J Herbmed Pharmacol. 2019; 8(2): 114-119.

http://www.herbmedpharmacol.com doi: 10.15171/jhp.2019.18

Journal of Herbmed Pharmacology

Collagenase inhibition by water-pepper (*Polygonum hydropiper* L.) sprout extract

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 12 July 2018
Accepted: 24 January 2019

Keywords:
Collagenase
Hyperoside
Polygonum hydropiper L.
Water-pepper

ABSTRACT

Introduction: Collagenase plays an important role in the degradation of dermal matrix proteins leading to wrinkle formation. The objectives of this study were to evaluate the inhibitory effect of water-pepper (*Polygonum hydropiper* L.) sprout extract on the activity of collagenase and to identify the inhibitory compounds.

Methods: Collagenase inhibitory activity was measured by spectrophotometric assay. Activity-guided fractionation was performed using liquid-liquid extraction of water and *n*-butanol and Diaion HP-20 column chromatography, followed by high-performance liquid chromatography (HPLC) fraction collection.

Results: A methanolic extract of water-pepper sprout inhibited collagenase activity in a concentration-dependent manner with an IC_{50} value of 156.7 µg/mL. Collagenase inhibitory activity (IC_{50} = 23.5 µg/mL) was found in 50% methanol eluate from the HP-20 column chromatography of the *n*-butanol soluble fraction. The active compound (IC_{50} = 1.9 µg/mL) in the eluate was isolated by HPLC and identified as quercetin-3-*O*-galactoside (hyperoside) from comparing retention time, UV-Vis absorption, and mass spectra with those of the standard. Lineweaver-Burk plots revealed that hyperoside was an uncompetitive inhibitor against collagenase. Hyperoside was also the most abundant flavonoid present in the methanolic extract.

Conclusion: These results suggest that water-pepper sprouts could be beneficial as a natural source of collagenase inhibitor which might be used for the treatment of skin aging.

Implication for health policy/practice/research/medical education:
Water-pepper sprout possesses collagenase inhibitory activity which may be beneficial in the treatment of skin aging.
Please cite this paper as: Kawaguchi T, Nagata K. Collagenase inhibition by water-pepper (*Polygonum hydropiper* L.) sprout extract. J Herbmed Pharmacol. 2019;8(2):114-119. doi: 10.15171/jhp.2019.18.

Introduction

Collagen is one of the major constituents of the extracellular matrix in human skin and provides tensile strength to the skin (1). Collagenase, the matrix-metalloprotease that cleaves collagen, plays an important role in the turnover of connective tissues in the skin by degrading defective dermal matrix proteins (2). However, the unbalanced high levels of collagenase observed in inflamed or UV-irradiated skin involve the formation of wrinkles in the skin (3). Therefore, there is a growing interest in the use of collagenase inhibitors as therapeutic agents for treating skin aging (4,5).

Water-pepper (*Polygonum hydropiper* L.) contains bioactive compounds such as polygodial, hydropiperoside, and several flavonoid compounds, such as quercetin, quercitrin, kaempferol, taxifolin, rutin, persicarin, rhamnazin, and hyperoside (6,7). In traditional medicine, water-pepper has been used for treating cancer (8), hypertension, cardiovascular (9), hemostatic, diuretic, inflammatory (10) and skin diseases (11). The biological activities of water-pepper have been reported to include antioxidative (12), anti-tumor (13), anti-bacterial (14), anti-inflammatory (15), anti-tyrosinase (16), and anti-cholinesterase (17) activities. However, no study has yet been conducted to evaluate the collagenase inhibitory activity of water-peppers. In particular, little is known about the bioactivity of the sprouts of water-pepper, which is used as a Sashimi Garnish in Japan (16). Therefore, the main objective of the present study was to evaluate the collagenase inhibitory activity of water-pepper sprouts.

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Materials and Methods

Materials

Collagenase from *Clostridium histolyticum* type IV and quercetin were obtained from Sigma-Aldrich (St. Louis, MO, USA), Pz-Pro-Leu-Gly-Pro-D-Arg-OH (Pz-peptide) from Bachem (Bubendorf, Switzerland), hyperoside from Extrasynthese (Genay, France), isoquercitrin, quercitrin, and quercetin 4-glucoside from Tokiwa Phytochemical (Chiba, Japan), and rutin from Tokyo Chemical Industry (Tokyo, Japan). Water-pepper sprouts were obtained from JA Chikuzen Asakura (Fukuoka, Japan).

