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作成者（著者）	Hiroko, Izumi-Nakaseko / Wei, Li / Xin Cao / Yuji, Nakamura / Kentaro, Ando / Koichiro, Tanaka / Azjargal, Enkhsaikhan / Choijamts, Gotov / Batkhuyag, Purevjav / Yeruult, Chultemsuren / Khaliun, Nyambayar / Narantungalag, Dorjsuren / Mihoko, Hagiwara-Nagasawa
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Original Article

Assessment of Pharmacological Effects of Mongolian Medicinal Plant *Adonis mongolica* in Guinea Pigs *in vivo* and *in vitro*

Hiroko Izumi-Nakaseko[1)] Wei Li[2)] Xin Cao[3)]
Yuji Nakamura[1)] Kentaro Ando[1)] Koichiro Tanaka[4)]
Azjargal Enkhsaikhan[5)] Choijamts Gotov[6)] Batkhuyag Purevjav[7)]
Yeruult Chultemsuren[7)] Khaliun Nyambayar[7)] Narantungalag Dorjsuren[7)]
Mihoko Hagiwara-Nagasawa[1)] Atsuhiko T. Naito[1)] Kazuo Koike[2)]
and Atsushi Sugiyama[1)]*

[1)]Department of Pharmacology, Faculty of Medicine, Toho University
[2)]Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Toho University
[3)]Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Toho University
[4)]Division of Traditional Japanese Medicine, Department of General Medicine and Emergency Care, Faculty of Medicine, Toho University
[5)]Department of Pharmacology, Toho University Graduate School of Medicine
[6)]Central Research Laboratory, Mongolian Traditional Medical and Training Centre "Manba Datsan" Otoch Manramba University
[7)]Department of Pharmacology, School of Pharmacy and Bio-Medicine, Mongolian National University of Medical Sciences

ABSTRACT

Background: *Adonis mongolica* (Ranunculaceae) is one of the endemic plants in Mongolia and has been used as a medicinal herb in Mongolian traditional medicine to treat patients with congestive heart failure showing tachycardia and edema. Although the plant has been empirically used in the last three decades, the precise information regarding its cardiovascular profile is still limited.

Methods: We assessed the cardiohemodynamic and electrophysiological profile of the water-soluble extract of *A. mongolica* using the guinea pig *in vivo* model ($n = 4$) and *in vitro* preparation ($n = 5-17$). In addition, the onset mechanism of the extract-induced effects on the heart rate and blood pressure *in vivo* ($n = 4$), and the atrial rate and contractile force *in vitro* ($n = 4-5$) were pharmacologically analyzed.

Results: The extract exerted the positive chronotropic, negative dromotropic, and vasopressor effects in addition to the proarrhythmic action *in vivo*. Meanwhile, it modestly decreased the atrial rate and aortic tension but increased the atrial contractile force *in vitro*. The pretreatment of *dl*-propranolol and prazosin significantly suppressed the positive chronotropic and vasopressor actions induced by the extract *in vivo*, indicating that the extract increased the sympathetic tone. Also, liquid chromatography-mass spectrometry analysis showed that water-soluble extract of *A. mongolica* contained eight kinds of cardiac glycosides.

1, 5) 5–21–16 Omorinishi, Ota, Tokyo 143–8540, Japan
2, 3) 2–2–1 Miyama, Funabashi-shi, Chiba 274–8510, Japan
4) 6–11–1 Omorinishi, Ota, Tokyo 143–8541, Japan
6) 48/59 Ulaanbaatar, Mongolia
7) S.Zorig street, Ulaanbaatar 14210, Mongolia
*Corresponding Author: tel: +81–(0)3–3762–4151 (Ext. 2361)
e-mail: atsushi.sugiyama@med.toho-u.ac.jp
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Conclusions: These results indicate that cardiac glycosides in the water-soluble extract of *A. mongolica* may explain currently observed various cardiovascular effects.

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KEYWORDS: cardiovascular, *Adonis mongolica*, Mongolian traditional medicine, LC-MS

Introduction

Adonis mongolica Simonovich belonging to the family of Ranunculaceae is one of the endemic plants in Mongolia (Fig. 1), which has been used as a medicinal herb in Mongolian traditional medicine for thousands of years, and is categorized to "Cardiac barbad." The plant is perennial with small roots, and produces cardiac glycosides like other *Adonis* species. The aerial parts have been used for traditional medicine in Mongolia and Russia. In the last 30 years, patients with congestive heart failure showing tachycardia and edema have been treated with its infusion formula made from the aerial parts; namely, orally 3 times a day for 4 days as home therapy like western medicine. The formulation has been known to induce diuresis, reducing peripheral and pulmonary edema; suppress tachycardia; and lower blood pressure in such patients. The chemical constituents from *A. mongolica* have been reported to contain cardiac glycosides, such as cymarin, adonitoxin, corchoroside A, k-strophanthin-β, k-strophanthoside, eryslmoside, olitoriside, strophanthidin and glucoolitoroside,[1–5] in the phytochemical investigations carried out in the 1970s. Extracts of *A. mongolica* have been empirically used in clinical practice; however, the cardiovascular profile of the extract has not been fully elucidated.[6–8] In this study, we systematically analyzed the cardiohemodynamic, electrophysiological and proarrhythmic profiles of the water-soluble extract of *A. mongolica* using the guinea-pig *in vivo* model and *in vitro* preparation. In addition, we performed liquid chromatography-mass spectrometry (LC-MS) analysis for cardiac glycosides in the extracts.

Materials and Methods

All experiments in this study were approved by the Animal Research Committee for Animal Experimentation of Toho University (No. 17-55-251) and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Toho University and ARRIVE guidelines for reporting experiments involving animals.[9, 10] Animals were housed in a controlled environment at 22℃ with a 12-h light/12-h dark cycle and provided with standard chow and water *ad libitum*. Animals were obtained through Japan SLC, Inc. (Hamamatsu, Japan).

Preparation of the extract of *A. mongolica*

Adonis mongolica bears white flowers from May through July, and the pharmacological potency of the plant is the highest in the flowering time (Fig. 1). The aerial parts of the plants during anthesis stage were collected by Choijamts Gotov at Uushig Mountain in Darkhan-Uul Province, Mongolia in July 5th, 2013 and were identified at the Institute of Biology, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia. The plant materials were dried at room temperature in darkness for 2 weeks, which were then kept desiccated in a cool place (5℃ – 15℃).

