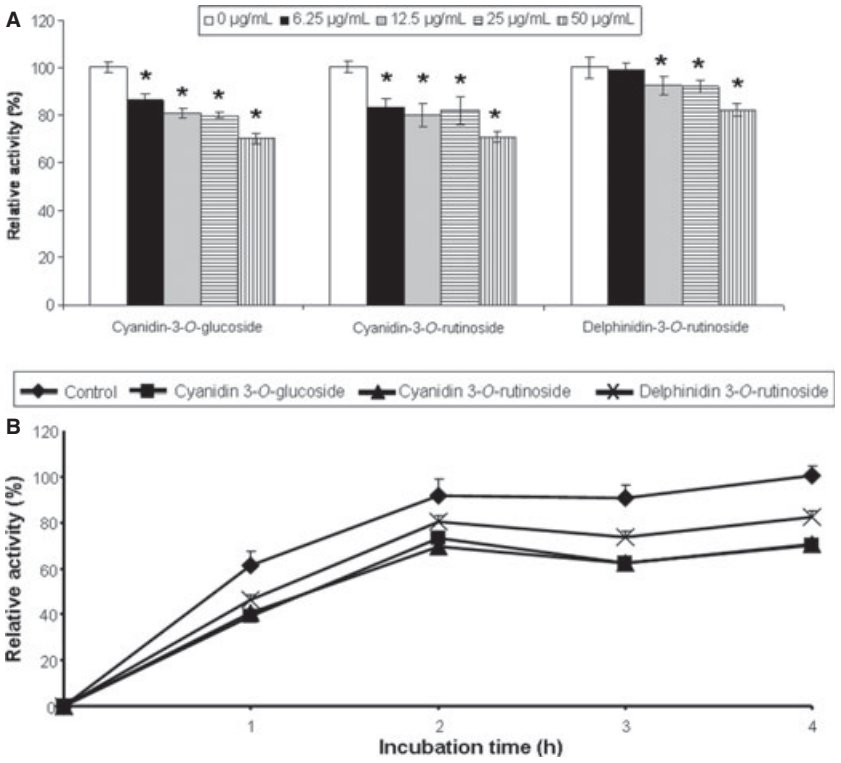

Fig. 4. Inhibitory effect of anthocyanins on neutrophil elastase activity. Elastase was incubated with or without anthocyanins and the chromogenic substrate. Enzyme activity was recorded to 4 h using a spectrophotometer. (A) Degradation observed after 4 h of incubation. Bars marked with an asterisk (*) indicate significant inhibition of enzyme activity when compared with the untreated control ($p < 0.05$). (B) Time-dependent inhibition of elastase activity by anthocyanins (50 μg/mL).

Discussion

Scaling and root planing procedures are standards of practice when treating periodontitis patients. In spite of being highly effective in most cases, there are still some situations in which this therapeutic approach cannot yield satisfactory clinical outcomes. In such cases, adjunctive therapies may be appropriate, more specifically the use of antimicrobial and anti-inflammatory agents. Several therapeutic strategies, based on targeting different pathways of the pathogenesis of periodontal disease, have been put forward. In this regard, a number of authors proposed that periodontitis progression can be hampered by successfully inhibiting both bacteria- and host-derived proteinases involved in connective tissue destruction of the periodontium (6,17).

This study first demonstrated that a crude extract of blackcurrant possesses a marked capacity to inhibit various proteolytic enzymes. As blackcurrant is known to have a high content of anthocyanins, we further tested the antiproteinase property of its major anthocyanins cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside. A marked time-dependent inhibitory effect of anthocyanins on both MMP-1 and MMP-9 was observed. Similar outcomes have previously been reported for other phenolic compounds, espe-

