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## Chotosan Extract

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Chotosan Extract contains not less than 24 mg and not more than 72 mg of hesperidin, not less than 8 mg and not more than 24 mg of glycyrrhizic acid ( $C_{42}H_{62}O_{16}$ : 822.93), and not less than 0.3 mg of the total alkaloid (rhyncophylline and hirsutine), per extract prepared with the amount specified in the Method of preparation.

### Method of preparation

	1)	2)
Uncaria Hook	3 g	3 g
Citrus Unshiu Peel	3 g	3 g
Pinellia Tuber	3 g	3 g
Ophiopogon Root	3 g	3 g
Poria Sclerotium	3 g	3 g
Ginseng	2 g	3 g
Saposhnikovia Root and Rhizome	2 g	3 g
Chrysanthemum Flower	2 g	3 g
Glycyrrhiza	1 g	1 g
Ginger	1 g	1 g
Gypsum	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** Chotosan Extract is a light brown to yellow-brown powder or blackish brown viscous extract. It has a slight odor, and has a pungent and slightly sweet first, then bitter taste.

**Identification (1)** Shake 2.0 g of a dry extract (or 6.0 g of the viscous extract) with 20 mL of water and 2 mL of ammonia TS, and then shake with 20 mL of diethyl ether, separate the diethyl ether layer, evaporate the layer under reduced pressure, add 1 mL of methanol to the residue, and use this solution as the sample solution. Separately, dissolve 1 mg each of rhyncophylline for thin-layer chromatography and hirsutine 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  $\mu$ L of the sample solution and 2  $\mu$ L of the standard solution on a plate of silica gel with fluorescent indicator 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 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): one of the spot among the several spots obtained from the sample solution has the same color tone and *R<sub>f</sub>* value with one of the two dark purple spots obtained from the standard solution (Uncaria Hook).

**(2)** Shake 2.0 g of a dry extract (or 6.0 g of the viscous extract) with 10 mL of water, add 10 mL of 1-butanol, and shake, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 1 mg of hesperidin 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 20  $\mu$ L of the sample solution and 10  $\mu$ L of the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, acetone, water and acetic acid (100) (10:6:3:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly 2,6-dibromo-*N*-chloro-1,4-benzoquinone monoimine TS on the plate, allow to stand in an ammonia gas: one of the spot among the several spots obtained from the sample solution has the same color tone and *R<sub>f</sub>* value with the blue spot obtained from the standard solution (Citrus Unshiu Peel).

**(3)** Shake 2.0 g of a dry extract (or 6.0 g of the viscous extract) with 10 mL of water, add 5 mL of 1-butanol and shake, centrifuge, remove the 1-butanol layer, and use the aqueous layer as the sample solution. Separately, heat 3.0 g of ophiopogon root in 50 mL of water under a reflux condenser for 1 hour. After cooling, shake 20 mL of the extract with 5 mL of 1-butanol, centrifuge, remove the 1-butanol layer, and use the aqueous layer as the standard solution. Perform the test with these solutions as directed under Thin-layer Chromatography <2.03>. Spot 2  $\mu$ L of the sample solution and 5  $\mu$ L of the standard solution as bands on original line on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethanol (99.5), water and acetic acid (100) (120:80:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly 4-methoxybenzaldehyde-sulfuric acid TS on the plate, heat at 105°C for 5 minutes: one of the spot among the several spots obtained from the sample solution has the same color tone and *R<sub>f</sub>* value with the dark blue-green spot (around *R<sub>f</sub>* value 0.3) obtained from the standard solution (Ophiopogon Root).

**(4)** Shake 2.0 g of a dry extract (or 6.0 g of the viscous extract) with 10 mL of sodium hydroxide TS, add 5 mL of 1-butanol, shake, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 1 mg of Ginseno-

side Rb<sub>1</sub> RS or ginsenoside Rb<sub>1</sub> 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  $\mu$ L of the sample solution and 2  $\mu$ L 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 10 cm, and air-dry the plate. Spray evenly vanillin-sulfuric acid TS on the plate, heat at 105°C for 5 minutes, and allow to cool: one of the spot among the several spots obtained from the sample solution has the same color tone and R<sub>f</sub> value with the purple spot obtained from the standard solution (Ginseng).

