Byakkokaninjinto Extract

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Byakkokaninjinto Extract contains not less than 9 mg and not more than 36 mg of mangiferin, not less than 13 mg and not more than 39 mg of glycyrrhizic acid ($C_{42}H_{62}O_{16}$: 822.93), and not less than 0.9 mg (for preparation prescribed 1.5 g of Ginseng) or not less than 1.8 mg (for preparation prescribed 3 g of Ginseng) of ginsenoside Rb_1 ($C_{54}H_{92}O_{23}$: 1109.29), per extract prepared with the amount specified in the Method of preparation.

Method of preparation

	1)	2)
Anemarrhena Rhizome	5 g	5 g
Gypsum	15 g	15 g
Glycyrrhiza	2 g	2 g
Brown Rice	8 g	8 g
Ginseng	1.5 g	3 g

Prepare a dry extract or viscous extract as directed under Extracts, according to the prescription 1) or 2), using the crude drugs shown above.

Description Byakkokaninjinto Extract occurs as a very pale yellow-brown to light brown powder, or blackish brown viscous extract. It has a slight odor, and has a slightly sweet and slightly bitter taste.

Identification **(1)** To 2.0 g of the dry extract (or 6.0 g of the viscous extract) add 10 mL of sodium hydroxide TS, shake, then add 5 mL of 1-butanol, shake, centrifuge, and use the supernatant liquid as the sample solution. Separately, to 1 g of pulverized Anemarrhena Rhizome add 10 mL of water, shake, then add 10 mL of 1-butanol, shake, centrifuge, and use the supernatant liquid as the standard solution. Perform the test with these solutions as directed under Thin-layer Chromatography *<2.03>*. Spot 5 μL of the sample solution and 1 μL of the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, 1-propanol, water and acetic acid (100) (7:5:4:1) to a distance of about 7 cm, and air-dry the plate. Spray evenly 4-dimethylaminobenzaldehyde TS for spraying on the plate, and heat at 105℃ for 2 minutes, and allow to cool: one of the several spots obtained from the sample solution has the same color tone and *R*f value with the yellowish red to dark red spot (at an *R*f value of about 0.3) from the standard solution (Anemarrhena Rhizome).

(2) Place 2.0 g of the dry extract (or 6.0 g of the viscous extract) in a porcelain crucible, and ignite to incinerate at 500 – 550℃. Add 60 mL of water to the residue, shake, centrifuge, and use the supernatant as the sample solution. Add ammonium oxalate TS to the sample solution: a white precipitate is formed. The precipitate does not dissolve in diluted acetic acid, but dissolves on the addition of diluted hydrochloric acid (Gypsum).

(3) To 1.0 g of the dry extract (or 3.0 g of the viscous extract) add 10 mL of water, shake, then add 10 mL of 1-butanol, shake, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 1 mg of liquiritin for thin-layer chromatography in 1 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under Thin-layer Chromatography *<2.03>*. Spot 1 μL each of the sample solution and standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, methanol and water (20:3:2) to a distance of about 7 cm, and air-dry the plate. Spray evenly dilute sulfuric acid on the plate, heat at 105℃ for 5 minutes, and examine under ultraviolet light (main wavelength: 365 nm): one of the several spots obtained from the sample solution has the same color tone and *R*f value with the yellow-green fluorescent spot from the standard solution (Glycyrrhiza).

(4) To 5.0 g of dry extract (or 15 g of the viscous extract) add 15 mL of water, shake, then add 5 mL of diethyl ether, shake, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 1 mg of cycloartenyl ferulate for thin-layer chromatography in 1 mL of ethyl acetate, and use this solution as the standard solution. Perform the test with these solutions as directed under Thin-layer Chromatography *<2.03>*. Spot 30 μL of the sample solution and 5 μL of the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of hexane, acetone and acetic acid (100) (50:20:1) to a distance of about 7 cm, and air-dry the plate. Spray evenly a mixture of sulfuric acid and ethanol (99.5) (1:1), heat at 105℃ for 5 minutes, and examine under ultraviolet light (main wavelength: 365 nm): one of the several spots obtained from the sample solution has the same color tone and *R*f value with the light yellow-white to yellow fluorescent spot from the standard solution. (Brown Rice).