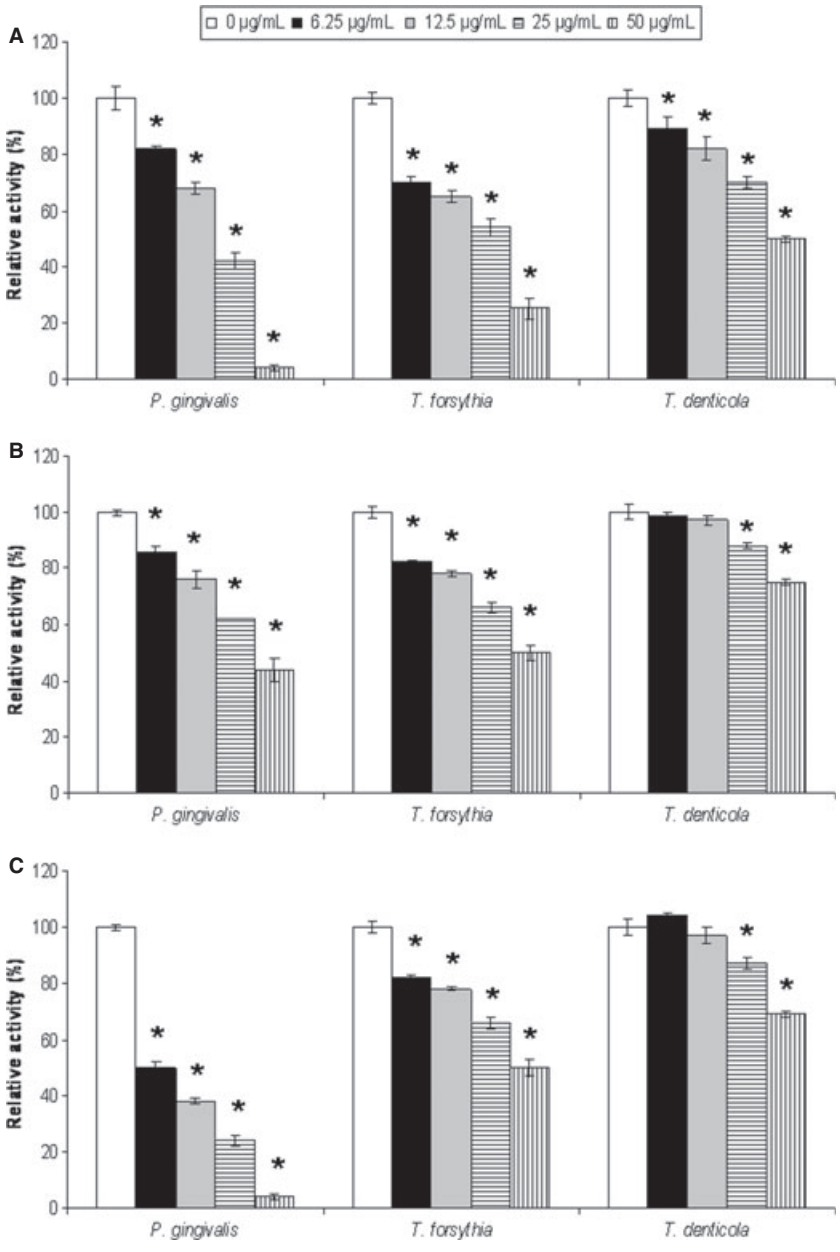

Fig. 5. Inhibitory effect of anthocyanins on bacterial proteinase activity. A value of 100% was assigned to degradation observed after incubation (for 4 h) of substrate with *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*) or *Treponema denticola* (*T. denticola*) proteinases in the absence of anthocyanins. Bars marked with an asterisk (*) indicate significant inhibition of enzyme activity when compared with the untreated control ($p < 0.05$). (A) Cyanidin-3-*O*-glucoside. (B) Cyanidin-3-*O*-rutinoside. (C) Delphinidin-3-*O*-rutinoside.