(5) Shake 2.0 g of a dry extract (or 6.0 g of the viscous extract) with 10 mL of sodium hydroxide TS, add 5 mL of 1-butanol, shake, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 1 mg of 4'-O-glycosyl-5-O-methylvisaminol 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 5  $\mu$ L each of the sample solution and standard solution on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of 1-butanol, water and acetic acid (100) (7:2:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): one of the spot among the several spots obtained from the sample solution has the same color tone and R<sub>f</sub> value with the blue spot obtained from the standard solution (Saposhnikovia Root and Rhizome).

(6) Shake 2.0 g of a dry extract (or 6.0 g of the viscous extract) with 10 mL of water, add 20 mL of diethyl ether, and shake. Separate the diethyl ether layer, evaporate the layer under reduced pressure, add 1 mL of methanol to the residue, and use this solution as the sample solution. Separately, dissolve 1 mg of luteolin 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  $\mu$ L of the sample solution and 3  $\mu$ L of the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, hexane and formic acid (5:5:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly iron (III) chloride-methanol TS on the plate: one of the spot among the several spots obtained from the sample solution has the same color tone and R<sub>f</sub> value with the yellow-brown spot obtained from the standard solution (Chrysanthemum Flower).

(7) Shake 2.0 g of a dry extract (or 6.0 g of the viscous extract) with 10 mL of water, 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 5  $\mu$ L 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 10 cm, and air-dry the plate. Spray evenly dilute sulfuric acid on the plate, heat at 105°C for 5 minutes: one of the spot among the several spots obtained from the sample solution has the same color tone and R<sub>f</sub> value with the yellow-brown spot obtained from the standard solution (Glycyrrhiza).

(8) Shake 1.0 g of a dry extract (or 3.0 g of the viscous

extract) with 10 mL of water, add 25 mL of diethyl ether, and shake. Separate the diethyl ether layer, evaporate the layer under reduced pressure, add 2 mL of diethyl ether to the residue, and use this solution as the sample solution. Separately, dissolve 1 mg of [6]-gingerol 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  $\mu$ L of the sample solution and 5  $\mu$ L of the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate and hexane (1:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly vanillin-sulfuric acid TS on the plate, heat at 105°C for 5 minutes: one of the spot among the several spots obtained from the sample solution has the same color tone and R<sub>f</sub> value with the red-purple spot obtained from the standard solution (Ginger).

(9) Shake 1.0 g of a dry extract (or 3.0 g of the viscous extract) with 30 mL of methanol, centrifuge, and separate the supernatant liquid. Shake the residue with 30 mL of water, centrifuge, and separate the supernatant liquid. Add ammonium oxalate TS to this solution: a white precipitate is formed, and it does not dissolve by addition of dilute acetic acid, but it dissolves by addition of dilute hydrochloric acid. (Gypsum)

**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 7.5% (1 g, 105°C, 5 hours).

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

**Total ash** <5.01> Not more than 15.0%, calculated on the dried basis.

**Assay (1)** Hesperidin—Weigh accurately about 0.1 g of the dry extract (or an amount of the viscous extract, equivalent to about 0.1 g of dried substance), add exactly 50 mL of diluted tetrahydrofuran (1 in 4), shake for 30 minutes, centrifuge, and use the supernatant liquid as the sample solution. Separately, weigh accurately about 10 mg of hesperidin for assay, previously dried in a desiccator (silica gel) for 24 hours, dissolve in methanol to make exactly 100 mL. Pipet 10 mL of this solution, add diluted tetrahydrofuran (1 in 4) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 10  $\mu$ L 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<sub>T</sub> and A<sub>S</sub>, of hesperidin in each solution.

$$\text{Amount (mg) of hesperidin} = M_S \times A_T/A_S \times 1/20$$

M<sub>S</sub>: Amount (mg) of hesperidin for assay taken

**Operating conditions—**

Detector: An ultraviolet absorption photometer (wavelength: 285 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  $\mu$ m in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of water, acetonitrile and acetic acid (100) (82:18:1).

