

Title

CRF receptor 1 antagonism and brain distribution of active components contribute to the ameliorative effect of rikkunshito on stress-induced anorexia

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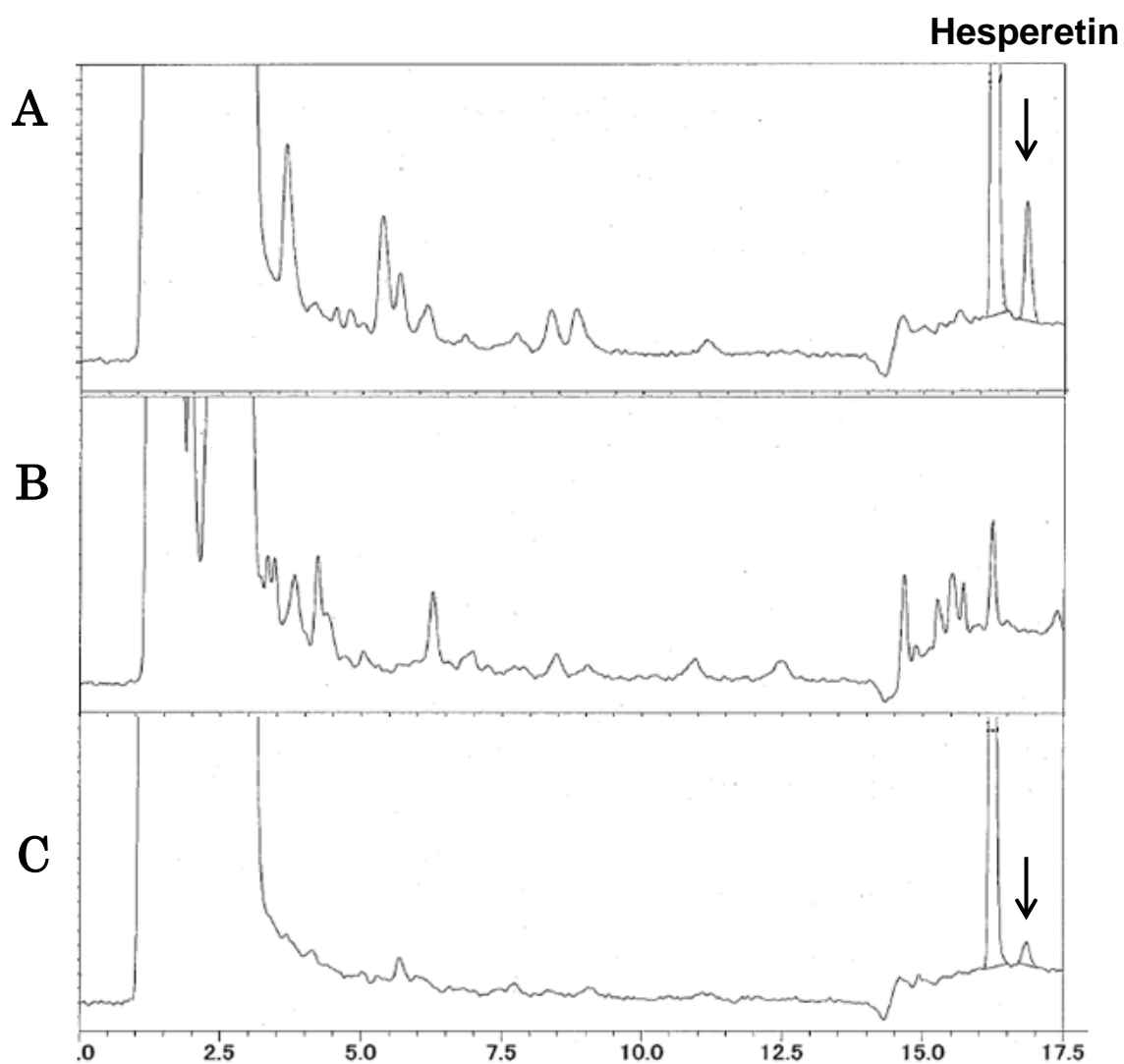
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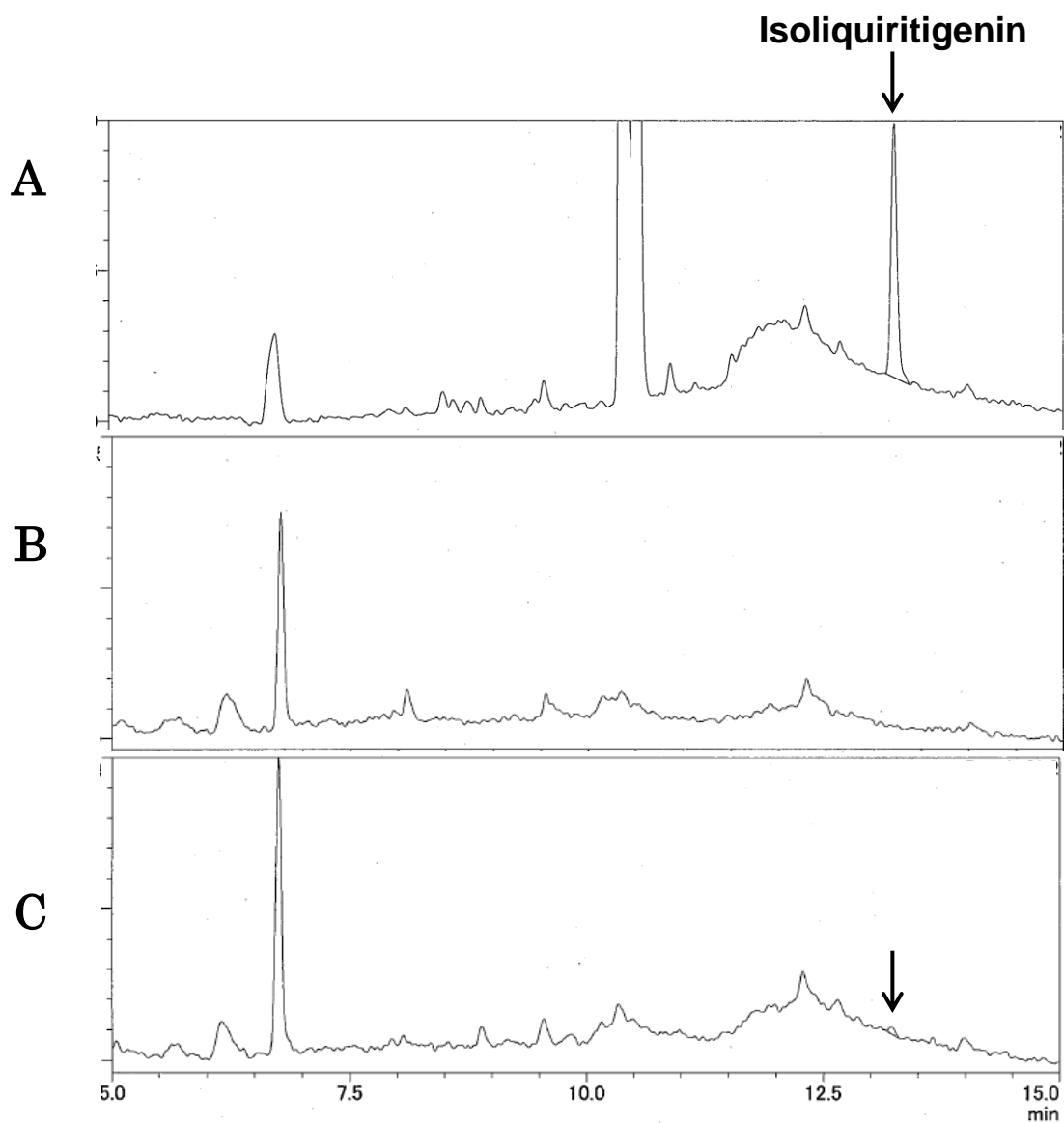
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Supplementary Figure S1.



