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Efficacy of horse chestnut leaf extract ALH-L1005 as a matrix metalloproteinase inhibitor in ligature-induced periodontitis in canine model

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Matrix metalloproteinases (MMPs) are the main proteinases associated with periodontal tissue destruction and remodeling. Therefore, inhibition of host-derived MMPs has a key role in the prevention and reduction of periodontitis progression. Horse chestnut (*Aesculus hippocastanum* L.) extracts have been used as treatments for inflammatory disease, traditionally. This study assessed the clinical effect as a MMP inhibitor of horse chestnut leaf extract ALH-L1005 on periodontitis. ALH-L1005 was obtained from horse chestnut leaf and its MMP inhibitory activities estimated. Periodontitis was induced in beagles assigned to 4 groups and medicated for 6 weeks: low dose test (LT; ALH-L1005, 100 mg/kg/day), high dose test (HT; ALH-L1005, 200 mg/kg/day), positive control (PC; doxycycline, 10 mg/kg/day), or negative control (NC; placebo). Before and after administration, clinical indices of the teeth and MMP quantity in gingival tissues using zymography were measured. Clinical conditions of the LT, HT, and PC groups were significantly improved after 6 weeks. In zymographic evaluations, gelatinolytic and caseinolytic activities were suppressed in LT, HT, and PC groups but not in the NC group. The results suggest that ALH-L1005 could be an effective agent for clinical prevention and treatment of periodontitis by inhibiting the gelatinase and collagenase activities, which can detach periodontal ligaments from alveolar bone.

Keywords: dog, doxycycline, horse chestnut leaf extract, matrix metalloproteinase, periodontal disease

Introduction

Periodontal diseases are caused by periodontal bacterial colonization (plaque) and stimulation of the host's immune responses due to bacterial by-products [1]. Several researchers have shown pharmacological modulation of exaggerated host inflammatory and immune responses in addition to microbial elimination to be beneficial in the treatment of periodontitis [9,23]. It has been reported that matrix metalloproteinases (MMPs) have important roles in the proteolysis of components of the extracellular matrix [4]. Under progression of periodontal disease, the host and microbial-derived proteolytic enzymes mediate extracellular matrix degradation [23]. Recent studies have shown that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) can produce periodontal tissue destruction; moreover, polymorphonuclear leukocyte (PMN)-derived MMPs (MMP-8, MMP-9) have been reported to be the main proteinase involved in periodontal tissue destruction and remodeling [5,11,18]. Other studies have shown that gingival crevicular fluid samples from periodontitis contain MMP-13, which was not found in samples from healthy gingiva [10]. Thus, inhibition of host-derived MMPs is may have a key role in the prevention and reduction of periodontitis progression.

In folk medicine, horse chestnut (*Aesculus hippocastanum* L.) products and their extracts had been used as remedies for cough, congestion, arthritis, and rheumatism. In particular, horse chestnut seed extract contains aescin, an acid triterpene glycoside that possesses anti-inflammatory, anti-edematous, anti-secretory, and tonic effects [19]. Horse chestnut seed extracts are available commercially, worldwide, for the management of chronic venous insufficiency. Horse chestnut leaf extracts contain only traces of aescin but are rich in flavonoids including quercetin and kaempferol, as well as flavonol glycosides such as quercitrin. Such extracts are commercially available for

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injection and are used in the treatment of edema and venous insufficiency [2].

The purpose of this study was to assess the clinical effect of the horse chestnut leaf extract ALH-L1005 as an MMP inhibitor on periodontitis progression in beagle dogs with experimentally induced periodontitis.

Materials and Methods

The protocol of this study was approved by the Institutional Animal Care and Use Committee (SNU-080118-2; Seoul National University, Korea). All experimental operations and examinations were performed under general anesthesia with a commercial combination of zolazepam and tiletamine (5 mg/kg, intramuscular [IM]; Virbac, France).

Chemical preparation

Horse chestnut leaf (*Aesculus hippocastanum* L; article No. 38135000) was purchased from Kräuter Mix (Germany) and the ALH-L1005 extract prepared as follows: Ten kilograms of dried horse chestnut leaves were extracted with 200 L of 25% aqueous ethanol for 2 h and filtrated. The filtrates were concentrated under vacuum and dried to obtain 1.73 kg of ALH-L1005. The obtained ALH-L1005 was standardized with the reference compound by using high-performance liquid chromatography (HPLC). For oral administration, 150 mg ALH-L1005 extract capsules were formed.

