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(54) Title: CITRUS PEEL EXTRACT AS 3-HYDROXY-3-METHYLGLUTARYL COA(HMG-COA) REDUCTASE INHIBITOR

(57) Abrégé/Abstract:
A pharmaceutical composition for inhibiting the activity of 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase in mammals comprises an effective amount of citrus peel extract as an active ingredient together with a pharmaceutically acceptable carrier, and a food or beverage composition for inhibiting the 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase activity comprises an effective amount of citrus peel extract.

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(57) Abstract

A pharmaceutical composition for inhibiting the activity of 3–hydroxy–3–methylglutaryl CoA(HMG–CoA) reductase in mammals comprises an effective amount of citrus peel extract as an active ingredient together with a pharmaceutically acceptable carrier, and a food or beverage composition for inhibiting the 3–hydroxy–3–methylglutaryl CoA(HMG–CoA) reductase activity comprises an effective amount of citrus peel extract.

CITRUS PEEL EXTRACT AS 3-HYDROXY-3-METHYLGLUTARYL COA(HMG-COA) REDUCTASE INHIBITOR

FIELD OF THE INVENTION

The present invention relates to a pharmaceutical composition for inhibiting the activity of 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase in a mammal, which comprises an effective amount of citrus peel extract as an active ingredient together with a pharmaceutically acceptable carrier, and a food or beverage composition for inhibiting the HMG-CoA reductase activity, which comprises an effective amount of citrus peel extract.

BACKGROUND OF THE INVENTION

In recent years, coronary cardio-circulary diseases, e.g., atherosclerosis and hypercholesterolemia, have increasingly become a major cause of deaths. It has been reported that an elevated plasma cholesterol level causes the deposition of fat, macrophages and foam cells on the wall of blood vessels, such deposit leading to plaque formation and then to atherosclerosis(Ross, R., Nature, 362, 801-809(1993)). One of the methods for decreasing the plasma cholesterol level is alimentotherapy to reduce the ingestion of cholesterol and lipids. Another method is to lower the rate of cholesterol biosynthesis which takes place in the liver. It has been reported that hypercholesterolemia can be treated effectively by reducing the rate of cholesterol biosynthesis through the inhibition of HMG-CoA reductase which mediates the synthesis of mevalonic acid, an intermediate in the biosynthesis of sterols or isoprenoids(Cardiovascular Pharmacology, William W. Parmley and Kanu Chatterjee Ed., Wolfe Publishing, pages 8.6-8.7, 1994).

Therefore, numerous efforts have been made to develop

medicines to inhibit HMG-CoA reductase; and, as a result, several compounds derived from Penicillium sp. and Aspergillus sp. have been commercialized. Specifically, Lovastatin® and Simvastatin® developed by Merck Co., U.S.A., and Pravastatin® developed by Sankyo Co., Japan, have been commercialized(C.D.R. Dunn, Stroke: Trends, Treatment and Markets, SCRIPT Report, PJB Publications Ltd., 1995). However, these medicines are very expensive and a long-term administration thereof is known to induce an adverse side effect of increasing creatine kinase in the liver. Accordingly, there has continued to exist a need to develop an inexpensive and non-toxic inhibitor of HMG-CoA reductase.

Citrus peel has been used as a digestion improving agent. However, there has been no report on the HMG-CoA reductase inhibitory activity of the citrus peel extract.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a pharmaceutical composition for inhibiting the HMG-CoA reductase activity in mammals.

It is another object of the present invention to provide a food or beverage composition for inhibiting the HMG-CoA reductase activity in mammals.

In accordance with one aspect of the present invention, there is provided a pharmaceutical composition for inhibiting the activity of 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase in mammal, which comprises an effective amount of citrus peel extract as an active ingredient together with a pharmaceutically acceptable carrier.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a pharmaceutical

composition for inhibiting the HMG-CoA reductase activity, which comprises citrus peel extract as an active ingredient, in combination with pharmaceutically acceptable excipients, carriers or diluents.

The citrus may be tangerins, oranges, lemons, grapefruits and Poncirus trifoliata.

The citrus peel extracts may be prepared by any of the conventional methods using alcohols or water. For instance, 5 to 100 ℓ alcohol or water is added to 1 kg of dried citrus peel and the mixture is allowed to stand at a temperature ranging from 5 to 80 °C for alcohol or from 20 to 140 °C for water for a period ranging from 10 min. to 48 hours. This extraction process may be repeated 1 to 3 times. The resulting extract is concentrated, e.g., by vacuum, to obtain a concentrated peel extract. Further, it can be prepared by treating citrus peels with calcium hydroxide solution, adding hydrochloric acid thereto to adjust solution to a pH ranging from 4.0 to 4.5, and then centrifuging the resulting solution to obtain the precipitates, flavonoides, as citrus peel extract(FDA Report No. FDA-BF-82/62, "Evaluation of health aspects of hesperidin, naringin and citrus bioflavonoide extracts as food ingredient", Natl. Technical Information Service PB 82-192931(1982)).

