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A new steroidal saponin from *Allium ampeloprasum* var. *porrum* with antiinflammatory and gastroprotective effects

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ABSTRACT

A new steroidal saponin was isolated from the bulbs of *Allium ampeloprasum* var. *porrum*. On the basis of chemical conversions and detailed analyses of ^{1}H and ^{13}C NMR spectra including 2D NMR spectroscopic techniques, its structure was established as 3-[(*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 2)-*O*-[*O*-β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranosyl)oxy]-2,6-dihydroxy-(2α,3β,5α,6β,25*R*)-spirostane. Results of the present study indicated that the steroidal saponin showed haemolytic effects in the *in vitro* assays and demonstrated antiinflammatory activity and gastroprotective property using *in vivo* models.

1. Introduction

Allium ampeloprasum L. var. *porrum* (Alliaceae), a bulbous perennial plant, is one of the daily edible green vegetables for Brazilian people. The bulbs have been used for treating inflammatory symptoms. The crushed bulb is used to treat initial stages of cough, mucous secretion and sore throat. The fresh juice is taken orally as a stomachic and antispasmodic and is also reputed to possess digestive properties (Corrêa, 1926).

The *Allium* plants have been found to be rich in steroidal saponins, as well as organosulfur compounds (Kravets et al., 1990; Matsuura, 2001). As part of our ongoing efforts in discovering potentially bioactive and/or new steroidal saponins from Brazilian medicinal plants (Parente and da Silva, 2008), we describe the isolation, structural elucidation and evaluation of the antiinflammatory and antiulcerogenic activities of a new steroidal saponin from the methanolic extract of *A. ampeloprasum* var. *porrum*.

2. Results and discussion

The MeOH extract of *A. ampeloprasum* var. porrum was suspended in H_2O and partitioned with *n*-BuOH. The *n*-BuOH extract was subjected to chromatographic purification steps to afford compound **1**, a colorless amorphous powder, which was positive to Liebermann–Burchard test. It revealed a quasi-molecular weight ion peak at *m/z* 1282.3401 $[M+Na]^+$ in the positive-ion mode MALDI-TOFMS. In the ^{13}C NMR spectrum, fifty-seven carbon signals observed belong to four methyl groups, fourteen methylene groups (six of which were oxygenated), thirty-six methine groups (twenty-nine of which were oxygenated) and three quaternary carbon atoms (one of which was oxygenated). The number of hydrogen atoms attached to each individual carbon atom was determined by DEPT spectrum. On the basis of the above mentioned MS and ^{13}C NMR spectral data (Table 1), compound **1** was assumed to be a saponin with the molecular formula $C_{57}H_{94}O_{30}$, bearing a chain of five monosaccharide moieties.

In addition to this, the spirostanol glycosidic nature of compound **1** was indicated by the strong absorption band at 3400 cm^{-1} for the hydroxyl group and characteristic 25*R*-spiroketal absorption bands at 890 and 910 cm^{-1} (intensity, 910 < 890 cm^{-1}) in the IR spectrum (Wall et al., 1952). The 25*R* stereochemistry of the Me-27 group was confirmed by hydrogens and carbons resonances at positions 25, 26 and 27 in comparison with data reported in the literature (Carotenuto et al., 1999; Agrawal, 2003). The A/B *trans*-ring fusion was deduced from the signals at δ 47.32 (CH, C-5), 54.03 (CH, C-9) and 16.72 (CH_3, C-19) indicating that the aglycone of **1** is a 5α*H* steroidal sapogenin, confirmed by one hydrogen at δ 1.27 (*td*, *J* = 2.8, 2.8, 12.0 Hz, 5α*H*) (Carotenuto et al., 1997). On inspection of the ^{1}H and ^{13}C NMR spectral data of compound **1**, the structure of the aglycone moiety was identical with (2α,3β,5α,6β,25*R*)-2,6-dihydroxyspirostan-3-yl.

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Table 1
^{13}C NMR data of compound **1** (150 MHz, pyridine-d_5).