Twenty g of the dried aerial parts were segmented (Fig. 1), and immersed with 20 mL of freshly purified water for 15 min. Then, 180 mL of boiling purified water was added to the mixture, which was incubated for overnight at room temperature. Finally, the mixture was centrifuged at 50 *g* for 10 min, the supernatant was filtered with a filter paper (Qualitative Filters Papers, Grade No. 2; Advantec Toyo Kaisha, Ltd., Tokyo, Japan), and the filtrate was dispensed into 1 – 2 mL aliquots and was frozen at –20℃. The extract of the herb showed green color, and was used as a stock solution in this experiment, of which concentration was 100 g/L (weight of dried plants/volume of water) in the present study. The stock solution was diluted with physiological solution into optimal concentration for each experiment before use.

Experiment 1: Cardiovascular effects of the extract in the *in situ* heart preparation

Four male Hartley guinea pigs weighing 335 – 670 g were used. They were initially anesthetized with thiopental sodium (50 mg/kg, i.p.). After a tracheal cannula was inserted, 1% halothane vaporized with 100% oxygen was inhaled with a rodent ventilator (SN-480-7; Shinano Manufacturing Co., Ltd., Tokyo, Japan). The tidal volume and respiratory rate were set at 10 mL/kg and 60 strokes/min, re-

Fig. 1 Photos of wild *Adonis mongolica* (left), one of the endemic plants in Mongolia, and its dried aerial parts (right). Scale bar represents 1 cm.

spectively, and the body temperature was kept at 37℃ with a heating pad (BWT-100A; Bio Research Center Co., Ltd., Nagoya, Japan). The surface lead I electrocardiogram was recorded from the limb electrodes. The corrected QT interval was calculated with the Fridericia's formula: $QTcF = QT/(RR/1{,}000)^{1/3}$.[11] The left jugular vein was cannulated for the extract administration, and the heparinized catheter was placed in the aorta through the left carotid artery to monitor the aortic pressure.

Electrocardiogram and aortic pressure were monitored using the polygraph system (RM-6000; Nihon Kohden, Tokyo, Japan) and recorded and analyzed by a real-time, fully automatic analysis system (MP/VAS3 ver 1.1R24v; Physio-Tech, Tokyo, Japan). The mean of three consecutive complexes was taken for each measurement of the electrocardiogram variables and aortic pressure. These cardiovascular variables were assessed under sinus rhythm. After the basal control assessment, 10 mg/kg of the extract was intravenously administered over 10 min, and each variable was assessed at 5, 10, 15, 20 and 30 min after the start of infusion. Then, 100 mg/kg of the extract was additionally infused over 10 min, and each variable was assessed in the same manner.

In addition, the same experiment was performed in the presence of β-blocker *dl*-propranolol and α_1-blocker prazosin ($n = 4$). First, basal responses of the heart rate and blood pressure to β_1-agonist (−)-isoproterenol in doses of 1.0 or 5.0 μg/kg and α_1-agonist noradrenaline in a dose of 1.0 μg/kg were sequentially examined. (−)-Isoproterenol in either dose of 1 or 5 μg/kg was selected, which could increase the heart rate by >100 beat/min. Next, *dl*-propranolol in a dose of 1 mg/kg and prazosin in a dose of 0.03 mg/kg were administered, and the blocking actions were confirmed by the administration of (−)-isoproterenol and noradrenaline. Then, the extract was infused at a dose of 100 mg/kg/10 min, and the changes of the parameters were assessed at 5 and 10 min after the start of the administration. Finally, the actions of the blockers were confirmed again by the injection of (−)-isoproterenol and noradrenaline.

Experiment 2: *In vitro* cardiovascular effects of the extract

Ten male Hartley guinea pigs weighing 320−350 g were used. They were deeply anesthetized with pentobarbital sodium (65 mg/kg, i.p.), and heparin calcium (1,000 U/kg, i.p.) was administered.

Isolated atrial preparation: The hearts were isolated and placed in Krebs-Henseleit solution containing (in mmol/L): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.18; KH_2PO_4, 1.18; $NaHCO_3$, 25.0; and glucose, 11.1, which was oxygenized with 95% O_2/5% CO_2. The whole atrium was dissected from the heart. The left atrial appendage was attached to a rigid support, whereas the right atrial appendage was connected to a force transducer (MLT0210/A; ADInstruments, Dunedin, New Zealand) with a silk thread. The atrium was bathed using surface perfusion in 20 mL of Krebs-Henseleit solution in the organ bath with the resting tension of 0.5 g. The bathing solution was maintained at 31℃ and continuously aerated with 95% O_2/5% CO_2. The solution was changed every 10 min during 30−60 min for stabilization before the start of experiments. According to our experience with the isolated atrial preparation, the mechanical and electrical properties of the atria could be kept within the physiological range for at least 3 h.

Developed tension was amplified by PowerLab 2/25 (ADInstruments) with bridge pod (ADInstruments), re-

corded and analyzed by LabChart (ver 5, ADInstruments). Atrial rate (beat/min) was automatically calculated with the frequency of the contraction. Atrial preparations (n = 10) were divided into two groups. In 5 preparations, *A. mongolica* extract in final concentrations of 1 μg/L to 1 g/L was added logarithmically and cumulatively with an interval of 5 to 20 min, watching the stabilization of the evoked changes. The developed tension and atrial rate at each concentration were measured at a steady state. In the rest of 5 preparations, the following experiment was performed; initially, β-adrenoceptor agonist (−)-isoproterenol in final concentrations of 1 and 10 nmol/L was sequentially added to confirm the positive inotropic and chronotropic actions; and the drug was washed out by changing the bathing solution ≥3 times until the parameters recovered to their baseline. Then, β_1-selective adrenoceptor blocker (±)-atenolol in a concentration of 10 μmol/L was added; isoproterenol in final concentrations of 1 and 10 nmol/L was sequentially added to confirm the inhibition of isoproterenol-induced responses; and the drugs were washed out in the same manner. Finally, (±)-atenolol in a final concentration of 10 μmol/L was added to the preparation again, and *A. mongolica* extract in final concentrations of 1 μg/L to 1 g/L was added logarithmically and cumulatively. The developed tension and atrial rate at each concentration were measured at a steady state.