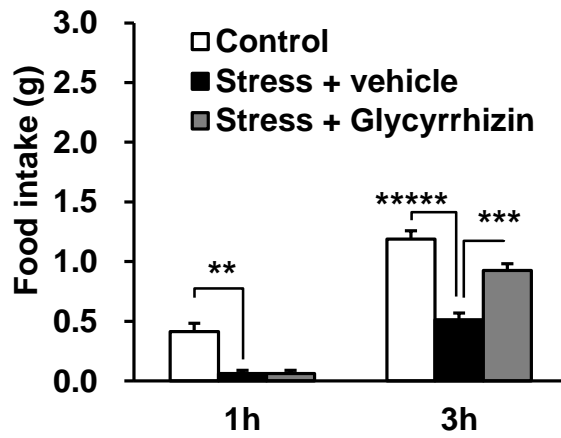
HPLC chromatograms of authentic standards, blank, and brain sample for hesperetin. (A) Brain sample spiked with standard solution. (B) Brain sample obtained from rats not treated with rikkunshito. (C) Brain sample after oral administration of rikkunshito.

Supplementary Figure S2.



HPLC chromatograms of authentic standards, blank, and brain sample for isoliquiritigenin. (A) Brain sample spiked with standard solution. (B) Brain sample obtained from rats not treated with rikkunshito. (C) Brain sample after oral administration of rikkunshito.