Sample preparation

Water-pepper sprout powder (30 g) manufactured by NLA Co., Ltd. (Fukuoka, Japan) was extracted with 300 mL of methanol for 1 hour at room temperature. The extract was then filtered and dried under reduced pressure. The dried extract was dissolved in water and mixed with an equal volume of water-saturated *n*-butanol to obtain water and butanol layers. This liquid-liquid extraction was repeated four times. Each layer was dried under reduced pressure. Part of the butanol layer dissolved in water was loaded onto a Diaion HP-20 column (Mitsubishi Kagaku, Tokyo, Japan). The column was eluted with water, 25% methanol, 50% methanol, 75% methanol, and methanol. Each fraction was dried under reduced pressure.

The 50% methanol eluate (0.1 mg) was further fractionated using an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent Poroschell 120 EC-C18 column (4.6 mm × 50 mm, 2.7 μm), online photodiode array detector (DAD), and automated analytical-scale fraction collector with time-based collection mode. The elution was achieved with (A) 0.1% formic acid in water, and (B) acetonitrile at a flow rate of 0.5 mL/min under these conditions; 5% B was held isocratically for 10 minutes then increased linearly to 45% B over 60 minutes. The detection wavelength was 250 nm. Spectra were recorded in the wavelength range 190–600 nm at sampling intervals of 2 nm with an integration time of 1 second. The column temperature was 30°C. Purification of the peaks of interest and hyperoside standard was achieved using the peak-based fraction collection mode. The fractions and purified compound were dried under reduced pressure. The collections were repeated to obtain a sufficient amount for assay.

Collagenase inhibition assay

Collagenase activity was measured using a previously described method (18) with a slight modification. Collagenase (10 μg/mL) was reacted with Pz-peptide (0.4 mM) in the presence or absence of the test samples at 37°C for 30 minutes in 0.1 M Tris-HCl buffer (pH 7.1) containing 20 mM $CaCl_2$ (total volume of 0.5 mL). The reaction was stopped by adding 1 mL of 25 mM citric acid. The product of the hydrolysis of the peptide was extracted with 5 mL of ethyl acetate. The absorbance of the ethyl acetate layer was measured at 320 nm using a UV-Vis spectrometer (Evolution 220, Thermo Fisher Scientific, Waltham, MA, USA). The percent inhibition was calculated according to the following formula:

% inhibition = (1 − absorbance of sample / absorbance of control) × 100

LC-MS analysis

Water-pepper sprout extract was separated and analyzed by reversed-phase HPLC equipped with the DAD coupled to a quadrupole mass spectrometry (MS). The separation was performed under the conditions mentioned above. The MS system included an electrospray ionization interface. The MS analysis was performed in the negative and positive modes under the following conditions: drying gas flow, 12 L/min; nebulizer gas pressure, 343 kPa; drying gas temperature, 350°C; capillary voltage, 2.5 kV; and fragmentor voltage, 100 V. The molecular ions were scanned from 200 to 800 *m/z*. The identity of the compounds was confirmed by comparing their retention time, UV-Vis and mass spectra to those of standards (hyperoside, quercetin 4-glucoside, isoquercitrin, quercetin, quercitrin, and rutin). The wavelength for quantification of the compounds was 250 nm.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons using R version 3.3.2 (The R Foundation for Statistical Computing, http://www.r-project.org). All P values < 0.05 were considered as statistically significant.

Results

Inhibitory effects of a methanolic extract and its different fractions of water-pepper sprout on collagenase

The collagenase inhibition activity of the methanolic extract of water-pepper sprout was evaluated according to a published method using Pz-peptide as a substrate (18). Collagenase from *Clostridium histolyticum*, which hydrolyzes both triple-helical collagen in physiological conditions and synthetic peptides as substrates was used (19). Ethylenediamine tetraacetate (EDTA), a known collagenase inhibitor that removes metal ions required for enzyme stability and activity, was used as a positive control. Figure 1 indicates that the methanolic extract inhibited collagenase-mediated Pz-peptide degradation. Activity-guided fractionation was used to determine the active fraction in the methanolic extract. The extract dissolved in water was divided into two layers by adding water-saturated *n*-butanol. The *n*-butanol layer showed significantly higher activity than the water layer with a 61% inhibition, whereas the water layer only exhibited a 17.6% inhibition. Subsequently, further fractionation of the butanol layer was performed using Diaion HP-20 column chromatography. The collagenase inhibition activity of the

