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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 6 mg and not more than 18 mg of glycyrrhizic acid (C₄₂H₆₂O₁₆: 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 black-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 the dry extract (or 6.0 g of the viscous extract) with 20 mL of water and 2 mL of ammonia TS, add 20 mL of diethyl ether, and shake. Separate the diethyl ether layer, evaporate the solvent under low pressure (in vacuo), 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 µL of the sample solution and 2 µL 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 7 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): one of the several spots obtained from the sample solution has the same color tone and R_f value with one of the two dark purple spots

from the standard solution (*Uncaria Hook.*).

(2) Shake 2.0 g of the 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 1-butanol layer 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 μL of the sample solution and 10 μL 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 7 cm, and air-dry the plate. Spray evenly 2,6-dibromo-*N*-chloro-1,4-benzoquinone monoimine TS on the plate, and allow to stand in an ammonia gas: one of the several spots obtained from the sample solution has the same color tone and *R_f* value with the blue spot from the standard solution (*Citrus Unshiu Peel*).

(3) Shake 2.0 g of the dry extract (or 6.0 g of the viscous extract) with 10 mL of water, add 5 mL of 1-butanol, shake, centrifuge, remove the 1-butanol layer, and use the aqueous layer as the sample solution. Separately, heat 3.0 g of pulverized 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 μL of the sample solution and 5 μL of the standard solution as bands on the 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 7 cm, and air-dry the plate. Spray evenly 4-methoxybenzaldehyde-sulfuric acid TS on the plate, and heat the plate at 105°C for 5 minutes: one of the several spots obtained from the sample solution has the same color tone and *R_f* value with the dark blue-green spot (around *R_f* value 0.3) from the standard solution (*Ophiopogon Root*).

(4) Shake 2.0 g of the 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 1-butanol layer as the sample solution. Separately, dissolve 1 mg of Ginsenoside *R_{b1}*, *RS* or ginsenoside *R_{b1}* 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 2 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 the plate at 105°C 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 spot from the standard solution (*Ginseng*).

(5) Shake 2.0 g of the 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 1-butanol layer as the sample solution. Separately, dissolve 1 mg of 4'-*O*-glycosyl-5-*O*-methylvisamminol 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 μL 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 7 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): one of the several spots obtained from the sample solution has the same color tone and *R_f* value with the blue spot from the standard solution (*Saposhnikovia Root* and *Rhizome*).

(6) Shake 2.0 g of the 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 solvent under low pressure (in vacuo), 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 μL of the sample solution and 3 μL 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 7 cm, and air-dry the plate. Spray evenly iron (III) chloride-methanol TS on the plate: one of the several spots obtained from the sample solution has the same color tone and *R_f* value with the yellow-brown spot from the standard solution (*Chrysanthemum Flower*).

(7) Shake 2.0 g of the 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 1-butanol layer 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 the plate at 105°C 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*).

(8) Shake 1.0 g of the 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 solvent under low pressure (in vacuo), 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 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 and hexane (1:1) to a distance of about 7 cm, and air-dry the plate. Spray evenly vanillin-sulfuric acid TS on the plate, heat the plate at 105°C for 5 minutes: one of the several spots obtained from the sample solution has the same color tone and *R_f* value with the red-purple spot 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 μ 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 hesperidin in each solution.

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

M_S : 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 octadecylsilylated silica gel for liquid chromatography (5 μ 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 μ 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 μ 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 the dried substance), add 20 mL of ethyl acetate and 10 mL of water, and shake for 10 minutes. After centrifugation, remove the ethyl acetate layer, add 20 mL of ethyl acetate, proceed in the same manner as described above, and remove the ethyl acetate layer. To the resultant aqueous layer add 10 mL of methanol, shake for 30

minutes, centrifuge, and take the supernatant liquid. To the residue add 20 mL of diluted methanol (1 in 2), shake for 5 minutes, centrifuge, and take the supernatant liquid. Combine these supernatant liquids, add diluted methanol (1 in 2) to make exactly 50 mL, and use this solution 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 μ 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 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 octadecylsilylated silica gel for liquid chromatography (5 μ m in particle diameter).

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

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 (the retention time of glycyrrhizic acid is about 15 minutes).

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 μ L 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 μ 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 (rhynchophylline 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 remove the diethyl 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 diethyl ether layer. To the residue add 20 mL of diethyl ether, proceed in the same manner, and repeat the procedure twice. Combine all the extracts, evaporate the solvent under low pressure (in vacuo) 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 rhynchophylline 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 μ 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 μ 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 μ 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 μ 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.

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