Supplementary Figure S3.



Effect of glycyrrhizin (glycoside form of glycyrrhetic acid) on novelty stress-induced hypophagia. Six week-old male mice were deprived of food for 24 h and orally administered with glycyrrhizin (4mg/ kg). Immediately after administration, the mice were isolated and cumulative food intake was determined at 1 and 3 h after the stress exposure. Data are presented as the mean  $\pm$  SEM (n = 8). \*\*, \*\*\*, \*\*\*\* p < 0.01, 0.001, 0.00001 vs. stress group by Steel test at 1 h and Dunnett test at 3 h.

## **Supplementary Method**

### **Determination of plasma levels of active components after the oral administration of RKT in rats**

Twenty five microliters of methanol was added to 500  $\mu\text{L}$  plasma samples followed by mixing. Atractylenolide III or digoxin (internal standard [IS]) solution (50  $\mu\text{L}$ ) was added to the solutions followed by mixing. Ammonium acetate solution (100  $\text{mmol L}^{-1}$ , 100 or 400  $\mu\text{L}$ ) was added to the solutions followed by mixing. A solid-phase cartridge (OASIS HLB, 30 mg/1 cc; Waters, Milford, MA, USA) was conditioned with methanol and 10  $\text{mmol L}^{-1}$  ammonium acetate solution, and the samples were loaded onto the cartridge. The cartridge was washed with water: methanol (9:1, v/v), and 1.5 mL of methanol and 30  $\mu\text{L}$  of propylene glycol were added to the cartridge to elute the analytes. The mixtures were dried at 25°C under a stream of nitrogen gas. Furthermore, 100  $\mu\text{L}$  of 10  $\text{mmol L}^{-1}$  ammonium acetate: methanol (8:2, v/v) was added to the dried samples followed by mixing and sonication. The solutions were filtered (0.22  $\mu\text{m}$ ) and followed by injection into an LC–MS/MS system. This system comprised an LC-20A system (Shimadzu, Kyoto, Japan) connected to an API5000 triple quadrupole mass spectrometer fitted with a TurboIonSpray electrospray ionization interface (AB Sciex, Framingham, MA, USA). Those analytical conditions are shown in Supplementary Tables S1 and S2. The standard components contained in RKT were supplied by Tsumura & Co.

**Table S1 Methods of LC-MS/MS: Ion parameters of rikkunshito components and internal standards**

<b>Compound name</b>	<b>Q1Mass (<i>m/z</i>)</b>	<b>Q3Mass (<i>m/z</i>)</b>	<b>Polarity</b>	<b>LC methods ID</b>
<b>Glycyrrhetic acid</b>	471	149	Positive	1
<b>Nobiletin</b>	403	373	Positive	1
<b>Tangeretin</b>	373	343	Positive	1
<b>[8]-Shogaol</b>	303	167	Negative	2
<b>Liquiritin apioside</b>	550	255	Negative	2
<b>Liquiritin</b>	417	255	Negative	2
<b>Isoliquiritigenin</b>	255	119	Negative	2
<b>Glycoumarin</b>	367	309	Negative	2
<b>Hesperetin</b>	301	164	Negative	2
<b>Digoxin (internal standard)</b>	780	651	Negative	2

**Table S2 Methods of LC-MS/MS: HPLC conditions for analyzing RKT components**

<b>Methods ID</b>		<b>HPLC condition</b>
1	Column: Mobile phase Gradient elution program (%B in A) flow rate column temperature injection volume	shim-pack XR-ODS II (2.0 mm I.D., × 100 mm L., 2.2- $\mu$ m particle size; Shimadzu GLC Ltd., Tokyo, Japan) (A) 10 mM ammonium acetate, (B) methanol 0.01–0.50 min, 20%; 0.50–2.50 min, 20–40%; 2.50–17.00 min, 40–85%; 17.00–30.00 min, 85–95%; 30.00–34.00 min, 95%; 34.10–40.00 min, 20% 0.2 mL/min 40°C 10 $\mu$ L
2	Column: Mobile phase Gradient elution program (%B in A) flow rate column temperature injection volume	shim-pack XR-ODS II (2.0 mm I.D., × 100 mm L., 2.2- $\mu$ m particle size; Shimadzu GLC Ltd., Tokyo, Japan) (A) 10 mM ammonium acetate, (B) methanol 0.01–0.50 min, 20%; 0.50–2.50 min, 20–40%; 2.50–17.00 min, 40–85%; 17.00–30.00 min, 85–95%; 30.00–34.00 min, 95%; 34.10–40.00 min, 20% 0.2 mL/min 40°C 30 $\mu$ L