MMP assay of ALH-L1005

To establish the most effective formula, the MMP inhibitory activities of doxycycline, commercial seed extract (Finzelberg, Germany), commercial leaf extract (Dr. Willmar Schwabe, Germany) and ALH-L1005 were estimated experimentally. Before the MMP assay, recombinant human MMP-2 and MMP-9 (R&D Systems, USA) were activated with 1 mM APMA. The MMP activities were measured on a spectrofluorometer (Perkin-Elmer, USA) using 2,4-dinitrophenyl-Pro-Leu-Gly-Met-Trp-Ser-Arg (Calbiochem, USA) as a substrate. The MMP (10 nM) and substrate (1 μM) were mixed in 2 mL of reaction buffer (50 mM Tricine, pH 7.5, 10 mM $CaCl_2$, 200 mM NaCl) along with each test material. Fluorescence intensity was measured at room temperature using a 280 nm excitation wavelength and a 360 nm emission wavelength.

Preparation for healthy gingiva

Sixteen, approximately 1.5-year-old, beagle dogs were used for the study. All teeth of each beagle were scaled and polished. In the following 2-week period, tooth brushing was performed twice daily without anesthesia to obtain optimal periodontal status. During these 2 weeks, the beagles were fed a pellet-type hard diet to maintain gingival health by reducing the potential for plaque formation [20].

Experimentally induced periodontitis

After preparation of healthy gingiva, experimental periodontitis was induced as follows: Sutures using 2-0 silk (Ailee, Korea) with dental ligature wire (ClassOne Orthodontics, USA) were tied around the cervical region of the right upper PM2, PM3, and PM4, and right lower PM3, PM4, and M1 teeth. Tooth brushing was stopped after ligation and soft moistened food was given for 8 weeks. Teeth that were unfastened within the 8 weeks were excluded from the study. The ligatures were removed after the periodontitis-induction period, and the dogs were divided randomly into 4 groups: negative control (NC; excipient only), positive control (PC; doxycycline; DongKoo Bio & Pharma, Korea), ALH-L1005 low dose test (LT), and ALH-L1005 high dose test (HT) (Table 1). All medications were administrated perorally, twice a day following 6 weeks. After ligature removal, periodontal status indicators, including plaque index (PI), gingival index (GI), periodontal pocket depth (PPD), clinical attachment level (CAL) and bleeding on pressure (BoP), were examined. The standards were modified as summarized in Table 2 [17,26,31]. All measurements were taken by one experienced clinician (SE Kim) using a sterile color-coded periodontal probe (Helmut Zepf Medizintechnik, Germany).

Gingival sample collection and processing

At 6 weeks after the start of medication, gingival tissue samples were obtained from the buccal gingival margin of the right upper PM4 by means of a biopsy punch (Miltex, Germany). The collected tissues were washed immediately in normal saline (0.9% NaCl) to remove debris, including plaque and blood, and stored frozen at −80°C in coded microcentrifuge tubes until analyzed. Subsequently, the gingival tissues were thawed, weighed, and extracted at 4°C with 5 M urea buffer (20

Table 1. Experimental design

Group	Treatment	Dosage (mg/kg/d)	Duration (wk)	Number of animal	Number of teeth
Negative control (NC)	Excipient	100	6	3	14
Positive control (PC)	Doxy-cycline	10	6	3	14
Low dose test (LT)	ALH-L1005*	100	6	5	22
High dose test (HT)	ALH-L1005*	200	6	5	20

*25% aqueous alcohol extract of horse chestnut leaf (*Aesculus hippocastanum* L.).

Table 2. Scoring* of periodontal parameters

Parameter	Score	
PI	0	No plaque
	1	Cervical marginal plaque (within normal range)
	2	Thin and lineal film of plaque at gingival margin (< 1 mm)
	3	Lineal film of plaque less than one-third of crown (> 1 mm)
	4	Moderate amount of plaque more than one-third and less than two-thirds of crown
	5	Severe amount of plaque more than two-thirds of crown
GI	0	Absence of inflammation
	1	Mild inflammation - slight change in color of gingival margin and little change in texture
	2	Moderate inflammation − moderate change in color and hypertrophy in gingival papilla
	3	Severe inflammation − glazing, redness, edema, and hypertrophy
	4	Severe inflammation − marked redness and hypertrophy, bleeding, and ulceration
PPD	The distance between the gingival margin and the bottom of the probeable pocket	
CAL	The distance between the cementoenamel junction and the bottom of the probeable pocket	
BoP	0	Absence of bleeding within 10 sec following probing
	1	Presence of bleeding within 10 sec following probing

*Some scoring indices were modified from the original references. PI, plaque index; GI, gingival index; PPD, periodontal pocket depth; CAL, clinical attachment level; BoP, bleeding on pressure.

mg wet weight gingival tissue per 1 mL buffer). Protein content in the gingival extracts was quantified by using the bicinchoninic acid assay method [29].