The citrus peel extract exerts an inhibitory effect on the HMG-CoA reductase at a dose of 10 mg/kg/day or more, the inhibitory effect increasing with the dose thereof.

Moreover, in spite of their potent efficacies, the citrus peel extract shows little toxicity or mitogenicity in tests using mice. More specifically, the citrus peel extract exhibits no toxicity when it is orally administered to a mouse at a dosage of 1,000 mg/kg, which corresponds to oral administration dose of 50 to 100 g/kg body weight of the citrus peel extract for a person weighing 50 kg. Further, the citrus peel extract exerts no adverse effects on the liver function.

A pharmaceutical formulation may be prepared by using the composition in accordance with any of the conventional procedures. In preparing the formulation, the active ingredient is preferably admixed or diluted with a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet or other container. When the carrier serves as a diluent, it may be a solid, semi-solid or liquid material acting as a vehicle, excipient or medium for the active ingredient. Thus, the formulations may be in the form of a tablet, pill, powder, sachet, elixir, suspension, emulsion, solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged powder and the like.

Examples of suitable carriers, excipients, and diluents are lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, alginates, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoates, propylhydroxybenzoates, talc, magnesium stearate and mineral oil. The formulations may additionally include fillers, anti-agglutinating agents, lubricating agents, wetting agents, flavoring agents, emulsifiers, preservatives and the like. The compositions of the invention may be formulated so as to provide quick, sustained or delayed release of the active ingredient after their administration to a mammal by employing any of the procedures well known in the art.

The pharmaceutical formulation of the present invention can be administered via various routes including oral, transdermal, subcutaneous, intravenous and intramuscular introduction. In case of human, a typical daily dose of citrus peel extract may range from about 10 to 500 mg/kg body weight, preferably 20 to 100 mg/kg body weight, and can be administered in a single dose or in divided doses. However, it should be understood that the amount of the active ingredient actually administered ought to be

determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's symptom; and, therefore, the above dose should not be intended to limit the scope of the invention in any way.

Moreover, the citrus peel extract can be incorporated in foods or beverages for the purpose of inhibiting the HMG-CoA reductase activity. Accordingly, the present invention also provide a food or beverage composition for inhibiting the HMG-CoA reductase activity, which comprises an effective amount of citrus peel extract. In this case, the content of the citrus peel extract in a food or beverage may range from 0.1 to 50%.

As described above, the citrus peel extract can be used as an effective, non-toxic pharmaceutical agent for inhibiting HMG-CoA reductase activity.

The following Examples are intended to further illustrate the present invention without limiting its scope.

Further, percentages given below for solid in solid mixture, liquid in liquid, and solid in liquid are on a wt/wt, vol/vol and wt/vol basis, respectively, and all the reactions were carried out at room temperature, unless specifically indicated otherwise.

Example 1: Preparation and Analysis of Citrus Peel Extract

Citrus peels were dried at room temperature and to 6.7kg of the dried peels was added 80 ℓ of 95% ethanol. The mixture was allowed to stand at room temperature for 24 hours and filtered to obtain an extract and a solid residue. The solid residue was extracted one more time by the same procedure. The extract thus obtained were combined, concentrated under a reduced pressure using a large capacity evaporator(EYELA Rotary vacuum evaporator N-11) to obtain

1.7 kg of concentrated extract(d=1.3g/ml).

The concentrated extract was diluted in DMSO/methanol(1:1) mixture at a concentration of 10 mg/ml. 100μl of the resulting solution was subjected to high performance liquid chromatography(HPLC) using Phenomenex Prodigy column(5μ ODS(3) 100Å, 4.6x250mm) which was pre-equilibrated with 0.01M phosphoric acid/methanol(70:30) mixture. The sample was eluted with 0.01M phosphoric acid/methanol(70:30) mixture for 55 min. with increasing gradually the concentration of methanol to 45% at a flow rate of 0.6 ml/min. 100μl(1mg/ml) of hesperidin and naringin(Sigma Chemical Co., USA) were used as standard materials. The eluates were detected at 280nm and it was discovered that 1kg of the citrus peel extract contains 5,950mg of hesperidin and 280mg of naringin.

