Supporting Information

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Novel Pharmacological Effects of Syringin on Anxiety Behavior and Autonomic Nervous System Activity

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1. Materials and Methods

1.1. Animals

Male Sprague-Dawley rats (6 weeks old) were purchased from SLC, Inc. (Shizuoka, Japan), individually housed in standard polycarbonate cages for 7 days, and subjected to serial 7-day handling. Food (MF, Oriental Yeast Manufacturing Co., Ltd., Tokyo, Japan) and water were available *ad libitum*. Room temperature was maintained at 23 ± 2°C. The test schedule is described in Figure S1.

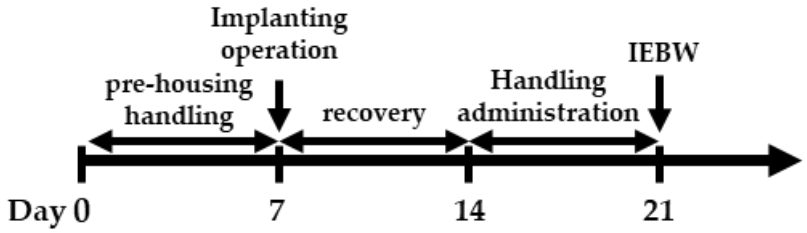

Figure S1. The schedule of the improved elevated beam walking (IEBW) test. After 1 week of pre-housing, the rats were implanted with a telemeter followed by 1 week of recovery. After the recovery period, the rats were administered 1 mL of each reagent. On the test day, rats were administered the reagents 30 min before the IEBW test.

1.2. Implantation of Wireless Telemeter for Electrocardiogram (ECG)Recording

After a 7-day acclimatization period, the rats were treated with a local anesthetic (ropivacaine hydrochloride hydrate) and general anesthesia (sodium pentobarbital, 40 mg/kg, i.p.), and a wireless telemeter (model TR50BB; Kaha Sciences Ltd., Auckland, New Zealand) was implanted in the abdomen of each animal. The rats were treated with antibiotics (imipenem hydrate and cilastatin sodium, 8.3 mg/kg, i.m.) to prevent postoperative infection at the end of the surgery. After the surgery, the rats were allowed to recover for 7 days. We checked the receiving sensitivity using a receiver and normal operation of the charge function using a smart pad (models TR181, TR190, PL3516, and MLS260; Kaha Sciences Ltd.) during the recovery and adaptation periods.

1.3. Administration

Based on the test food consumption in our previous study of ASH extract [12 in the main text], we determined the dose (isofraxidin: 5 mg/kg, 099-03651, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, syringin: 16 mg/kg, S920050, Toronto Research Chemicals, Toronto, Canada). The dose and timing of administration of 1 mL of isofraxidin, syringin, or distilled water (Control) was administered through a probe once a day for 7 days. In the behavioral test day, rats were administered 1 mL of each solution once 30 min before the behavioral test.

1.4. IEBW Test

To examine the anxiolytic effects of isofraxidin and syringin, we performed behavioral experiments using high-fear stress. In generally, the elevated plus maze (EPM) test was conducted to evaluate animal anxiety. However, the EPM test revealed large standard error of the measured data for ANS activity. We created a simpler apparatus by reducing the numbers of the arms and increasing the height from the ground in the EPM test. The variation of ANS activity data in our developed IEBW test

was lower than that measured using the EPM test (see reference [12 in the main text] in the manuscript for details).

After 7 days of administration, rats were placed in the tip of the open arm 140 cm away from the closed arm 30 min after the last administration and allowed to explore the area freely for 3 min. Their behavior and ECG were recorded. We assessed the time spent in the open arm.

1.5. Assessment of Cardiac ANS Activity (Heart Rate Variability [HRV])

ECG data were recorded using a radio-telemetry system comprising the implanted device, a battery-charging and telemetry-receiving device underneath the cage, and a data acquisition system (PowerLab16/35, PL3516, AD Instruments, Sydney, New South Wales, Australia) interfaced with a computer. Cardiac autonomic activity was assessed via the spectral analysis of R-R interval variability. Data were stored and analyzed using LabChart Pro (ver. 8.0, AD Instruments). Frequency domain analysis and the power spectra of R-R interval variability were obtained using the fast Fourier transform algorithm. High (HF, 0.6–3.0 Hz), low (LF, 0.2–0.6 Hz), and very low frequencies (≤0.2 Hz) were determined. LF and HF components were expressed in normalized units (LFnu and HFnu). The power of the HF component indicates cardiac parasympathetic activity, whereas that of the LF component indicates the sympathetic activity with parasympathetic modulation. LF/HF is an index of the cardiac sympathetic-parasympathetic tone balance. We analyzed LF/HF, LFnu, and HFnu in the IEBW test and home cage conditions at the time of the IEBW test before the IEBW test.

1.6. Ethics Statement

This study was conducted according to the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 85–23, revised in 1996). All experimental protocols were approved by the Ethics Committee on Animal Use of the Suzuka University of Medical Science (No. 1 of April 1, 2016).

1.7. Statistics Analysis

Data were expressed as the mean ± standard error and derived from measurements of five rats. Statistical analysis was performed using SPSS statistics 25 (IBM Japan, Ltd., Tokyo, Japan). The homogeneity of variances was examined using Levene's test. One-way ANOVA was used for intergroup comparisons. When ANOVA revealed significant differences, Dunnett's *t*-test or T3 post hoc test was used to identify significant differences versus the Cont group. Between-group differences were analyzed using a paired Student's *t*-test. Differences between Cont and ASH groups were analyzed using a unpaired Student's *t*-test. $P < 0.05$ denoted statistical significance.