Zymography

Gelatinolytic and caseinolytic activities in gingival extracts were determined by zymography. Gingival extracts were mixed with 2× zymography sample buffer containing 63 mM Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.0025% bromophenol blue without heat denaturation. Electrophoresis was performed on 10% SDS-polyacrylamide gels containing 0.1% gelatin and on 12% SDS-polyacrylamide gels containing 0.25% casein at 125 V. After electrophoresis, the gels were incubated in 2.5% renaturing buffer (Sigma-Aldrich, USA) for 30 min at room temperature with gentle agitation and then equilibrated in developing buffer containing 50 mM Tris base, 40 mM HCL, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Brij 35 for 30 min at room temperature with gentle agitation. Subsequently, the gels were incubated in developing buffer overnight at 37°C. The gels were stained with 0.1% Coomassie blue R250 (Sigma-Aldrich, USA) and destained with 10% acetic acid in 40% methanol. Human MMP-2 and -9 standards were run on gel for gelatin zymography and on MMP-13 standard for casein zymography as positive controls.

Table 3. Comparison of matrix metalloproteinases (MMP) inhibitory activities (%)

Sample (25 μg/mL)	MMP-2	MMP-9
ALH-L1005*	88.50	91.84
Commercial seed extract†	18.51	27.48
Commercial leaf extract‡	49.98	55.73
Doxycycline	93.21	93.72

*25% aqueous alcohol extract of horse chestnut leaf (*Aesculus hippocastanum* L.). †Horse chestnut seed powdered extract (Finzelberg). ‡Venoplant (Dr. Willmar Schwabe).

Statistical analysis

All data were statistically analyzed by using SPSS software (ver. 21.0; SPSS, USA). Changes in all periodontal parameters from Week 0 to Week 6 within each group were assessed via paired Student's *t*-test. For intergroup comparison, the values at Week 0 and 6 were analyzed by one-way analysis of variance (ANOVA). Dunnett's test was used as a *post hoc* test. *P* values of less than 0.05 were considered statistically significant.

Results

MMP Inhibitory activities of ALH-L1005

The MMP inhibitory activity of ALH-L1005 was compared with those of other horse chestnut extracts and doxycycline (Table 3). At the same concentrations, ALH-L1005 showed greater MMP-2 inhibition (88.50%) than the 18.51% inhibition of commercial horse chestnut seed extract (Finzelberg, Germany) and the 49.98% inhibition of commercial horse chestnut leaf extract for injection (Dr. Willmar Schwabe). In addition, ALH-L1005 showed excellent MMP-9 inhibition (91.84%) compared to the 27.48% and 55.73% inhibitions of the commercial seed extract and commercial leaf extract for injection, respectively. Compared with doxycycline, ALH-L1005 had similar inhibitory activity levels against MMP-2 and MMP-9.

Periodontal status

After the 8 week induction period, periodontitis was induced markedly on the experimental teeth compared to that on the untreated teeth. The ligatures placed on the cervical region

resulted in progression of gingival inflammation caused by an accumulation of plaque with food debris. Of the 96 teeth ligated by silk and wire ligatures, 70 continued the ligation at the end of the induction period and were included in the statistical analysis. The numbers of included teeth in each group were as follows: NC (n = 14), PC (n = 14), LT (n = 22) and HT group (n = 20).

During the experimental period, the periodontal parameters of the NC group did not recover significantly from the baseline status ($p > 0.05$), except for GI score ($p = 0.000$). However, the PC and LT groups showed significant improvements in all periodontal parameters ($p < 0.05$). The HT group also significantly improved overall ($p < 0.05$), but that group's CAL score improvement did not reach significance ($p = 0.260$). The means ± SD of the periodontal parameters at baseline and at week 6 are summarized in Table 4.

Fig. 1 shows an intergroup comparison of clinical parameters before and after the 6 week study period. Baseline values of all clinical parameters in the PC, LT, and HT groups were not significantly different from those in the NC group ($p > 0.05$; Fig. 1). At week 6, the PI and BoP scores of the LT and HT groups were significantly low compared to those of the NC group ($p < 0.05$; panel A and E in Fig. 1). Similarly, GI scores of the PC, LT, and HT groups were also significantly low compared with that of the NC group at week 6 ($p < 0.05$; panel B in Fig. 1).