Isolated aortae preparation: Aortae were isolated and skeletonized by forceps and cross-sectioned into 2 to 4 segments to obtain ring preparations. Each preparation was bathed in 10 mL of Tyrode solution containing (in mmol/L): NaCl, 137; KCl, 5.4; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4; and glucose, 5.6, in Magnus apparatus with a resting tension of 1 g. The bathing solution was maintained at 31℃ and continuously aerated with 95% O_2/5% CO_2. Developed tension was measured with force transducers (TBM4M; World Precision Instruments, Inc., Sarasota, FL, USA), amplified by PowerLab 4SP (ADInstruments), and recorded and analyzed with LabChart (ver 5; ADInstruments).

The basal condition of ring preparations was assessed initially as follows: K^+-rich Tyrode solution containing (in mmol/L): NaCl, 92.4; KCl, 49.6; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4; and glucose, 5.6, was applied to confirm their contractile responses. Next, the bathing solution was replaced by normal Tyrode solution, and an α_1-adrenoceptor agonist *R*-(−)-phenylephrine in final concentrations of 0.1 to 1 μmol/L was added to confirm their contractile responses; and then acetylcholine in final concentrations of 10 to 20 μmol/L was sequentially added to confirm their relaxation response. After the assessment of basal condition, the drugs were washed out in the same way. The preparations that showed pharmacological intervention-induced contraction and relaxation were used for the following experiments. Finally, the extract in final concentrations of 0.1, 1, 10, and 100 mg/L was cumulatively added with an interval of 10 − 15 min. The developed tension was measured at a steady state.

LC-MS analysis for cardiac glycosides in the extract

The stock solution (1 mL) of *A. mongolica* extract (100 g/L) was loaded onto a Sep-Pak C18 cartridge column and washed with H_2O and MeOH (each 10 mL). The MeOH eluate was filtered through a 0.22-μm membrane filter and used as a sample solution for LC-MS analysis. LC-MS analysis was conducted on a Shimadzu LCMS-8040 Triple Quadrupole LC/MS/MS Mass Spectrometer (Shimadzu, Kyoto, Japan) using a column, YMC Triart C18 Plus column (150 × 2.1 mm); of which temperature and flow rate were set at 35℃ and 0.2 mL/min, respectively. The mobile phase was composed of A (acetonitrile) and B (0.1% formic acid in aqueous solution) with a gradient elution: 0 − 5 min, 10% A; 5 − 30 min, 10 − 40% A; 30 − 40 min, 40 − 100% A; 40 − 50 min, 100% A. The column was equilibrated for 20 min under the initial conditions. The injection volume was 5 μL for qualitative analysis.

Mass-spectrometry (MS) detection was performed on a quadrupole MS/MS (Shimadzu) equipped with an electrospray ionization source. The temperatures were set as follows: 250℃ for the interface, 250℃ for the CDL, and 200℃ for the heat block. The nebulizing gas was 1.5 L/min, and the voltages were set as follows: (1) in the positive-ion mode, 4.5 kV for the interface, 30 V for the Q-array DC, 150 V for the Q-array RF, 30 V for the CDL, and 1.6 kV for the detector; (2) in the negative-ion mode, −3.5 kV for the interface, −30 V for the Q-array DC, 150 V for the Q-array RF, −25 V for the CDL, and −1.6 kV for the detector. The mass range was set to 100 − 2,000 *m/z* in the Q1 scan mode, with acquisition of both the positive- and negative-ion MS data for identification.

Drugs

The following drugs were purchased: (±)-atenolol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), (−)-isoproterenol hydrochloride (Sigma-Aldrich, St Louis, MO, USA), *R*-(−)-phenylephrine hydrochloride (Sigma-Aldrich), acetylcholine (Ovisot Inj™; Daiichi Sankyo, Tokyo, Japan),

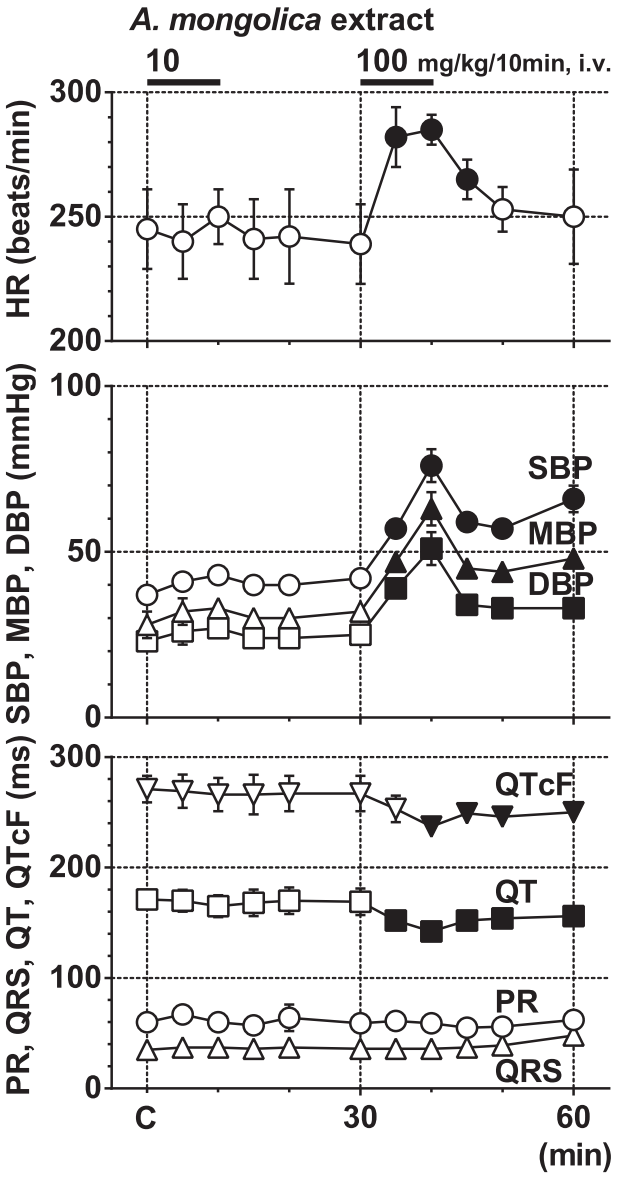

Fig. 2 The time courses of changes in the heart rate (HR; beats/min, top); the systolic, diastolic and mean blood pressure (SBP, DBP and MBP; mmHg, middle); the QT interval (ms), corrected QT interval with Fridericia's formula (QTcF), QRS (ms) width and PR interval (ms) of guinea pigs before and after the intravenous administration of *Adonis mongolica* extract at doses of 10 and 100 mg/kg/10 min ($n=4$, each). Data are presented as mean ± SE. Closed symbols represent significant changes from the respective pre-drug control values (C) by p-value < 0.05.