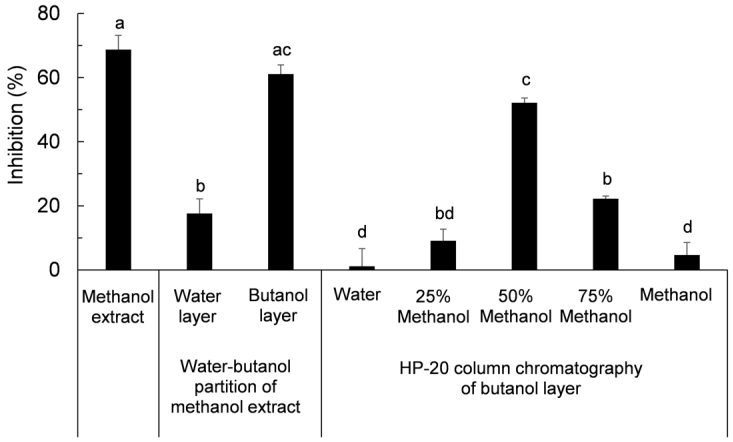

Figure 1. Inhibitory effects of the methanolic extract of water-pepper sprout and its fractions against collagenase activity. Results are expressed as the mean ± SD of triplicate experiments. Concentrations assayed were based on the yields of fractions and were 200, 136, 60, 18, 3, 24, 13 and 2 μg/mL for methanol extract, water layer, butanol layer, water, 25% methanol (MeOH), 50% MeOH, 75% MeOH and MeOH, respectively. Different letters above the columns represent a statistically significant difference ($P < 0.05$) by Tukey's test.

50% methanol eluate (MB50M) was significantly higher than that of the other eluates, but not significantly higher than that of the butanol layer of the methanolic extract. This suggested that active compounds were present in MB50M.

Isolation of the active compounds in MB50M by HPLC

Time- and peak-based fraction collections using reversed-phase HPLC-DAD was used to isolate the active compounds in the MB50M fraction. The time-based collection (4 min/fraction) revealed that the active compounds were present in fraction 8 (Figure 2a) corresponding to a retention time between 28 and 32 minutes (Figure 2b). This fraction was then subjected to a peak-based collection. The collagenase inhibition activities of peaks labeled 1–4 in Figure 2b were measured. The contribution of peak 2, the main peak in the chromatogram, to collagenase inhibition was highest for all peaks (Figure 2a inset). These results indicated that the constituents of peak 2 contributed most to the collagenase inhibition activity of MB50M.

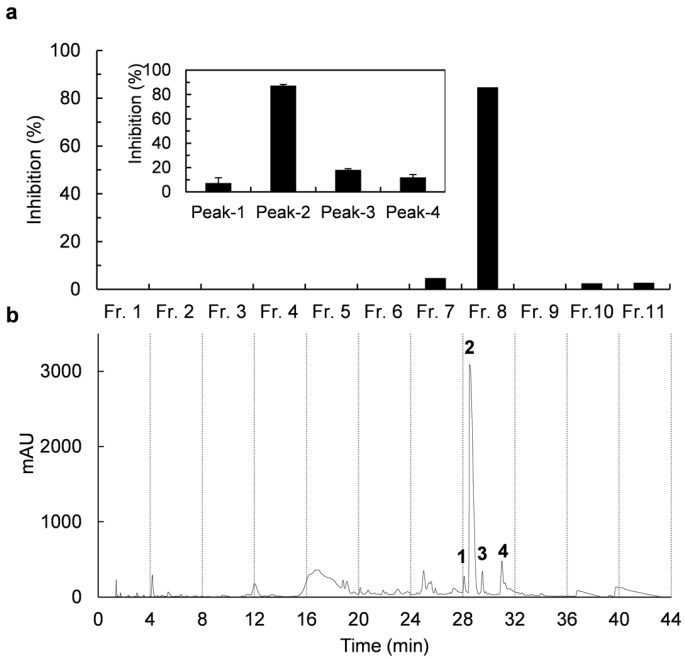

Figure 2. HPLC-based activity profiling of MB50M for collagenase inhibition. (a) Inhibition of collagenase activity by fractions obtained from HPLC of 0.1 mg MB50M (n = 1). Fractions were freeze-dried and dissolved in 400 μL of water before assay. Inset represents the inhibition by peaks labeled 1–4 on chromatogram (n = 3). Results are expressed as the mean ± SD of triplicate experiments. (b) Chromatogram of the separation. Time-based fractionation of 4 min each was performed to obtain fractions 1–11 as indicated with dashed lines. Peaks labeled 1–4 were further fractioned by peak-based fraction collection. MB50M, the active fraction from HP-20 column chromatography of the butanol soluble fraction of the methanolic extract; Fr, fraction number.