cially proanthocyanidins derived from cranberry (*Vaccinium macrocarpon*) (23,24). MMPs are thought to play an important role in periodontal tissue destruction (8,10). As a collagenase, MMP-1 may trigger degradation of connective tissue by breaking-down type I collagen fibrils into smaller, three-quarter- and quarter-length fragments, thus allowing other MMPs, for instance MMP-9 (gelatinase-2), to further hydrolyze this broken collagen into smaller peptides and amino acids (8). Both by-products may represent effective nutrients for asaccharolytic periodontopathogens, such as *P. gingivalis*, and thus may promote their multiplication in subgingival sites (25). Therefore, the MMP inhibitory effect presented by anthocyanins may be of potential therapeutic use in controlling MMP-mediated tissue destruction in periodontitis. Inhibition of collagenolytic enzymes in gingival crevicular fluid by polyphenols of green tea has been previously reported (26). It was found that epicatechin gallate and epigallocatechin gallate strongly inhibited collagenase activity, whereas catechin, epicatechin, gallocatechin and epigallocatechin had no effect. Although the exact mechanism of MMP inhibition by anthocyanins is not known, it may be related to either direct competition with the substrate by binding to the active site or to a conformational change in the enzyme by binding to a remote site. Interestingly, Madhan *et al.* (27) recently showed that the green tea flavonoid epigallocatechin gallate could induce changes in the secondary structure of collagenase, resulting in catalytic inhibition.

The elevated levels and high activity of neutrophil elastase observed in diseased periodontal sites are considered as biomarkers for periodontal disease progression (11,28). Once secreted, elastase can degrade different extracellular matrix proteins, including elastin, collagen, proteoglycan, fibronectin and laminin (29), thus contributing to tissue degradation. Accordingly, a reduction in the levels of elastase is usually detected as an outcome of efficient periodontal therapy and stabilization of the inflammation process (13). In this study we demonstrated that anthocyanins are able to inhibit elastase activity, indicating that these natural compounds may help to control the destructive effect of elevated elastase activity on periodontal tissues.

Proteinases produced by periodontopathogenic bacteria are considered as major virulence factors because they may participate in colonization, tissue invasion and evasion from host defence (25). The broad proteolytic activity exhibited by bacteria of the red complex (*P. gingivalis*, *T. forsythia* and *T. denticola*) is likely to contribute to periodontitis progression and may explain why this group of pathogens is frequently associated with more severe forms of the