(5) To 2.0 g of dry extract (or 6.0 g of the viscous extract) add 10 mL of sodium hydroxide TS, shake, then add 5 mL of 1-butanol, shake, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 1 mg of ginsenoside Rb_1 for thin-layer chromatography in 1 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under Thin-layer Chromatography *<2.03>*. Spot 10 μL of the sample solution and 5 μL of the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, 1-propanol, water and acetic acid (100) (7:5:4:1) to a distance of about 7 cm, and air-

dry the plate. Spray evenly vanillin-sulfuric acid-ethanol TS for spraying on the plate, heat at 105℃ for 5 minutes, and allow to cool: one of the several spots obtained from the sample solution has the same color tone and *R*f value with the blue-purple to dark purple spot from the standard solution (Ginseng).

Purity **(1)** Heavy metals <*1.07*>—Prepare the test solution with 1.0 g of the dry extract (or an amount of the viscous extract, equivalent to 1.0 g of the dried substance) as directed under Extracts (4), and perform the test (not more than 30 ppm).

(2) Arsenic <*1.11*>—Prepare the test solution with 0.67 g of the dry extract (or an amount of the viscous extract, equivalent to 0.67 g of the dried substance) according to Method 3, and perform the test (not more than 3 ppm).

Loss on drying <*2.41*> The dry extract: Not more than 10.0% (1 g, 105℃, 5 hours).

The viscous extract: Not more than 66.7% (1 g, 105℃, 5 hours).

Total ash <*5.01*> Not more than 20.0%, calculated on the dried basis.

Assay **(1)** Mangiferin Weigh accurately about 0.5 g of the dry extract (or an amount of the viscous extract, equivalent to about 0.5 g of the dried substance), add exactly 50 mL of diluted methanol (1 in 2), shake for 15 minutes, centrifuge, and use the supernatant as the sample solution. Separately, weigh accurately about 10 mg of mangiferin for assay, dissolve in diluted methanol (1 in 2) to make exactly 200 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and standard solution as directed under Liquid Chromatography <*2.01*> according to the following conditions, and determine the peak areas, A_T and A_S, of mangiferin in each solution.

Amount (mg) of mangiferin $= M_S \times A_T / A_S \times 1/4$

M_S: Amount (mg) of mangiferin for assay taken, calculated on the basis of the content obtained by qNMR

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 367 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40℃.

Mobile phase: A mixture of water, acetonitrile and phosphoric acid (1780:220:1).

Flow rate: 1.0 mL per minute.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of mangiferin are not less than 5000 and not more than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of mangiferin is not more than 1.5%.

(2) Glycyrrhizic acid—Weigh accurately about 0.5 g of the dry extract (or an amount of the viscous extract, equivalent to about 0.5 g of dried substance), add exactly 50 mL of diluted methanol (1 in 2), shake for 15 minutes, filter, and use the filtrate as the sample solution. Separately, weigh accurately about 10 mg of Glycyrrhizic Acid RS (separately determine the water <*2.48*> by coulometric titration, using 10 mg), dissolve in diluted methanol (1 in 2) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and standard solution as directed under Liquid Chromatography <*2.01*> according to the following conditions, and determine the peak areas, A_T and A_S, of glycyrrhizic acid in each solution.

Amount (mg) of glycyrrhizic acid ($C_{42}H_{62}O_{16}$)
$= M_S \times A_T / A_S \times 1/2$

M_S: Amount (mg) of Glycyrrhizic Acid RS taken, calculated on the anhydrous basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40℃.

Mobile phase: Dissolve 3.85 g of ammonium acetate in 720 mL of water, and add 5 mL of acetic acid (100) and 280 mL of acetonitrile.

Flow rate: 1.0 mL per minute.