noradrenaline (NOR-ADRENALIN INJECTION 1 mg, Daiichi Sankyo), prazosin hydrochloride (Sigma-Aldrich), *dl*-propranolol hydrochloride (Sigma-Aldrich), thiopental sodium (Ravonal® 0.5 g for Injection; Mitsubishi-Tanabe Pharma Co., Osaka, Japan), pentobarbital sodium (Somnopentyl®; Kyoritsu Seiyaku Co., Tokyo, Japan), and heparin calcium (Caprocin®; Sawai Pharmaceutical Co., Ltd., Osaka, Japan).

Statistics

Data are presented as the mean ± SE. The statistical analysis was performed by the software GraphPad Prizm 6 (ver6.03; GraphPad Software, Inc., La Jolla, CA, USA). The statistical significances within a parameter of a group were evaluated with one-way, repeated-measures analysis of variance (ANOVA) followed by a post-hoc test for mean values comparison, whereas those of paired data were evaluated with two-way repeated-measures ANOVA and those between the groups were analyzed with two-way factorial ANOVA. A p-value < 0.05 was considered to be statistically significant.

Results

***In vivo* cardiovascular effects of the extract**

The time courses of changes in the cardiovascular variables are summarized in Fig. 2. The pre-drug control values (C) of the heart rate, systolic/diastolic/mean blood pressure, PR interval, QRS width, QT interval and QT interval corrected by Fridericia's formula (QTcF) were 245± 16 beat/min, 37±3/23±3/28±4 mmHg, 60 ± 8 ms, 35 ± 2 ms, 171 ± 9 ms, and 271 ± 12, respectively. The extract in a low dose of 10 mg/kg/10 min did not alter any of the variables. That in a high dose of 100 mg/kg/10 min increased the heart rate for 5 − 15 min with a peak response of 285 ± 6 beat/min at 10 min ($p < 0.01$); elevated the systolic, diastolic and mean blood pressure for 5 − 30 min with peak responses of 76 ± 5, 51 ± 5 and 63 ± 5 mmHg ($p < 0.01$) at 10 min, respectively; but shortened the QT interval for 5 − 30 min and QTcF for 10 − 30 min with peak responses of 142 ± 6 ($p < 0.01$) and 237 ± 8 ($p < 0.01$) ms at 10 min, respectively, after the start of infusion. No significant change was detected in the PR interval or QRS width in these observation periods. During the next 30 min, prolongation of the PR interval and QRS width occurred followed by the onset of complete atrioventricular block, leading to cardiohemodynamic collapse in each animal. Typical traces showing the lethal effects of the extract on the electrocardiogram and blood pressure are depicted in Fig. 3.

Pharmacological analysis of the chronotropic and vasopressor actions *in vivo* induced by the extract of *A. mongolica*

The changes in the heart rate induced by (−)-isoproterenol and the extract of *A. mongolica* were compared between the absence and presence of *dl*-propranolol

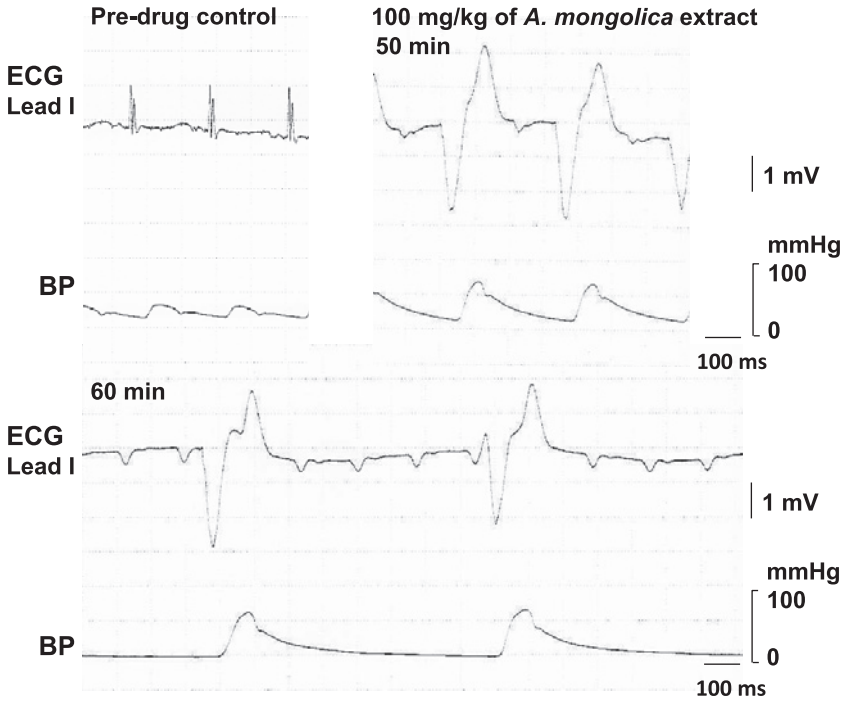

Fig. 3 Representative traces of the lead I electrocardiogram (ECG) and aortic blood pressure (BP) before (Pre-drug control) and 50 and 60 min after the start of intravenous administration of 100 mg/kg of *Adonis mongolica* extract into an anesthetized guinea pig. The extract delayed the intraventricular conduction and prolonged the PR interval, resulting in the onset of second-degree atrioventricular block at 50 min followed by complete atrioventricular block at 60 min.