There were different trend directions among the groups in CAL scores. In contrast to the CAL increment in the NC group, the other groups showed decreased CAL scores at week 6. The LT group showed significant CAL improvement over that in the NC group ($p < 0.05$; panel D in Fig. 1). Although the CAL scores of the PC and HT groups showed greater reduction than the NC group, the differences did not reach significance ($p > 0.05$). The PPD scores of all groups were not significantly different at week 6 ($p > 0.05$; panel C in Fig. 1).

Table 4. Changes in periodontal parameters after 6 week of treatment

Treatment group	Index	Baseline	Week 6
NC	PI	3.69 ± 1.09	3.64 ± 1.28
	GI	3.14 ± 0.47	2.62 ± 0.49*
	PPD	2.10 ± 0.98	1.86 ± 0.75
	CAL	1.60 ± 1.30	1.86 ± 1.07
	BoP	0.79 ± 0.42	0.76 ± 0.43
PC	PI	4.24 ± 0.85	3.74 ± 1.01*
	GI	3.40 ± 0.59	2.24 ± 0.53*
	PPD	2.40 ± 1.06	1.88 ± 0.63*
	CAL	2.19 ± 1.25	1.86 ± 1.12*
	BoP	0.90 ± 0.30	0.57 ± 0.50*
LT	PI	3.41 ± 1.40	2.89 ± 1.50*
	GI	3.17 ± 0.83	1.68 ± 0.66*
	PPD	2.45 ± 1.14	1.74 ± 0.56*
	CAL	1.50 ± 1.65	1.17 ± 1.10*
	BoP	0.61 ± 0.49	0.21 ± 0.41*
HT	PI	3.15 ± 1.12	2.57 ± 1.11*
	GI	3.03 ± 0.66	1.60 ± 0.62*
	PPD	2.57 ± 1.00	1.72 ± 0.71*
	CAL	1.45 ± 1.40	1.30 ± 1.28
	BoP	0.70 ± 0.46	0.23 ± 0.43*

*Statistically significant difference compared to baseline score ($p < 0.05$ by paired Student's *t*-test). NC, negative control group (excipient); PC, positive control group (doxycycline, 10 mg/kg/d); LT, low dose test group (ALH-L1005, 100 mg/kg/d); HT, high-dose test group (ALH-L1005, 200 mg/kg/d); PI, plaque index; GI, gingival index; PPD, periodontal pocket depth; CAL, clinical attachment level; BoP, bleeding on pressure. Data are expressed as mean ± SD values.

MMP inhibitory activity in the gingival tissue

The gelatin zymograms showed that the NC group contained gelatinolytic activity in bands at 92 kDa, which is the same molecular weight area as that in human MMP-9 standards. The NC group showed bands at 82 kDa, as is shown by pro-MMP-9. However, similar bands were not detected in the samples from the PC and HT groups, and were only detected faintly in the LT group (panel A in Fig. 2). None of the groups showed gelatinolytic bands in the area indicating MMP-2.

Similar to the gelatin results, the casein zymograms showed that samples of the NC group expressed caseinolytic activity in bands at 48 kDa, same as the band area for the MMP-13 standards, whereas the bands in the PC and LT groups were detected only faintly. In the samples of the HT group, the band was barely detectable.

Discussion

The present study was designed to identify the clinical efficacy of ALH-L1005 in periodontitis. In this study, clinical signs of periodontitis were significantly resolved in the groups medicated with ALH-L1005. The LT and HT groups showed significant decrements in PI, GI, CAL, and BoP scores compared to those of the NC group at week 6, although the CAL variation of the HT group did not reach statistical significance. Additionally, the NC group showed a PPD decrement concurrent with a CAL increment, whereas the other groups showed decreased CAL scores during the experimental period. These results indicate that the PPD decrement in the NC group may be regarded as gingival recession after periodontal inflammation without improvement of periodontitis. This study showed that ALH-L1005 treatment clinically improved overall periodontal status compared to that in the control group.