Flow rate: 1.0 mL per minute (the retention time of hesperidin is about 15 minutes).

*System suitability—*

System performance: Dissolve 1 mg each of hesperidin for assay and naringin for thin-layer chromatography in diluted methanol (1 in 2) to make 100 mL. When the procedure is run with 10  $\mu$ L of this solution under the above operating conditions, naringin and hesperidin are eluted in this order with the resolution between these peaks being not less than 1.5.

System repeatability: When the test is repeated 6 times with 10  $\mu$ L of the standard solution under the above operating conditions, the relative standard deviation of the peak area of hesperidin 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  $\mu$ L 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.

$$\begin{aligned} &\text{Amount (mg) of glycyrrhizic acid (C}_{42}\text{H}_{62}\text{O}_{16}) \\ &= M_S \times A_T/A_S \times 1/2 \end{aligned}$$

$M_S$ : Amount (mg) of Glycyrrhizic Acid RS taken, calculated on the dried 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  $\mu$ m in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of diluted acetic acid (31) (1 in 15) and acetonitrile (13:7).

Flow rate: 1.0 mL per minute (the retention time of glycyrrhizic acid is about 12 minutes).

*System suitability—*

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

System repeatability: When the test is repeated 6 times with 10  $\mu$ L 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) Total alkaloid (rhyncophylline and hirsutine)—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 20 mL of diethyl ether, shake, add 3 mL of 1 mol/L hydrochloric acid TS and 7 mL of water, shake for 10 minutes, centrifuge, and separate the ether layer. To the aqueous layer add 20 mL of diethyl ether, and repeat the

above process. To the aqueous layer add 10 mL of sodium hydroxide TS and 20 mL of diethyl ether, shake for 10 minutes, centrifuge, and separate the supernatant liquid. Repeat the above process twice more with the residue using 20 mL portion of diethyl ether. Combine all supernatant liquids, evaporate to dryness under reduced pressure at not more than 40°C, and dissolve the residue in the mobile phase to make exactly 10 mL, and use this solution as the sample solution. Separately, weigh accurately about 5 mg of rhyncophylline for assay and about 5 mg of hirsutine for assay, and dissolve in a mixture of methanol and dilute acetic acid (7:3) to make exactly 100 mL. Pipet 10 mL of this solution, add a mixture of methanol and dilute acetic acid (7:3) to make exactly 50 mL, and use this solution as the standard solution. Perform the test with exactly 10  $\mu$ L 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 of rhyncophylline and hirsutine,  $A_{TR}$  and  $A_{TH}$ , and  $A_{SR}$  and  $A_{SH}$ , in each solution.

Amount (mg) of the total alkaloid (rhyncophylline and hirsutine)

$$= M_{SR} \times A_{TR}/A_{SR} \times 1/50 + M_{SH} \times A_{TH}/A_{SH} \times 1/50$$

$M_{SR}$ : Amount (mg) of rhyncophylline for assay taken

$M_{SH}$ : Amount (mg) of hirsutine for assay taken

*Operating conditions—*

Detector: An ultraviolet absorption photometer (wavelength: 245 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  $\mu$ m in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 5 g of sodium lauryl sulfate in 1150 mL of acetonitrile and 1350 mL of water, mix with 1 mL of phosphoric acid.

Flow rate: 1.0 mL per minute (the retention time of rhyncophylline is about 12 minutes and that of hirsutine is about 27 minutes).

*System suitability—*

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

System repeatability: When the test is repeated 6 times with 10  $\mu$ L of the standard solution under the above operating conditions, the relative standard deviations of the peak area of rhyncophylline and hirsutine are not more than 1.5%, respectively.

**Containers and storage** Containers—Tight containers.