in guinea pigs as shown in Fig. 4A (n = 4). Those in the mean blood pressure induced by noradrenaline and the extract were also compared between the absence and presence of prazosin as shown in Fig. 4B (n = 4). (−)-Isoproterenol in doses of 1 or 5 μg/kg increased the heart rate by 128±16 beat/min, and the extract in a high dose of 100 mg/kg/10 min did it by 38±12 and 40±12 beat/min at 5 and 10 min, respectively, after the start of its infusion. *dl*-Propranolol in a dose of 1 mg/kg significantly suppressed the positive chronotropic action induced by (−)-isoproterenol (to +24±7 beat/min, $p < 0.05$) and the extract at 5 min (to +3±2 beat/min, $p < 0.05$) and at 10 min (to +8±3 beat/min, $p < 0.05$) after the start of infusion (Fig. 4A). Meanwhile, noradrenaline in a dose of 1 μg/kg elevated the mean blood pressure by 20±3 mmHg, and the high dose of the extract did it by 19±5 and 35±5 mmHg at 5 and 10 min, respectively, after the start of its infusion. Prazosin in a dose of 0.03 mg/kg significantly diminished the vasopressor action induced by noradrenaline (to +7±2 mmHg, $p < 0.05$) and the high dose of the extract at 10 min (to +14±3 mmHg, $p < 0.05$) after the start of its infusion (Fig. 4B). After the examination of the extract at 10 min, (−)-isoproterenol and noradrenaline were injected again. Blocking effects of the antagonists were confirmed, although atrioventricular block had been induced.

***In vitro* cardiovascular effects of the extract**

Typical traces showing the effects of the extract on developed force and atrial rate in the isolated atria are depicted in Fig. 5, and their time courses of the changes are summarized in Fig. 6 (A, B). The control values (C) of the contractile force and atrial rate before the treatment of the extract were 111±23 beat/min and 0.52±0.08 g in the absence of (±)-atenolol (n = 5), and 146±5 beat/min and 0.77 ±0.09 g in the presence of (±)-atenolol (n = 5), respectively. The extract slightly decreased the atrial rate by 6−8% ($p < 0.01$) at concentrations of 1 μg/L to 0.1 g/L (Fig. 6A) but increased the contractile force by 37% at a concentration of 1 g/L ($p < 0.01$, Fig. 6B). In the presence of 10 μmol/L of (±)-atenolol, the extract increased the atrial rate by 6% at 1 g/L and increased the contractile force by 16% ($p < 0.01$) at 0.1 g/L and 65% ($p < 0.01$) at 1 g/L (Fig. 6A, 6B). No statistically significant interaction was detected between changes in the contractile force induced by the extract with and without pretreatment of 10

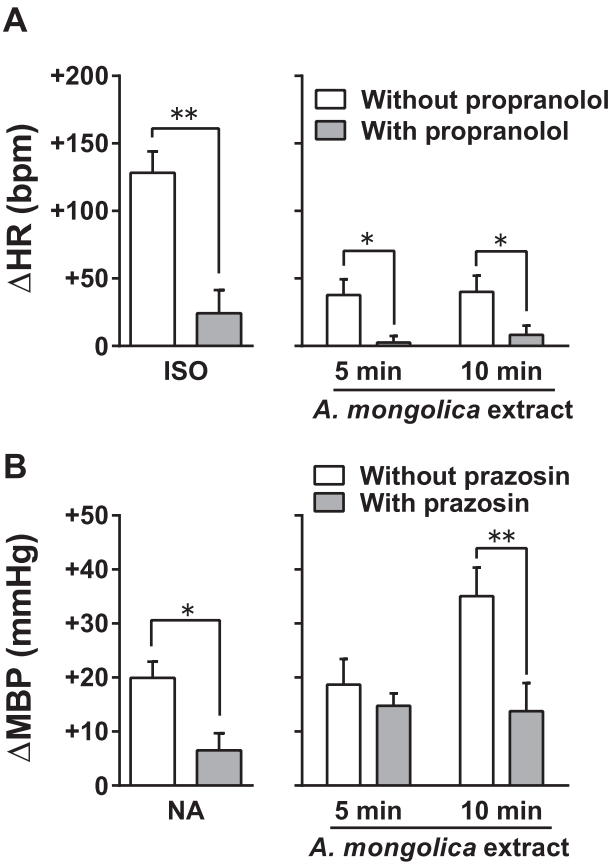

Fig. 4 Pharmacological analysis of *Adonis mongolica*-induced chronotropic and vasopressor actions in the anesthetized guinea pigs. (A) Increment in the heart rate (ΔHR) by β-adrenergic receptor agonist (−)-isoproterenol (1 or 5 μg/kg, ISO) (left) before (white column) and after (dark columns) *dl*-propranolol (1 mg/kg) treatment ($n=4$), whereas that by the extract of *A. mongolica* in a dose of 100 mg/kg/10 min (right) in the absence (white columns) and presence (dark columns) of *dl*-propranolol (1 mg/kg) ($n=4$ for each group). (B) Increments in the mean blood pressure (ΔMBP) by α-adrenergic receptor agonist noradrenaline (1 μg/kg, NA) (left) before (white columns) and after (dark columns) prazosin treatment (0.03 mg/kg) ($n=4$), whereas that by the extract of *A. mongolica* in a dose of 100 mg/kg/10 min (right) in the absence (white columns) and presence (dark columns) of prazosin (0.03 mg/kg) ($n=4$ for each group). Data are presented as mean ± SE. **p*-value < 0.05, ***p*-value < 0.01.

μmol/L of (±)-atenolol (Fig. 6A, 6B).

The application of 10 nmol/L of (−)-isoproterenol hydrochloride increased the atrial rate by 34% and contractile force by 62%, and pretreatment with 10 μmol/L of (±)-atenolol significantly diminished the response by (−)-isoproterenol in the atrial rate and contractile force in + 5% ($p < 0.01$) and + 5% ($p < 0.01$), respectively (Fig. 6C).

The effects of the extract on the ring preparations of aortae are summarized in Fig. 6D. The extract significantly decreased the contraction of the preparations by 2, 3, 4 and 3% at concentrations of 0.1, 1, 10 and 100 mg/L, respectively ($p < 0.01$).