Position	δ C	DEPT[a]	Position	δ C	DEPT[a]
1	46.7	CH_2	β-D-GlcI		
2	71.1	CH	1	104.3	CH
3	84.1	CH	2	80.0	CH
4	33.8	CH_2	3	87.4	CH
5	47.3	CH	4	69.2	CH
6	70.2	CH	5	77.5	CH
7	39.6	CH_2	6	62.5	CH_2
8	29.5	CH			
9	54.0	CH			
10	36.6	C	β-D-GlcII		
11	20.9	CH_2	1	103.3	CH
12	40.2	CH_2	2	74.3	CH
13	40.4	C	3	87.0	CH
14	55.8	CH	4	68.8	CH
15	31.7	CH_2	5	77.5	CH
16	80.7	CH	6	61.5	CH_2
17	62.5	CH			
18	16.1	CH_3			
19	16.7	CH_3	β-D-GlcIII		
20	41.5	CH	1	104.8	CH
21	14.6	CH3	2	74.7	CH
22	108.8	C	3	77.5	CH
23	31.3	CH_2	4	69.5	CH
24	29.5	CH_2	5	77.9	CH
25	30.1	CH	6	62.0	CH_2
26	66.7	CH_2			
27	16.9	CH_3			
β-D-Gal			β-D-GlcIV		
1	102.5	CH	1	103.8	CH
2	72.0	CH	2	74.7	CH
3	75.1	CH	3	77.9	CH
4	79.3	CH	4	69.8	CH
5	75.1	CH	5	77.9	CH
6	60.2	CH_2	6	62.0	CH_2

[a] Multiplicities were assigned from DEPT spectrum.

On acid hydrolysis, compound **1** gave galactose, glucose and an aglycone, which was identical to agigenin (**1a**) by comparative analysis of ^{1}H and ^{13}C NMR and EIMS spectra of **1a** with those of known agigenin (Kel'ginbaev et al., 1974; Carotenuto et al., 1997). Analysis of the sugars by GC/MS indicated the presence of galactose and glucose in a ratio of 1:4, respectively (Kamerling et al., 1975). Their absolute configurations were determined by GC of their trimethylsilylated (−)-2-butylglycosides (Gerwig et al., 1978). D-Galactose and D-glucose were detected. The ^{1}H NMR spectrum of compound **1** displayed five anomeric hydrogen atoms at δ 4.89 (*d*, *J* = 7.8 Hz), 5.05 (*d*, *J* = 7.5 Hz), 5.12 (*d*, *J* = 7.8 Hz), 5.22 (*d*, *J* = 7.8 Hz), 5.57 (*d*, *J* = 7.5 Hz) which gave correlations in the HSQC spectrum with five anomeric carbon atoms at δ 102.54, 104.84, 104.33, 103.77 and 103.29, respectively. Evaluation of chemical shifts and spin–spin couplings allowed the identification of one β-galactopyranosyl unit (β-Gal) and four β-glucopyranosyl units (β-GlcI, β-GlcII, β-GlcIII and β-GlcIV). The attachments of the sugar moieties to the aglycone moiety were established by ^{1}H–^{1}H-COSY, HMBC and HMQC experiments. The HMBC and HMQC spectra displayed long range couplings between Gal-H-1 at δ 4.89 and aglycone-C-3 at δ 84.07. In addition to this, long range couplings were observed between GlcI-H-1 at δ 5.12 and Gal-C-4 at δ 79.35, between GlcII-H-1 at δ 5.56 and GlcI-C-2 at δ 80.01, between GlcIII-H-1 at δ 5.05 and GlcII-C-3 at δ 87.01, between GlcIV-H-1 at δ 5.22 and GlcI-C-3 at δ 87.39, which accounted for the saccharide part linkage to the C-3 β OH group of agigenin. The NMR signals of compound **1** were assigned by 2D NMR experiments including COSY, HSQC, HMQC and HMBC and by comparing the NMR data of **1** with those reported in the literature (Mimaki et al., 1995; Carotenuto et al., 1999; Itabashi et al., 2000; Ohtsuki et al., 2004; da Silva and Parente, 2006).