System suitability—

System performance: Dissolve 5 mg of monoammonium glycyrrhizinate for resolution check in 20 mL of dilute ethanol. When the procedure is run with 10 μL of this solution under the above operating conditions, the resolution between the peak having the relative retention time of about 0.9 to glycyrrhizic acid and the peak of glycyrrhizic acid is not less than 1.5.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above

operating conditions, the relative standard deviation of the peak area of glycyrrhizic acid is not more than 1.5%.

(3) Ginsenoside Rb_1—Weigh accurately about 1 g of the dry extract (or an amount of the viscous extract, equivalent to about 1 g of dried substance), add 25 mL of diluted methanol (3 in 5), shake for 30 minutes, then allow to stand, and separate the supernatant liquid. To the residue add 8 mL of water, shake for 15 minutes, then add 12 mL of methanol, shake for 15 minutes, centrifuge, and separate the supernatant liquid. Combine all the supernatant liquids, and add diluted methanol (3 in 5) to make exactly 50 mL. Pipet 10 mL of this solution, add 3 mL of sodium hydroxide TS, allow to stand for 30 minutes, then add 3 mL of 1 mol/L hydrochloric acid TS, and add water to make exactly 20 mL. Apply exactly 10 mL of this solution to a column [about 10 mm in inside diameter, packed with 0.36 g of octadecylsilanized silica gel for pre-treatment (55 – 105 μm in particle size), and washed just before using with methanol and then diluted methanol (3 in 10)], and wash the column in sequence with 2 mL of diluted methanol (3 in 10), 1 mL of sodium carbonate TS and 10 mL of diluted methanol (3 in 10). Finally, elute with methanol to collect exactly 5 mL, and use this solution as the sample solution. Separately, weigh accurately about 10 mg of Ginsenoside Rb_1 RS (separately determine the water *<2.48>* by coulometric titration, using 10 mg), and dissolve in methanol to make exactly 100 mL. Pipet 10 mL of this solution, add methanol to make exactly 50 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and standard solution as directed under Liquid Chromatography *<2.01>* according to the following conditions, and determine the peak areas, A_T and A_S, of ginsenoside Rb_1 in each solution.

$$\text{Amount (mg) of ginsenoside } Rb_1\ (C_{54}H_{92}O_{23}) = M_S \times A_T / A_S \times 1/10$$

M_S: Amount (mg) of Ginsenoside Rb_1 RS taken, calculated on the anhydrous basis

For the preparation 1)

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 203 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with carbamoyl group bound silica gel for liquid chromatography (3.5 μm in particle diameter).

Column temperature: A constant temperature of about 60℃.

Mobile phase: A mixture of acetonitrile, water and phosphoric acid (1700:300:1).

Flow rate: 1.0 mL per minute.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of ginsenoside Rb_1 are not less than 5000 and not more than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of ginsenoside Rb_1 is not more than 1.5%.

For the preparation 2)

Operating conditions—

Detector, column temperature, and flow rate: Proceed as directed in the operating conditions in the preparation 1).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with carbamoyl group bound silica gel for liquid chromatography (5 μm in particle diameter).

Mobile phase: A mixture of acetonitrile, water and phosphoric acid (400:100:1).

System suitability—

System performance and system repeatability: Proceed as directed in the system suitability in the preparation 1).

Containers and storage Containers—Tight containers.

Add the following to 9.41 Reagents, Test Solutions:

Anemarrhena Rhizome [Same as the namesake monograph]

Mangiferin for assay $C_{19}H_{18}NO_{11}$ Yellow crystals or crystalline powder. Practically insoluble in water and in ethanol (99.5). It is used after correction based on the content obtained in the Assay.

Identification—Proceed as directed in the Assay: it exhibits a multiplet signal equivalent to one proton around δ 3.15 ppm, a multiplet signal equivalent to one proton around δ 3.19 ppm, a multiplet signal equivalent to one proton around δ 3.22 ppm, a multiplet signal equivalent to one proton around δ 3.43 ppm, a doublet-like signal equivalent to one proton around δ 3.71 ppm, a triplet-like signal equivalent to one proton around δ 4.07 ppm, a doublet signal equivalent to one proton around δ 4.61 ppm, a singlet signal equivalent to one proton around δ 6.40 ppm, a singlet signal equivalent to one proton around δ 6.89 ppm and a singlet signal equivalent to one proton around δ 7.40 ppm.