LC-MS analysis

The total-ion chromatogram of the sample was analyzed by collecting its MS spectrum in full-scan mode. The MS scan in both the positive- and negative-ion modes provided valuable information to confirm the molecular weights (MWs) of glycosides in the sample solution. As shown in Fig. 7, eight peaks were detected, which are most likely cardiac glycosides, MW of which was estimated as 590, 742, 754, 756, 892, 924, 1,006 and 1,006.

Discussion

In the present study, we examined the pharmacological effects of water-soluble extract of *A. mongolica* on the cardiovascular system and showed that it resembles the effects of cardiac glycosides. We also showed that the extract contained the molecules which were supposed to be cardiac glycosides.

Cardiac glycosides

Eight peaks of glycosides were detected, and the MW of each component was measured in MS analysis; 590, 742, 754, 756, 892, 924, 1,006 and 1,006 (Fig. 7). Their MWs were larger than those of previously reported cardiac glycosides which were isolated from *A. mongolica Simonovich*; namely, corchoroside A (MW = 534), cymarin (MW = 548), adonitoxin (MW = 550), k-strophanthin-β (MW = 696), eryslmoside (MW = 696), olitoriside (MW = 710), glucoolitoroside (MW = 858) and k-strophanthoside (MW = 872), respectively.[1–5] The larger MWs observed in this study might come from the difference in the length of the sugar chain that is added to the cardiac glycosides. Further phytochemical investigation should be carried out to clarify the chemical structure of these cardiac glycosides.

The rationale for the drug doses

In Mongolian traditional medicine, 6 g of the dried aerial parts are roughly segmented and immersed with 200 mL of purified water in a glass chamber. After the mixture is put in a water bath, the bath temperature is elevated and kept at 100℃ for 30 min, and the mixture is cooled down at room temperature for 30 − 45 min. It is filtered, and the filtrate is adjusted to 180 mL by adding purified water. The concentration of this infusion formula of a ratio of 1:30 can be estimated to be 33.3 g/L (weight of plant/volume of water). The infusion has been orally given 3 times a day

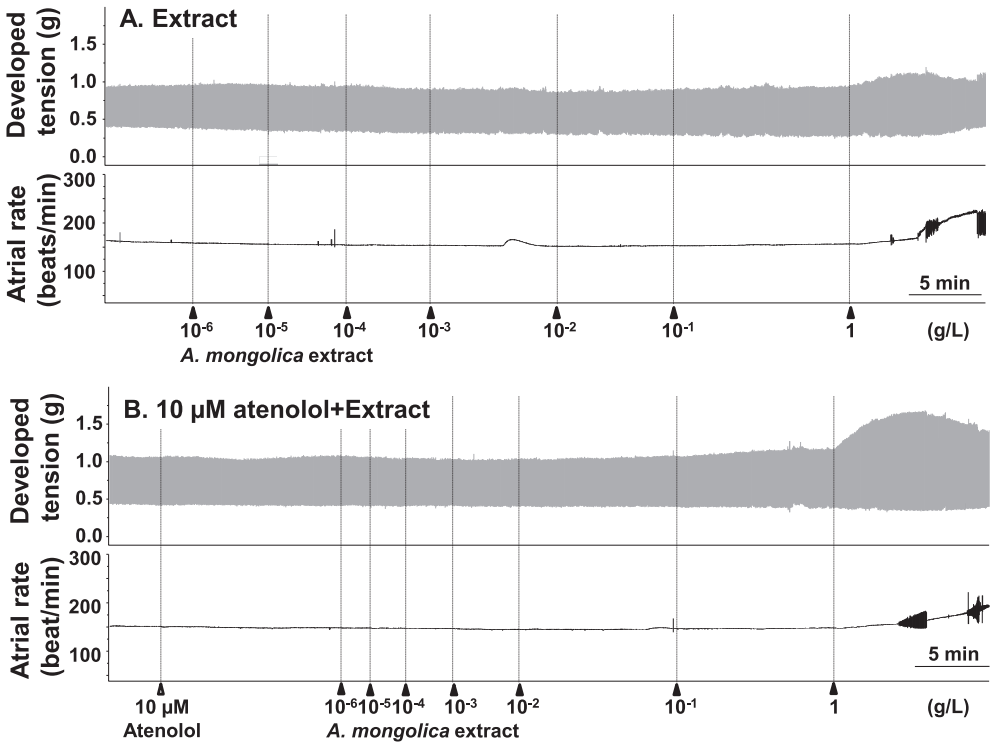

Fig. 5 Representative traces of developed tension and atrial rate in isolated guinea pig atria. The extract of *Adonis mongolica* was cumulatively applied in the range of 1 μg/L (10^{-6} g/L) to 1 g/L in the absence (A) and presence (B) of 10 μmol/L (±)-atenolol. Developed tension with basal tension of 0.5 g (upper) and atrial rate (lower) calculated online from the cycle length of the atrial contractions were shown in each panel. Positive inotropic effect was observed from 0.1 g/L. At 1 g/L, atrial tachycardia was observed in each condition.

before meals (45 mL/body/day) for 4 days to treat patients with heart failure showing tachycardia and edema. In a case of patient weighing 60 kg, 15 mL/body of the infusion can be calculated to be 8.3 mg/kg, which would provide peak plasma concentration of about 0.83 μg/mL, supposing that bioavailability is 10%. The formulation is empirically known to induce diuresis and reduce pulmonary edema, resulting in the reduction of respiratory rate, and to lower the blood pressure in such patients. In this study, the extract of *A. mongolica* was obtained with a concentration of 100 g/L (weight of plant/volume of water). In the *in vivo* experiment, the extract in doses of 10 and 100 mg/kg/10 min was administered intravenously to the anesthetized guinea pigs, of which peak plasma concentration may be about 10 and 100 times higher than that of the therapeutic one, respectively. In the *in vitro* experiments, the extract in concentrations of 1 μg/L − 1 g/L and 0.1 mg/L − 1 g/L was applied to isolated atria and aortae in the bath solution, of which concentration may be about 1/1,000 − 1,000 and 1/10 − 1,000 times of therapeutic plasma concentration, respectively. Thus, the doses and concentrations assessed in this study can be considered to be enough to analyze the pharmacodynamic effects of the extract.