Further, ingredients of the citrus peel extract were identified in accordance with a conventional method. For instance, moisture content was determined by dry method at 105°C; crude protein, by Kjeldahl method; crude lipid, by Soxhlet method; free saccharide, by Bertrand method; crude ash, by ashing at 550-600°C; and hesperidin and naringin, by HPLC method. The result is shown in Table I.

Table I

Ingredient		Content(%)
Moisture		39.1
Crude protein		2.7
Crude lipid		1.8
Free saccharide	Fructose	20.0
	Glucose	16.5
	Sucrose	8.6
Crude ash		1.0
Hesperidin		0.6
Naringin		0.03
Other saccharides		9.67

Example 2: Administration of Citrus Peel Extract to an Animal

20 four-week-old Sprague-Dawley rats(Taihan laboratory animal center, Korea) each weighing about 90 to 110 g were evenly divided into two dietary groups by a randomized block design. The rats of the two groups were fed with two different high-cholesterol diets, i.e., AIN-76 laboratory animal diet(ICN Biochemicals, Cleveland, OH, U.S.A.) containing 1% cholesterol(Control group), and 1% cholesterol plus 16.7% citrus peel extract, respectively. The compositions of diets fed to the two groups are shown in Table II.

Table II

Dietary group Component	Control group	Citrus peel extract*2 group
Casein	20	20
D,L-methionine	0.3	0.3
Corn starch	15	15
Sucrose	49	32.3
Cellulose powder*1	5	5
Mineral mixture*1	3.5	3.5
Vitamin mixture*1	1	1
Choline citrate	0.2	0.2
Corn oil	5	5
Cholesterol	1	1
Citrus peel extract		16.7
Total	100	100

*1 Purchased from TEKLAD premier Co.(Madison, WI, U.S.A.)
*2 0.1% hesperidin equivalent

The rats were allowed to feed freely on the specified diet together with water for six weeks, the ingestion amount

was recorded daily and the rats were weighed every 7 days, and then the record was analyzed. All rats showed a normal growth rate and there was observed no significant difference among the two groups in terms of the feed ingestion amount and the weight gain.

Example 3: Determination of Total Cholesterol, HDL-cholesterol and Neutral Lipid Content in plamsa

The effect of administering citrus peel extract to rats on the plasma cholesterol and neutral lipid content was determined as follows.

Blood samples were taken from the rats of the two dietary groups and plasma HDL fractions were separated therefrom by using HDL-cholesterol reagent(Sigma Chemical Co., Cat. No. 352-3) containing dextran-sulfate. Total cholesterol and HDL-cholesterol levels were determined by using Sigma Diagnostic Kit Cat. No. 352-100(Sigma Chemical Co., U.S.A.)(Allain et al., Clin. Chem., 20, 470-475(1974)). Neutral lipids level was determined by using Sigma Diagnostic Kit Cat. No. 339-50(Bucolo, G. and David, H., Clin. Chem., 19, 476-482(1973)). The result is shown in Table III, wherein the total plasma cholesterol level decreased by 35% in the citrus peel extract-fed rat group, as compared with that of the control group.

Table III

Group Lipids Conc.	Control group	Citrus peel extract group
Total-C (mg/dl)	147.8±34.8	94.2±23
HDL-C (mg/dl)	22.2	23.5
$\frac{\text{HDL-C}}{\text{Total-C}}$ (%)	15.7±5.3	26.2±7.5
TG (mg/dl)	99.2±18.9	108.5±15.9
Atherosclerosis index	6.3±3.4	3.1±1.2

* Total-C: Total-cholesterol
* HDL-C: HDL-cholesterol
* TG: Triglyceride

Example 4: Activity of Citrus Peel Extract in HMG-CoA Reductase Inhibition

(Step 1) Preparation of microsomes

To determine the effect of citrus peel extract feeding to rats on the activity of HMG-CoA reductase, a regulatory enzyme of the cholesterol synthesis in the liver, microsomes were separated from the liver tissue to be used as an enzyme source.

First, the rats of the two groups were sacrificed by decapitation and the livers were excised and immediately placed in an ice-cold homogenization medium(50 mM KH_2PO_4(pH 7.0), 0.2M sucrose, 2 mM dithiothreitol(DTT)). The livers were homogenized in the homogenization medium(2 ml medium/g of the liver) with a Waring blendor for 15 sec.(three strokes with a motor-driven Teflon pestle in a Potter-Elvehjem type glass homogenizer). The homogenate was centrifuged at 15,000xg for 10 min. and the supernatant thus obtained was centrifuged at 100,000xg for 75 min. to obtain microsomal pellets, which were then resuspended in the

homogenization medium containing 50 mM EDTA and centrifuged at 100,000xg for 60 min. The supernatant containing the microsome was used as an enzyme source.