The sequence of the sugar chain of **1** was confirmed by methylation analysis (Parente et al., 1985) which furnished 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl glucitol. Consequently, on the basis of the results described above, the structure of compound **1** was established as 3-[(*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 2)-*O*-[*O*-β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranosyl)oxy]-2,6-dihydroxy-(2α,3β,5α,6β,25*R*)-spirostane (**1**).

According to the literature, steroidal saponins isolated from medicinal plants have been reported to have important biological activities, such as stimulation of lymphocyte proliferation (Gautam et al., 2009) and inhibition of the production of inflammatory cytokines by activated macrophages (Kim et al., 2009). With the aim of evaluating the biological properties, we tested the in vivo antiinflammatory and gastroprotective activities of compound **1** (Fig. 1) in order to explore its contribution to the traditional medicinal use of this species. On the toxicological side, the in vitro haemolytic potential was also measured.

Generally, steroidal saponins possess powerful haemolytic capacity because steroids have higher affinities for cholesterol on erythrocyte membranes. The ability of compound **1** to induce haemolysis of human red blood cells was investigated and compared with the saponin QS-21 isolated from *Quillaja saponaria* (HD_{50} 5 μg/mL), a substance commonly used in animal and human experimental models. According to procedures described in the literature (Santos et al., 1997), compound **1** was shown to possess potent haemolytic capacity (HD_{50} 20 μg/mL), which can be explained by the amphipathic characteristic of its structure,

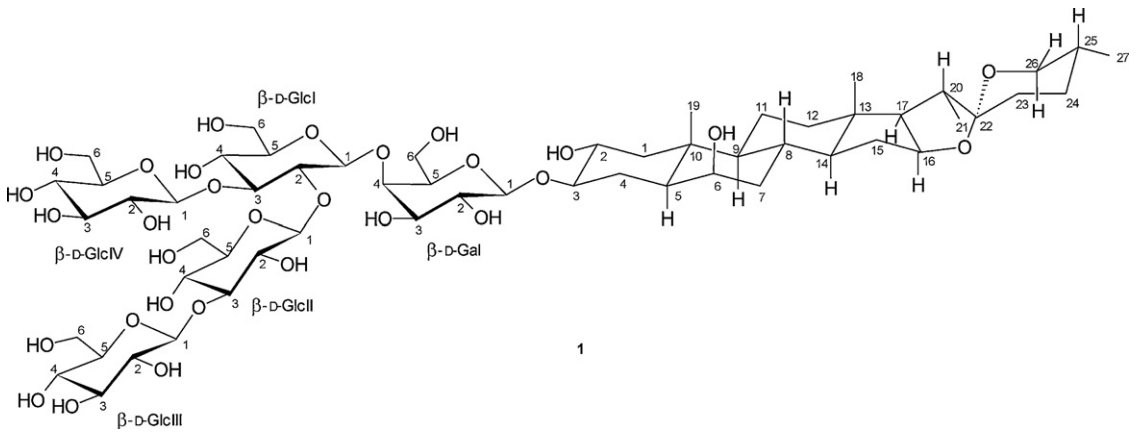

Fig. 1. Structure of compound **1**.

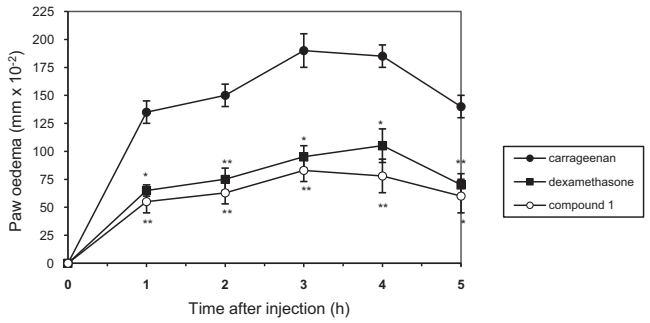

Fig. 2. Antiinflammatory activity of compound **1** (100 mg/kg) and the reference compound dexamethasone (25 mg/kg) against mouse paw oedema induced by carrageenan. Results are mean ± S.E.M. ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from the control group.

containing a hydrophobic steroidal nucleus and two hydrophilic carbohydrate moieties.