Numerous studies have investigated host response modulation for treatment of periodontal diseases, including studies into MMPs inhibitors such as tetracycline analogs and non-steroidal

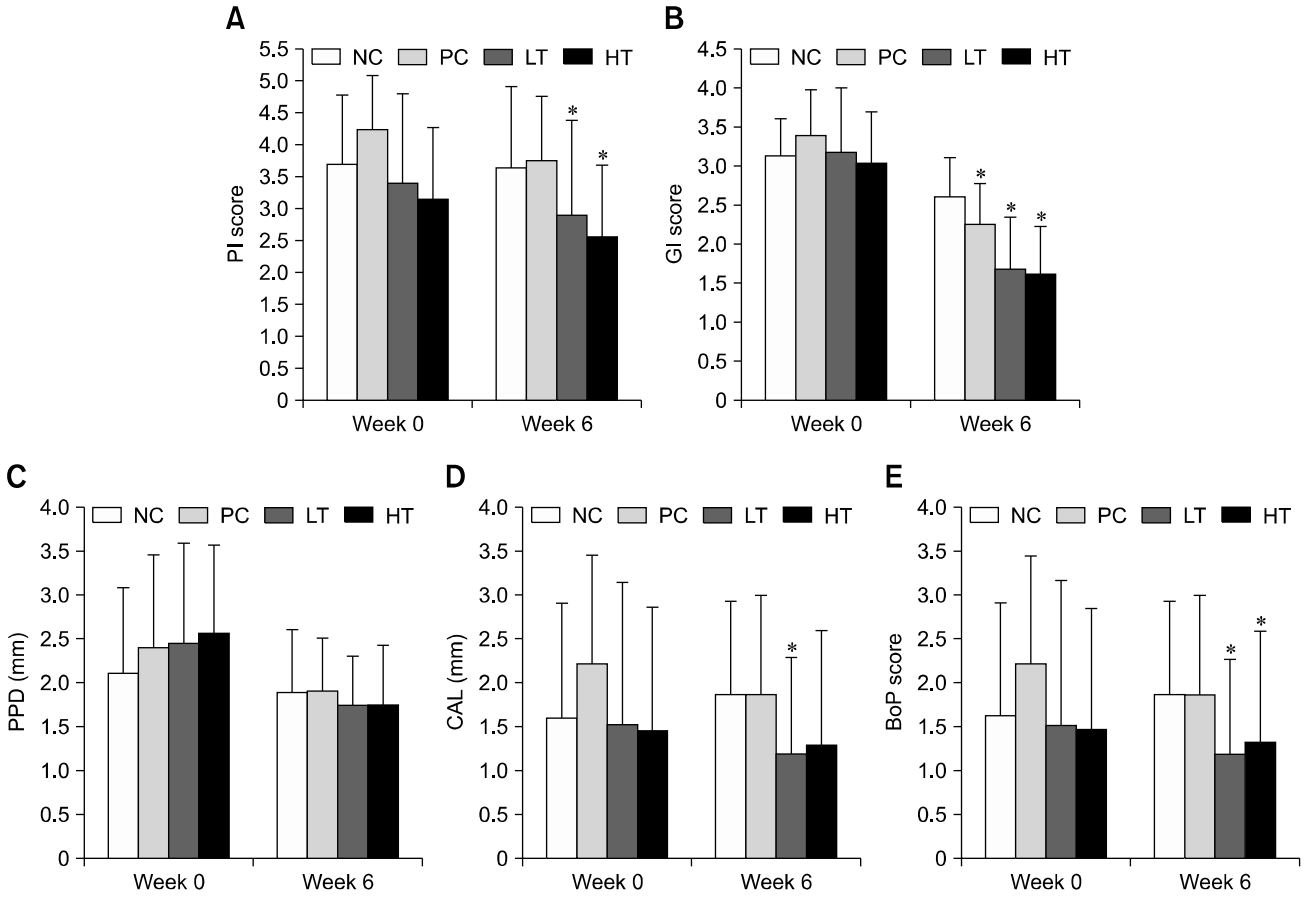

Fig. 1. Means of clinical parameters at Week 0 and Week 6 of treatment. (A) Plaque index. (B) Gingival index. (C) Periodontal pocket depth. (D) Clinical attachment loss. (E) Bleeding on pressure scores. *Significant improvement compared to the NC group at same week ($p < 0.05$).