Identification of the active compound

Identifying the active compounds in peak 2 was performed using HPLC-DAD-MS. The chromatogram of the isolated peak 2 showed a single peak (Figure 3a). The UV-Vis and mass spectra of this single peak are shown in Figure 3a (inset). The compound indicated by this peak had absorption maxima at 256 and 354 nm and shoulders at 266 and 294 nm in the UV-Vis spectrum, and molecular ions at *m/z* 465 $[M+H]^+$ and *m/z* 463 $[M-H]^-$ in the mass spectra. A fragment ion at *m/z* 303 $[M+H-162]^+$ observed in the positive ion mode, corresponding to the loss of a single hexose unit, and the UV-Vis absorption maxima of this peak were both characteristics of quercetin derivatives (20). Therefore, these data and comparisons with the authentic standard (Figure 3b) led to identifying this active compound as quercetin-3-*O*-galactoside (hyperoside).

The identity was also confirmed by comparing the collagenase inhibition activities of the active compound and authentic hyperoside (Table 1). The inhibitory activity of the active compound with an IC_{50} value of 1.9 μg/mL was similar to that of hyperoside (IC_{50} of 1.7 μg/mL) and was significantly higher than that of EDTA (IC_{50} of 110.6 μg/mL). These results have indicated that the active compound in the methanolic extract of water-pepper sprout was hyperoside.

Mechanism of collagenase inhibition

The inhibition mechanism of hyperoside was analyzed by Lineweaver-Burk plots. As shown in Figure 4, hyperoside exhibited uncompetitive inhibition with an inhibition constant (*Ki* value) of 0.3 μg/mL.

Discussion

Collagenases are the enzymes that initiate collagen turnover in connective tissue under normal physiological conditions. The activities of the collagenases are regulated by endogenous inhibitors, known as TIMPs (tissue inhibitors of metalloproteinases) (21). Overproduction of collagenase leads to the excessive destruction of collagen fiber and might thus be responsible for skin wrinkling (3). It has been suggested that retinoic acids possessing collagenase inhibitory activity might be useful therapeutic agents in recessive dystrophic epidermolysis bullosa, which is, in part, caused by connective tissue destruction (22). Therefore, inhibiting collagenase activity could be important in preventing excess connective tissue breakdown by blocking the action of collagenase.

Table 1. Collagenase inhibitory activities and hyperoside contents of water-pepper sprout extract, fractions, and compounds

	IC_{50} (μg/mL)	hyperoside (% w/w)
Methanol extract	156.7 ± 26.15[a]	6.9
MB50M	23.5 ± 0.23[b]	30.4
Active compound, isolated	1.9 ± 0.06[b]	-
Hyperoside	1.7 ± 0.04[b]	-
EDTA	110.6 ± 0.74 [c]	-

IC_{50} values are expressed as the mean ± SD of triplicate experiments. Different superscript letters represent a statistically significant difference ($P < 0.05$) by Tukey's test. MB50M, the active fraction from HP-20 column chromatography of the butanol soluble fraction of the methanolic extract.

The objectives of the present study were to evaluate whether a methanolic water-pepper sprout extract inhibited collagenase activity and to explore its active compounds. We have found for the first time that water-pepper sprout extract can inhibit collagenase activity. Currently, several plant-origin inhibitors for collagenase have been reported. Thring et al found that hot-water extracts at concentrations of 166.7 μg/mL from white tea, green tea, rose tincture, and lavender from 23 plant extracts provided more than 30% inhibition of collagenase activity (23). These activity values agreed well with those of the methanolic extract in the present study with an IC_{50} value of 156.7 μg/mL. White tea (87% inhibition) and green tea (47% inhibition), containing catechins at

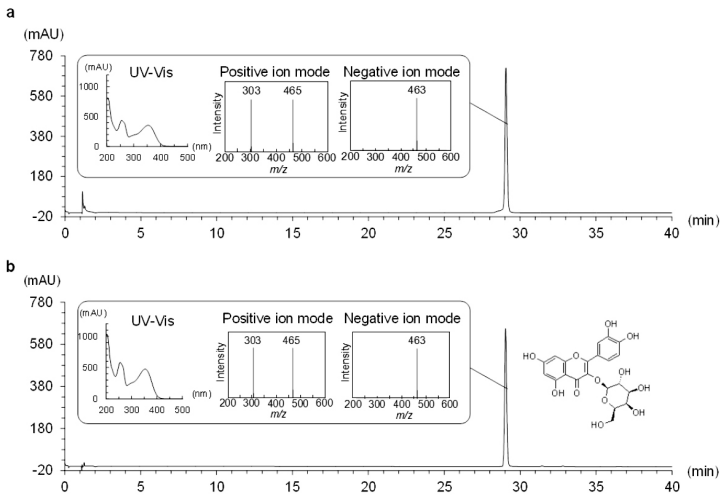

Figure 3 HPLC-DAD-MS chromatograms with UV-Vis and mass spectra for the isolated active compound (a) and hyperoside standard (b).