In addition to this, the antiinflammatory activity of compound **1** was investigated using an acute inflammation model. The results were measured by inhibition of carrageenan-induced mouse paw oedema (Levy, 1969). The carrageenan-induced inflammation is a biphasic phenomenon. The early phase of oedema is attributed to the release of histamine, serotonin and similar substances. The later phase results mainly from the potentiating effects of prostaglandins on mediator release. Compound **1** showed significant antiinflammatory potential, promptly controlling both phases of inflammation and provoking an inhibition of oedema formation similar to the reference compound dexamethasone (Fig. 2). In comparison with the literature reports, compound **1** showed antiedematous properties with potency similar to other bioactive compounds, such as anemarsaponin B, a steroidal saponin isolated from the rhizomes of *Anemarrhena asphodeloides*, a medicinal plant used against inflammatory disorders (Kim et al., 2009).

The gastroprotective property of compound **1** was investigated with the aim to confirm its utilization in the traditional medicine, since there are popular informations about the use of this plant for the treatment of digestive disorders. The antiulcerogenic activity was evaluated by measuring the inhibition of acute gastric lesions induced by acidified ethanol (Hamauzu et al., 2008). By macroscopic observations, in the control animals that received only water before acidified ethanol administration, intense and widespread gastric hyperemia and thickened lesions were evident. In contrast, the stomachs of the animals which received compound **1** showed an aspect close to normality, with significant reduction

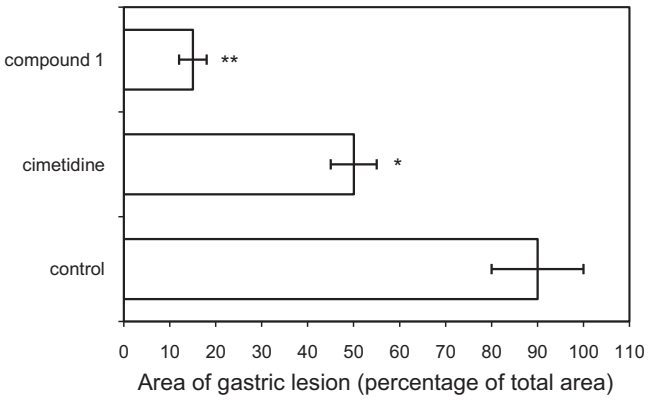

Fig. 3. Antiulcerogenic activity of compound **1** (100 mg/kg) and the reference compound cimetidine (100 mg/kg) against acidified ethanol induced gastric lesions (0.3 M HCl/EtOH). Results are mean ± S.E.M. ($n = 5$); $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from the control group.

in gastric hyperemia and in number and severity of lesions. This protective action is closely related to the reference compound cimetidine at the same dosage. The intensity of gastric ulcers was quantified by the percentage of the injury area in relation to the control group (Fig. 3). This result suggests that compound **1** probably interfere with the ulcerogenic mechanism, showing a cytoprotective property.

In conclusion, the investigation of the biological properties of compound **1** indicated that this substance may be the potential therapeutic agent involved in the gastroprotective property and the treatment of inflammatory conditions, justifying the use of this plant as a food source and in the traditional medicine.