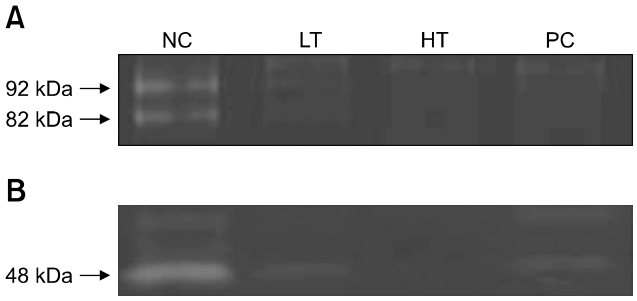

Fig. 2. Zymograms in extracts of sampled tissues after 6 weeks of medication. (A) Gelatinolytic activity in each group. The low dose test group (LT), high dose test group (HT), and positive control (PC) groups showed gelatinolytic inhibitory effects at 82 and 92 kDa molecular weights compared to that in the negative control (NC) group. (B) Caseinolytic activity in each group. The LT, HT, and PC groups showed caseinolytic inhibitory effects at 48 kDa molecular weight compared to that in the NC group.

anti-inflammatory drugs [23]. In particular, a subantimicrobial dose of doxycycline has been recognized as an effective treatment for periodontitis that could be applied clinically for pharmacological modulation of MMPs. However, doxycycline had side effects such as GI disturbance, photosensitivity reactions, and, rarely, hepatotoxicity. Additionally, it is contraindicated during pregnancy or childhood because of the possibility of retarding fetal skeletal development and discoloring teeth [16,22]. Non-steroidal anti-inflammatory agents also had adverse effects such as GI hemorrhage and hepatopathy [22].

Since their pharmacodynamics have been analyzed, an increasing number of anti-inflammatory agents derived from natural extracts are being applied in modern clinics. Previous herbal studies have shown that *Zea mays* L. extract, *Magnolia obovata thunberg* bark extract, and avocado/soybean unsaponifiable extracts are effective treatments for periodontal disease [3,14,27]. Horse chestnut seed extract contains anti-inflammatory agents, such as aescin and flavonoids, but it also contains glycoside aesculin that can have toxic effects [6]. The leaves of horse chestnut contains lower amounts of aescin and aesculin than the seeds, but a higher amount of rich flavonoids including quercetin and kaempferol, as well as

flavonol glycosides such as quercitrin, which have the additional effect of decreasing osteoclastic differentiation [2,19,30].

The sequence within the host's inflammatory response to periodontal disease have been well described in previous studies [8,21,23,24]. And there are several kinds of MMPs, such as MMP-1, 2, 3, 8, 9, and 13, involved in inflammatory responses that have been detected in tissue and gingival crevicular fluid samples from inflammatory gingiva [7,10,12,18,23,25,28]. In this *in vitro* study, ALH-L1005 showed notably better MMP-2 and MMP-9 inhibition effects than the commercially available horse chestnut seed and leaf extracts. In addition, compared with doxycycline, which inhibits MMP-2 and MMP-9, ALH-L1005 produced a similar inhibitory activity level.

In our study of gingival samples, MMPs activities were measured by using zymography to evaluate the actual anti-inflammatory activity *in vivo*. However, there is a limitation in this study because the molecular weights of canine MMPs have not been exactly determine. A previous study showed that canine MMP-2 and -9 had the same molecular weight level as the human standards, but the sequences of MMPs were not exactly homologous [15]. The zymographic results in the present study showed the periodontal tissues of ALH-L1005 administered (HT and LT) groups to have greater gelatinolytic and caseinolytic activity, related to MMP-9 and MMP-13 inhibition, respectively, than the levels in the NC group. Another previous study in human revealed that MMP-13 was able to degrade type I collagen, and it may activate pro-MMP-9 [13]. Therefore, it could be suggested that inhibition of MMP-13 activation might inhibit activation of pro-MMP-9 in our samples. In addition, the HT and LT groups had gelatinolytic and caseinolytic inhibitory activity levels similar to those of the PC group. Furthermore, both of the activities were suppressed dose dependently in the LT and HT groups. These results indicate that ALH-L1005 might be an effective agent to prevent and treat periodontal inflammation via its ability to suppress the activity of gelatinase and collagenase, which could detach periodontal ligaments from alveolar bone.

Based on the results, it is suggested that the ALH-L1005 horse chestnut leaf extract would be effective in the clinical treatment of periodontitis and would provide similar or better efficacy as that of doxycycline treatment in dogs. Additionally, the anti-inflammatory effects of ALH-L1005 are considered to act through a mechanism related to MMP-9 and MMP-13 inhibition in periodontal tissue. Thus, ALH-L1005 could be an effective treatment for periodontitis in dogs, and it might be applied to human periodontitis after further investigation into its safety and efficacy.

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Conflict of Interest

The authors declare no conflicts of interest.

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