(Step 2) HMG-CoA reductase assay

The activity of HMG-CoA reductase was determined by employing [^{14}C]HMG-CoA, in accordance with the method of Shapiro et al.(Biochemica et Biophysica Acta, 370, 369-377(1974)) as follows.

The enzyme in the supernatant containing the microsome obtained in (Step 1) was activated at 37°C for 30 min. Added to a reaction tube were 20 μl of HMG-CoA reductase assay buffer(0.25M KH_2PO_4(pH 7.0), 8.75 mM EDTA, 25 mM DTT, 0.45 M KCl and 0.25 mg/ml BSA), 5 μl of 50 mM NADPH, 5 μl of [^{14}C]HMG-CoA(0.05 μCi/tube, final conc. 120 μM), and 10 μl of activated microsomal enzyme(0.03-0.04 mg), and the mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 10 μl of 6 M HCl to the mixture, and the mixture was incubated at 37°C for 15 min. to allow complete lactonization of the product(mevalonate). The precipitate was removed by centrifugation at 10,000xg for 1 min. and the supernatant was applied to a Silica gel 60G TLC plate(Altech, Inc., Newark, U.S.A.) and then developed with benzene:acetone(1:1, v/v). A region having a Rf value ranging from 0.65 to 0.75 was removed by scraping with a disposable cover slips and assayed for radioactivity with 1450 Microbeta liquid scintillation counter(Wallacoy, Finland). Enzyme activities were calculated as picomoles mevalonic acid synthesized per min. per mg protein(pmoles/min/mg protein). The result is shown in Table IV.

Table IV

Group	Control group	Citrus peel extract group
HMG-CoA reductase activity (pmole/min/mg protein)	147 ±12.5	112.1 ±12.8

As can be seen from Table IV, the control group rats showed a relatively high HMG-CoA reductase activity, while the HMG-CoA activity observed with the citrus peel extract-fed rat group is lower than that of the control group by 34%.

Example 5: Toxicity of Orally Administered Citrus Peel Extract

7 to 8 week-old, specific pathogen-free ICR female mice(8 heads) each weighing about 25 to 29 g and male mice(8 heads) each weighing about 34 to 38 g were bred under a condition of temperature 22±1°C, moisture 55±5% and photoperiod 12L/12D. Fodder(Cheiljedang Co., mouse and rat fodder) and water were sterilized and fed to the mice.

The citrus peel extract was dissolved in 0.5% Tween 80 to a concentration of 100 mg/ml, and the solution was orally administered to the mice in an amount of 0.2 ml per 20 g of mouse body weight. The solution was administered once and the mice were observed for 10 days for signs of adverse effects or death according to the following schedule: 1, 4, 8, and 12 hours after the administration and, every 12 hours thereafter. The weight changes of the mice were recorded every day to examine the effect of citrus peel extract. Further, on the 10th day, the mice were sacrificed and the internal organs were visually examined.

All the mice were alive at day 10 and citrus peel extract showed no toxicity at a dose of 1,000 mg/kg. The

autopsy revealed that the mice did not develop any pathological abnormality, and no weight loss was observed during the 10 day test period. Accordingly, it was concluded that the citrus peel extract is not toxic when orally administered to an animal.

The following Formulation Example is for illustration only and not intended to limit the scope of the invention in any way.

Formulation Example

Hard gelatin capsules were prepared using the following ingredients:

	Quantity (mg/capsule)
Active ingredient(Citrus peel extract)	20
Starch, dried	160
Magnesium stearate	20
Total	200 mg

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

What is claimed is:

1. A pharmaceutical composition for inhibiting the activity of 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase in a mammal, which comprises an effective amount of citrus peel extract together with a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein the mammal is human.

3. The pharmaceutical composition of claim 1, wherein the citrus is tangerins, oranges, lemons, grapefruits and Poncirus trifoliata.

4. The pharmaceutical composition of claim 2, which the effective amount of citrus peel extract ranges from 10 to 500 mg/kg body weight/day.

5. A food composition for inhibiting the 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase activity, which comprises an effective amount of citrus peel extract.

6. A beverage composition for inhibiting the 3-hydroxy-3-methylglutaryl CoA(HMG-CoA) reductase activity, which comprises an effective amount of citrus peel extract.