Unity of peak—Dissolve 1 mg of mangiferin for assay in 20 mL of diluted methanol (1 in 2), and use this solution as the sample solution. Perform the test with 10 μL of the sample solution as directed under Liquid Chromatography *<2.01>* according to the following conditions, and compare the absorption spectra of at least 3 points including the top of mangiferin peak and around the two middle peak heights

of before and after the top: no difference in form is observed among their spectra.

Operating conditions

Column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay (1) under Byakkokaninjinto Extract.

Detector: A photodiode array detector (wavelength: 367 nm, measuring range of spectrum: 220 – 400 nm).

System suitability

System performance: When the procedure is run with 10 μL of the sample solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of mangiferin are not less than 5000 and not more than 1.5, respectively.

Assay—Weigh accurately 5 mg of mangiferin for assay and 1mg of DSS-d_6 for nuclear magnetic resonance spectroscopy using an ultramicrobalance, dissolve them in 1 mL of deuterated dimethylsulfoxide for nuclear magnetic resonance spectroscopy, and use this solution as the sample solution. Transfer the sample solution into an NMR tube 5 mm in outer diameter, measure ^{1}H-NMR as directed under Nuclear Magnetic Resonance Spectroscopy *<2.21>* and Crude Drugs Test *<5.01>* according to the following conditions, using DSS-d_6 for nuclear magnetic resonance spectroscopy as the internal reference compound. Calculate the resonance intensities, A_1 (equivalent to 1 proton), A_2 (equivalent to 1 proton) and A_3 (equivalent to 1 proton), of the signals around δ 6.40 ppm, δ 6.89 ppm and δ 7.40 ppm assuming the signal of the internal reference compound as δ 0 ppm.

Amount (%) of mangiferin ($C_{19}H_{18}O_{11}$)
$= M_S \times I \times P / (M \times N) \times 1.8824$

M: Amount (mg) of mangiferin for assay taken
M_S: Amount (mg) of DSS-d_6 for nuclear magnetic resonance spectroscopy taken
I: Sum of the signal resonance intensities, A_1, A_2 and A_3, based on the signal resonance intensity of DSS-d_6 for nuclear magnetic resonance spectroscopy as 9.000
N: Sum of the numbers of the proton derived from A_1, A_2 and A_3
P: Purity (%) of DSS-d_6 for nuclear magnetic resonance spectroscopy

Operating conditions

Apparatus: A nuclear magnetic resonance spectrometer having ^{1}H resonance frequency of not less than 400 MHz.

Target nucleus: ^{1}H.

Digital resolution: 0.25 Hz or lower.

Measuring spectrum range: 20 ppm or upper, including between −5 ppm and 15 ppm.

Spinning: off.

Pulse angle: 90°.

^{13}C decoupling: on.

Delay time: Repeating pulse waiting time not less than 60 seconds.

Integrating times: 8 or more times.

Dummy scanning: 2 or more times.

Measuring temperature: A constant temperature between 20℃ and 30℃.

System suitability

Test for required detectability: When the procedure is run with the sample solution under the above operating conditions, the SN ratio of the signal around δ 6.40 ppm, δ 6.89 ppm and δ 7.40 ppm are not less than 100.

System performance: When the procedure is run with the sample solution under the above operating conditions, the signals around δ 6.40 ppm, δ 6.89 ppm and δ 7.40 ppm are not overlapped with any signal of obvious foreign substances. Furthermore, the ratios of the resonance intensities, A_1/A_2, A_1/A_3 and A_2/A_3, are between 0.99 and 1.01.

System repeatability: When the test is repeated 6 times with the sample solution under the above operating conditions, the relative standard deviations of the ratios of the resonance intensities, A_1, A_2 and A_3 to that of the internal reference compound are not more than 1.0%.