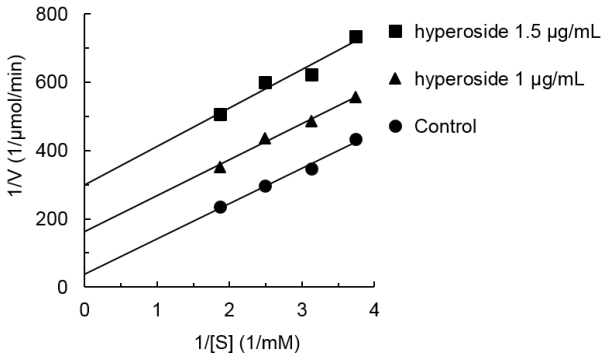

Figure 4. Lineweaver-Burk plots of collagenase inhibition by hyperoside. Collagenase inhibitory activities were measured in the presence or absence of hyperoside under different concentrations of Pz-peptide as cited. Results are expressed as the mean of duplicate experiments. Reaction velocity was estimated using an extinction coefficient of 21.0 cm^2/μmol at 320 nm for Pz-Pro-Leu, the product of the hydrolysis of the Pz-peptide. [S], Pz-peptide concentration; V, reaction velocity.

high concentrations, exhibited a higher activity than the other plant extracts. Some flavonoids such as kaempferol, quercetin and myricetin have exhibited considerable inhibition of collagenase over the concentration range of 25–500 μM (18). Phenolic compounds in higher plants have been considered as plausible candidates for collagenase inhibitors. In the present study, hyperoside, a flavonoid glycoside, was identified as an uncompetitive collagenase inhibitor (IC_{50} = 1.7 μg/mL, Ki = 0.3 μg/mL) from the active fraction of water-pepper sprout extract. This finding is consistent with and extends previous observations. Akkol et al have reported that *Rubus sanctus* Schreber and its active metabolite, hyperoside, can inhibit collagenase (24) but they provided no information on the activity and content of hyperoside itself in the active fraction. Based on the inhibitory activity (39.71% inhibition at 100 μg/mL) of the active fraction of *Rubus sanctus* Schreber, hyperoside was unlikely to have been the major constituent in the fraction, because its activity was considerably lower than that reported in the present study. On the other hand, the content of hyperoside (1 mg/g) present in the whole water-pepper plant has been reported to be lower than that of the other flavonoid compounds such as quercitrin (3.5 mg/g) and astragalin (1.6 mg/g) and higher than taxifolin (0.63 mg/g), quercetin (0.39 mg/g), and isoquercitrin (0.3 mg/g) (7,25). However, the major proportion of flavonoids present in the water-pepper sprouts was hyperoside (Table 1, 6.9% w/w) with minor constituents of quercetin (0.33% w/w), and isoquercitrin (0.19% w/w). No rutin or quercitrin was detected in the methanolic extract (data not shown). This is the first report of hyperoside being the most abundant flavonoid in the sprouts of water-pepper.

These findings give a new insight into the inhibitory activities of hyperoside and water-pepper sprouts rich in hyperoside against collagenase and suggest that their application for treating skin photo-aging might be effective, although further investigations are needed on its bioavailability and biodistribution.

Conclusion

Our findings have demonstrated that a methanolic extract of water-pepper sprout was effective for inhibiting collagenase activity. Hyperoside has been identified as an uncompetitive collagenase inhibitor and the most abundant flavonoid in water-pepper sprouts.

Authors' contributions

TK designed and performed experiments, analyzed data, and wrote the manuscript; KN designed experiments and gave technical support and advice. All authors discussed the results and implications and commented on the manuscript at all stages.

Conflict of interest

The present study was partly supported by NLA Co., Ltd. The company had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission redundancy) have been completely observed by the authors.

Funding/Support

This study was supported by Fukuoka Industrial Technology Center and partially by NLA Co., Ltd.

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