Effects of the extract on the cardiovascular systems of guinea pigs

Chronotropic effect: According to a classical literature,[6,7] intravenous injection of *A. mongolica* extract to healthy rabbits at 20 − 30 mg/kg or diseased ones at 5 − 20 mg/kg decreased the heart rate. On the other hand in this study using guinea pigs, the extract at a dose of 10 mg/kg hardly altered the heart rate, but that at 100 mg/kg increased it (Fig. 2). Although β_1-adrenoreceptor stimulation by 10 nmol/L (−)-isoproterenol increased the atrial rate of isolated atria of guinea pig (Fig. 6C), the extract hardly altered the atrial rate (Fig. 6A, Extract), indicating that the extract did not directly stimulate β_1-adrenoreceptors (Fig. 5, 6). Thus, the positive chronotropic effect of the extract observed *in vivo* may be an indirect action. The pretreatment of *dl*-propranolol significantly suppressed the positive chronotropic action induced by the extract *in vivo*

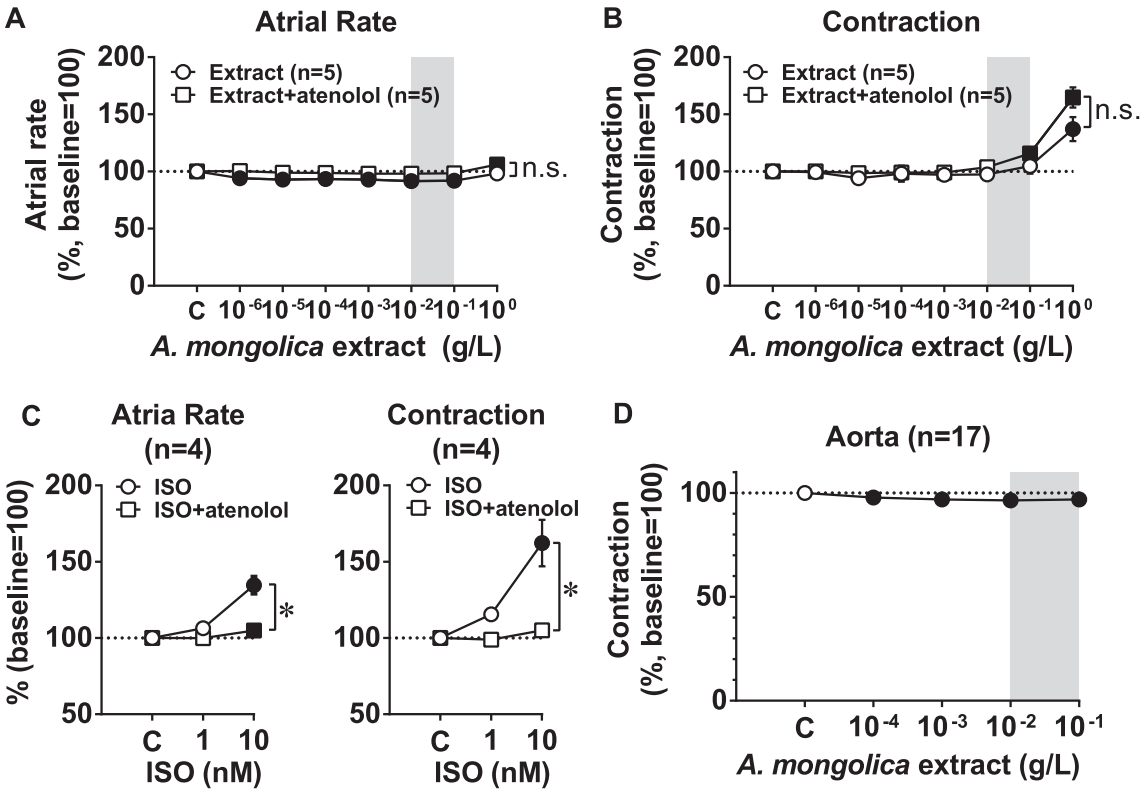

Fig. 6 Summary of the *in vitro* effects of *Adonis mongolica* extract on the atrial rate and developed tension of the atrial preparation, and on the contractile force of the aortae preparation. The effects of the extract at concentrations of 1 μg/L (10^{-6} g/L) to 1 g/L on atrial rate (A) and contraction (B) were calculated by percent changes from their respective pre-drug control values in the absence (circles) and presence (squares) of 10 μmol/L of (±)-atenolol. The effects of 1 and 10 nmol/L (−)-isoproterenol hydrochloride on the atrial rate and contraction in the absence (circles) and presence (squares) of 10 μmol/L (±)-atenolol are summarized (C). The effects of the extract on ring preparations of the aortae at concentrations of 0.1 mg/L (10^{-4} g/L) to 0.1 g/L (10^{-1} g/L) under basal tension of 1 g are summarized (D). Gray zones in the graphs indicate the predicted range of peak plasma concentrations of the extract in the *in vivo* experiment (Fig. 1). Data are presented as mean±SE. Closed symbols represent significant changes from the respective pre-drug control values (C) by p-value<0.05 within a group. *p-value<0.05 between groups with and without (±)-atenolol.

(Fig. 4A), indicating the extract would have increased the sympathetic tone. The positive chronotropic action with *A. mongolica* has not been reported in the previous studies *in situ* or *in vitro*;[6-8] however, in the *in vitro* experiment with rat brain, a cardiac glycoside ouabain at 1 mmol/L inhibited noradrenaline uptake by synaptosome via the inhibition of Na^+-K^+ ATPase,[12] which may partly support our hypothesis for the positive chronotropic effect (Fig. 2). Similarly, ouabain in a subcutaneous or intravenous dose of 100−200 μg/kg also increased the atrial beating rate by 20−40 beats/min from the baseline value of 240−310 beats/min in conscious guinea pigs,[13] and the positive chronotropic effect has been considered to be related with the increase of catecholamine and the mobilization of calcium.[14] In addition, the hypothesis could be supported by the observation that QT interval and QTcF were shortened in parallel with the increase of the heart rate, also reflecting the rise in sympathetic tone (Fig. 2).