3. Experimental

3.1. General experimental procedures

Carbohydrate content was analyzed by gas-chromatography–electron impact mass spectrometry (GC–EIMS) of the alditol acetates (Sawardeker et al., 1965). The experimental data were tested for statistical differences using the Student's *t*-test. Melting points were determined by an Electrothermal 9200 micro-melting point apparatus and are uncorrected. The optical rotations were measured on a Perkin Elmer 243B polarimeter. IR spectra were measured on a Perkin Elmer FT-IR 1600 spectrometer. ^{1}H and ^{13}C NMR, DEPT, COSY, HMBC, HSQC and HMQC experiments were performed in deuterated pyridine on a Bruker DRX-600 NMR spectrometer (600 MHz for δ_H and 150 MHz for δ_C). All chemical shifts (δ) are given in ppm units with reference to tetramethylsilane (TMS) as the internal standard and the coupling constants (J) are in Hz. Gas chromatography (GC) was carried out with flame ionization detector (FID), using a glass capillary column (0.31 mm × 25 m) SE-30. GC–EIMS were taken on a VG Auto SpecQ spectrometer operating at 70 eV. The MALDI-TOFMS was obtained using a perseptive Voyager RP mass spectrometer. Silica gel columns (230–400 mesh ASTM, Merck) and Sephadex LH-20 (Pharmacia) were used for column chromatography (CC). Thin-layer chromatography (TLC) of monosaccharides was performed on silica gel coated plates (Merck) using the following solvent systems: (A) $CHCl_3$–MeOH–H_2O (65:35:10, v/v/v, lower phase) for steroidal saponin **1**, (B) $CHCl_3$–MeOH (95:5, v/v) for sapogenin **1a**, and (C) *n*-BuOH–acetone–H_2O (4:5:1, v/v/v) for monosaccharides. Spray reagents were orcinol/H_2SO_4 for steroidal saponin **1** and monosaccharides and $CeSO_4$ for sapogenin **1a**.

3.2. Plant material

The bulbs of *A. ampeloprasum* var. *porrum* were purchased from a nursery in Rio de Janeiro, Brazil, in September 2007. The bulbs were cultivated, and a voucher specimen (LQPM 60) of the plant is on file in our laboratory.

3.3. Extraction and isolation

Fresh bulbs of *A. ampeloprasum* var. *porrum* (1.5 kg), previously cut into small pieces, were extracted with MeOH (6 L) for 72 h at rm. temp. The extract was concentrated under reduced pressure to remove most of the MeOH and the resulting aq. phase (500 mL) was extracted with *n*-BuOH (500 mL). This procedure was repeated and the resulting organic phase was evaporated *in vacuo* to give a crude material (4.93 g). It was dissolved in MeOH (70 mL) and roughly chromatographed (0.70 g/10 mL, each time) by column chromatography over Sephadex LH-20 (3.8 cm × 65 cm) using MeOH as eluent to yield fractions containing saponins (143 mg). The resulting residue containing saponins (1.0 g) was subjected to column chromatography over silica gel (2.8 cm × 90 cm), using

$CHCl_3$–MeOH–H_2O (65:35:10, lower phase) as eluent to afford compound **1** (186 mg).

3.4. 3-[(O-β-D-Glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 2)-O-[O-β-D-glucopyranosyl-(1 → 3)]-O-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranosyl)oxy]-2,6-dihydroxy-(2α,3β,5α,6β,25R)-spirostane (1)

Colorless amorphous powder (186 mg); m.p. 262–264 °C; $[\alpha]_D^{25}$ −21 (*c* 0.1, MeOH); IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 2930 (CH), 1445, 1365, 1235, 1145, 1045, 970, 910, 890, 855; ^1H NMR (C_5D_5N, 600 MHz) δ 5.57 (1H, *d*, *J* = 7.5 Hz, GlcII-H-1), 5.22 (1H, *d*, *J* = 7.8 Hz, GlcIV-H-1), 5.12 (1H, *d*, *J* = 7.8 Hz, GlcI-H-1), 5.05 (1H, *d*, *J* = 7.5 Hz, GlcIII-H-1), 4.89 (1H, *d*, *J* = 7.8 Hz, Gal-H-1), 4.84 (1H, *ddd*, *J* = 11.2, 8.9, 5.0 Hz, H-3), 4.55 (1H, *brq*, *J* = 7.1 Hz, H-16), 4.15 (1H, *ddd*, *J* = 11.5, 8.9, 4.7 Hz, H-2), 3.89 (1H, *brs*, H-6), 3.58 (1H, *dd*, *J* = 10.6, 3.7 Hz, H-26eq), 3.50 (1H, *dd*, *J* = 10.6, 10.6 Hz, H-26ax), 1.27 (1H, *td*, *J* = 2.8, 2.8, 12.0 Hz, 5αH), 1.13 (3H, *d*, *J* = 6.9 Hz, Me-21), 0.82 (3H, *s*, Me-18), 0.77 (3H, *s*, Me-19), 0.69 (3H, *d*, *J* = 5.8 Hz, Me-27). ^{13}C NMR (C_5D_5N, 150 MHz): see Table 1. MALDI-TOFMS *m*/*z*: 1282.3401 $[M+Na]^+$ (calcd for $C_{57}H_{94}NaO_{30}^+$, 1282.3564).