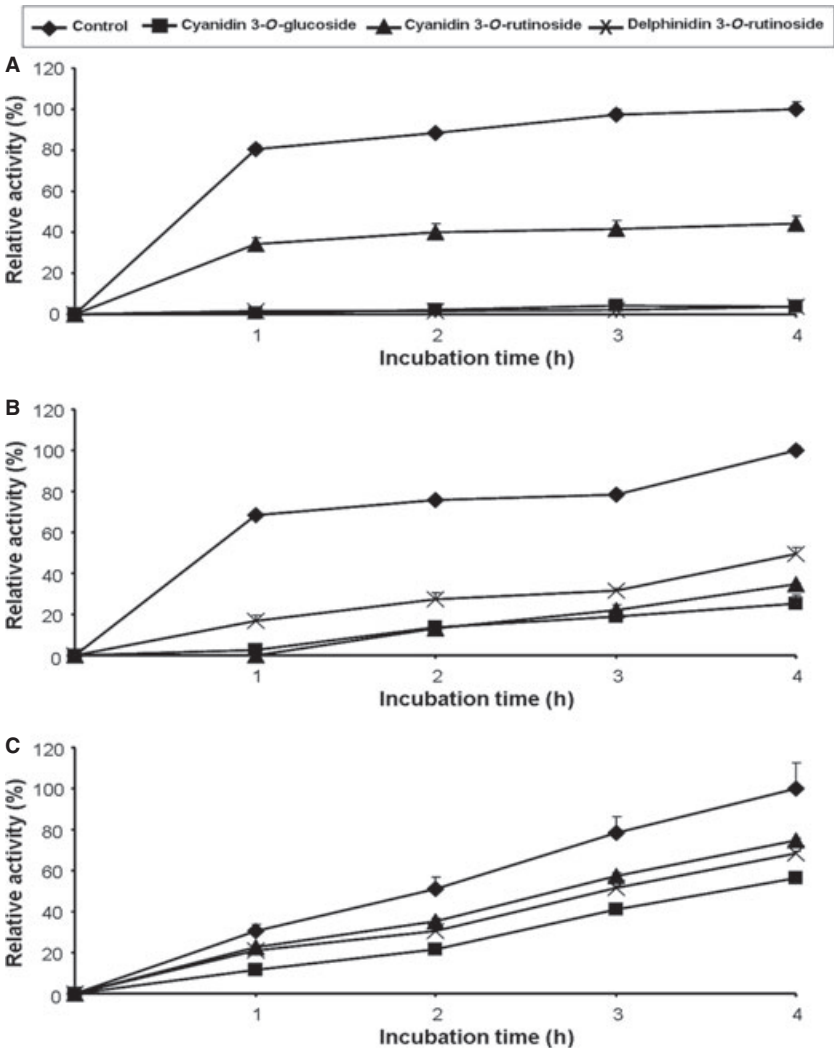

Fig. 6. Time-dependent inhibition of bacterial proteinases by anthocyanins (50 μg/mL). (A) *Porphyromonas gingivalis*. (B) *Tannerella forsythia*. (C) *Treponema denticola*.

disease (5). In view of the results obtained in the present study, it may be suggested that anthocyanins reduce the pathogenicity of red complex bacteria by inhibiting their proteolytic activity. As bacterial infection of host tissues triggers an inflammatory response that will further increase tissue damage, anthocyanins may also contribute to the reduction of host-mediated destructive processes. An uncharacterized high-molecular-weight proanthocyanidin fraction isolated from cranberry has demonstrated inhibitory effects on *P. gingivalis*, *T. forsythia* and *T. denticola* proteinases that are similar to those of the blackcurrant extracts in the present study (30).

In the search for adjunctive therapies that would improve the outcome of periodontal treatments, it is important to consider approaches that will target bacteria- and host-mediated mechanisms of tissue destruction. Anthocyanins have long been investigated for their diverse human health benefits. However, to the best of our knowledge, this is the first report on the potential benefits of anthocyanins for periodontal health. In this regard, this study clearly showed that the three tested anthocyanins – cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside – possess marked inhibitory activities on MMP-1, MMP-9, elastase and bacterial proteinases. Although additional studies are required, this group of polyphenols may be regarded as potential therapeutic agents with the ability to limit extracellular matrix degradation and bacterial virulence. It is not clear whether dietary intake of anthocyanin-rich berry fruits may allow a satisfactory therapeutic effect for periodontal disease. However, the topical application of anthocyanins to diseased periodontal sites may represent an effective strategy to obtain therapeutic concentrations of the bioactive compounds.

The inhibitory effects of anthocyanins on both host- and bacteria-derived proteinases, demonstrated in the present study, could be important not only in the context of periodontal disease but also in other proteinase-mediated diseases. Indeed, this rationale may be potentially extended to other oral and nonoral disorders involving proteolytic microbial pathogens and the host-mediated inflammatory response. Additionally, the broad-spectrum proteinase-inhibitory effect exhibited by the crude blackcurrant extract may be considered quite promising. As cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and delphinidin-3-*O*-rutinoside are the major anthocyanins in blackcurrant, these flavonoids were probably responsible for the antiproteolytic activity observed in the crude extract. The incorporation of such crude plant extracts could be interesting for industrial large-scale production of oral hygiene products as the use of purified molecules usually results in a high cost of the final product. In this sense, the present results indicate that blackcurrant fractions would be suitable as a cost-effective ingredient with potential to be used in oral health products.

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