Positive inotropic effect: In isolated atria of guinea pig, the contraction force was increased by the extract at a concentration of 1 g/L (Fig. 5, 6). This inotropic effect was not suppressed by (±)-atenolol, suggesting that the effect may be exerted via β_1-adrenoreceptor-independent pathway. Importantly, the positive inotropic effect was followed by repetitive premature atrial contractions and contracture (Fig. 5), suggesting the development of calcium overload in the cardiomyocytes, which was in good accordance with the phenotype of ouabain-induced digitalis intoxication.[15]

Pressor response: In guinea pigs *in vivo*, the extract ele-

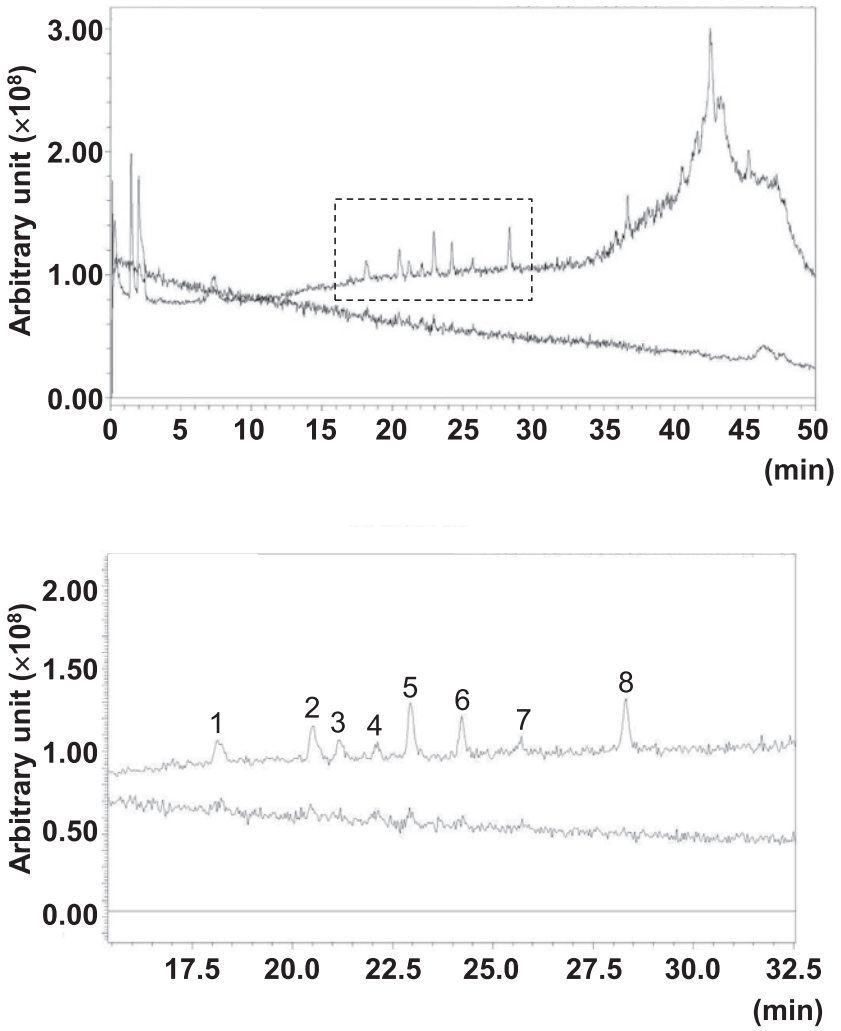

Fig. 7 Data of the water-soluble extract of *Adonis mongolica* obtained from liquid chromatography-mass spectrometry analysis. Whole (A) and enlarged (B) data in liquid chromatography analysis detected 8 peaks of glycosides. The molecular weight of each component was measured in mass spectrometry analysis, including (1) 924, (2) 892, (3) 754, (4) 742, (5) 756, (6) 1,006, (7) 1,006 and (8) 590.

vated systolic, diastolic and mean blood pressure in a dose-related manner, and the significant increase was detected at 5－60 min after the high dose (Fig. 2), which is different from clinical experiences with *A. mongolica*. The pretreatment of prazosin significantly suppressed the vasopressor action induced by the extract *in vivo* (Fig. 4B), indicating that the extract would have increased the sympathetic tone. Meanwhile, ouabain has been reported to directly increase peripheral vascular resistance in anesthetized dogs[16] and to elevate total peripheral resistance in conscious dogs with total arterial denervation.[17] Ouabain has been also reported to increase the blood pressure in guinea pig *in vivo*,[18] and endogenous ouabain has been considered to partly contribute to the pathophysiology in hypertension.[19] However, in this study with isolated aortae of guinea pigs, the extract did not increase the contraction force directly (Fig. 6). These results at least suggest that the elevation in blood pressure in our study may be caused by the rise in the sympathetic tone (Fig. 4B) in addition to the increase of the heart rate (Fig. 2, 4A) and myocardial contractile force (Fig. 5, 6).

Conduction block: The conduction block induced by the extract appeared >30 min after the administration of the high dose, consisting of intraventricular conduction delay, PR-interval prolongation (first-degree atrioventricular block) and second-degree atrioventricular block followed by complete atrioventricular block (Fig. 3), leading to cardiohemodynamic collapse in each animal. These results

were similar to that of a previous study in conscious guinea pigs with ouabain, which induced prolongation of PR interval, loss of T wave, sinoatrial block and atrioventricular block.[13] Cardiac glycoside has been reported to stimulate the arterial baroreceptors and potentiate parasympathetic sensitivity, which can suppress the atrioventricular conduction,[20] possibly explaining currently observed negative dromotropic effect of the extract.

Clinical implications

The pharmacological profile of the extract of *A. mongolica* observed in this study was the same as those of digitalis reported in the previous studies.[21] Because of the limited utility and well-known adverse effect of digitalis, it may not be practical to use the extract as a clinically available strategy in Japan. Importantly, the present study implies that excessive elevation of the sympathetic tone induced by its overdosing, leading to the onset of tachycardia and hypertension, can be treated by adrenergic receptor antagonists.

Conclusion

Water-soluble extract of *A. mongolica* contained 8 kinds of cardiac glycosides, which may explain currently observed various cardiovascular effects including positive chronotropic and inotropic, negative dromotropic, and vasopressor effects in addition to the proarrhythmic action.

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Conflicts of interest: The authors indicate no potential conflicts of interest.

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