3.5. Acid hydrolysis of compound 1

Compound **1** (100 mg) was dissolved in 1 M HCl soln./1,4-dioxane 1:1 (10 mL) and heated in a sealed tube at 100 °C during 2 h. After cooling, the mixture was neutralized with 1 M NaOH in MeOH and the salts that deposited on addition of MeOH were filtered off, and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel column (10 mm × 100 mm) eluted with $CHCl_3$–MeOH mixtures (19:1 → 7) to furnish the aglycone moiety (29 mg) and sugar mixture (47 mg). A sample of the sugar mixture (1 mg) was dissolved in pyridine (100 μL) and analyzed by TLC in comparison with D-glucose and D-galactose.

3.6. Molar carbohydrate composition and D,L configurations

The molar carbohydrate composition of compound **1** (1 mg) was determined by GC–MS analyses of their monosaccharides as their trimethylsilylated methylglycosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80 °C) and trimethylsilylation (Kamerling et al., 1975). The configurations of the glycosides were established by capillary GC and GC–EIMS of their trimethylsilylated (−)-2-butylglycosides (Gerwig et al., 1978).

3.7. Methylation analysis

Compound **1** (1 mg) was dissolved in dimethylsulfoxide (200 μL) in a Teflon-lined screw-cap tube. Lithium methylsulfinyl carbanion (200 μL) was added to the solution under an inert atmosphere and the mixture was sonicated for 60 min. After cooling to −4 °C, cold methyl iodide (400 μL) was added. Sonication was conducted in a sonication bath (20 °C) for 45 min. The methylation was terminated by addition of water (4 mL) containing sodium thiosulfate, and the permethylated product extracted with chloroform (3× 2 mL) and evaporated (Parente et al., 1985). The methyl ethers were obtained after hydrolysis (4 N TFA, 2 h, 100 °C) and analyzed as alditol acetates by GC–EIMS (Sawardeker et al., 1965).

3.8. Antiinflammatory activity

Antiinflammatory activity was evaluated by the carrageenan-induced oedema method (Levy, 1969). Male Swiss mice (three months old, 25–35 g) in groups of five were administered orally with 1 mL of saline solution as the negative control or compound **1** (100 mg/kg) or the reference compound dexamethasone (25 mg/kg) dissolved in saline solution as positive control. Acute inflammation was produced by subplantar injection of 50 (L of 1% freshly prepared colloidal suspension of carrageenan in physiological saline injected into the subplantar region of the right hind paw of the mice, 1 h after the oral administration of test sample as well as the negative and positive controls. The footpad thickness was measured with a spring-loaded dial gauge (Mitutoyo Corp., Tokyo, Japan) before and every 1 h during 5 h after induction of inflammation. Percent inhibition of the inflammation was determined by applying statistical methods followed by the calculation of percent inhibition for each group by comparing with control group.

3.9. Antiulcerogenic activity

Antiulcerogenic activity was evaluated by measuring acute gastric lesions induced by acidified ethanol. Male Swiss mice (three months old, 25–35 g) in groups of five were fasted for 24 h before the experiment and administered orally with 1 mL of pure water as the negative control, or compound **1** (100 mg/kg), or the reference compound cimetidine (100 mg/kg) dissolved in vehicle as positive control. One hour after the treatments, all animals received orally 200 (L of acidified ethanol solution (0.3 M HCl/EtOH) to induce gastric lesions. The animals were killed 1 h after treatment with the ulcerogenic agent and the stomachs removed, opened along the greater curvature and rinsed with physiological saline to determine the lesion damage. The degree of gastric mucosal damage was evaluated from digital pictures using a computerized image analysis system. The percentage of the total lesion area (hemorrhagic lesions) to the total surface area of the stomach was defined as the ulcer index (Hamauzu et al., 2008).

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