

USE OF WULING FORMULATION IN THE PREPARATION OF DRUG FOR PREVENTING OR TREATING NON-ALCOHOLIC FATTY LIVER DISEASE

Cross-reference to Related Applications

The present application claims the benefit of priorities of CN Patent Application No. 202310919939.7 entitle “Use of Wuling Formulation in the Preparation of Drug for Preventing or Treating Non-Alcoholic Fatty Liver Disease” filed on 25 July 2023 and CN Patent Application No. 202410263883.9 entitled “Use of Wuling Formulation in the Preparation of Drug for Preventing Or Treating Non-Alcoholic Fatty Liver Disease” filed on 7 March 2024, the contents of which are hereby incorporated by reference in their entirety for all purposes.

Field of the Invention

The present invention relates to the field of traditional Chinese medicines, in particular to use of Wuling formulation in the preparing a medicament for preventing or treating non-alcoholic fatty liver disease.

Background of the Invention

Fatty liver is characterized by excessive deposition of lipids in hepatocytes. When lipid accumulation in hepatocytes exceeds 5% of liver wet weight, or histologically when steatosis is observed in more than one-third of hepatocytes per unit area, it is referred to as fatty liver disease. Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome caused by factors other than alcohol and other definite liver-damaging factors, primarily characterized by diffuse hepatic macrovesicular steatosis. The accumulation of lipids in the liver is the main cause of NAFLD, with pathological manifestations including vacuolar steatosis of hepatocytes, inflammation within hepatic lobules, hepatocyte necrosis and balloon-like degeneration, and the like. With the significant rise in the global incidence of obesity and metabolic syndrome, NAFLD has now become the most common chronic metabolic liver disease worldwide and is currently the leading chronic liver disease in China.

Non-alcoholic fatty liver disease is one of the most common precursors to chronic liver

diseases (such as liver fibrosis and hepatocellular carcinoma) and metabolic disorders (such as obesity, type II diabetes, and atherosclerosis). Patients with NAFLD cannot recover from this condition; instead, the disease progresses to non-alcoholic steatohepatitis (NASH), hepatic fibrosis and cirrhosis. Moreover, as a significant member of metabolic syndrome, it is closely associated with cardiovascular and cerebrovascular diseases. This severely impacts people's physical health and quality of life, and also places a heavy burden on society.

Currently, the treatment for fatty liver disease primarily involves eliminating causative factors, aggressive treatment of underlying diseases, and adherence to a balanced diet, while medicament serves only an auxiliary role. At present, the drugs clinically used for treating fatty liver disease are mainly categorized into lipid-lowering agents, hepatoprotective and lipid-modifying agents and traditional Chinese herbal medicines. However, the traditional Chinese herbal medicines often have complex components and intricate manufacturing processes.

Summary of the Invention

The present application provides a use of a Wuling formulation in the preparation of a medicament for preventing or treating non-alcoholic fatty liver disease.

In the first aspect of the present invention, it provides the use of a Wuling formulation in the preparation of a medicament for preventing or treating non-alcoholic fatty liver disease.

In some embodiments, the Wuling formulation comprises, in parts by weight: 200 to 400 parts of *Bupleuri radix* (the root of *Bupleurum* species), 100 to 200 parts of *Ganoderma* (the sporocarp of *Ganoderma lucidum* (Leyss. ex Fr.) Karst. or *Ganoderma sinense* Zhao, Xu et Zhang), 200 to 350 parts of *Salviae miltiorrhizae radix et rhizome* (the root and rhizome of *Salviae miltiorrhiza* Bge.) and 250 to 350 parts of *Schisandrae fructus* (the fruit of *Schisandra chinensis* (Turcz.) Baill or *Schisandra sphenanthera* Rehd. et Wils.).

In some embodiments, the Wuling formulation comprises, in parts by weight: 300 to 350 parts of *Bupleuri radix*, 150 to 180 parts of *Ganoderma*, 300 to 350 parts of *Salviae*

miltiorrhizae radix et rhizome and 300 to 350 parts of Schisandrae fructus.

In some embodiments, the Wuling formulation comprises, in parts by weight: 342 parts of Bupleuri radix, 173 parts of Ganoderma, 342 parts of Salviae miltiorrhizae radix et rhizome and 342 parts of Schisandrae fructus.

In some embodiments, the Wuling formulation is prepared in a dosage form selected from the group consisting of a decoction, a pill, an oral liquid, a tablet, a capsule, a granule, a medicated electuary (i.e., Herbal Paste for oral use), and a powder.

In some embodiments, the Wuling formulation is selected from any one of Wuling Capsule, Wuling Pill and Wuling Powder.

In some embodiments, the Wuling formulation is administered to an adult in a unit dose of 3 g to 6 g, three times per day.

The effective components of the Wuling formulation comprise Bupleuri radix, Ganoderma, Salviae miltiorrhizae radix et rhizome and Schisandrae fructus. Despite its simple composition, the four medicinal components act synergistically to exert a therapeutic action in the prevention or treatment of non-alcoholic fatty liver disease (NAFLD).

Brief Description of Drawings

In order to more clearly illustrate the technical solutions of the embodiments of the present application, the figures required for describing the embodiments will be briefly introduced below. It is obvious that the figures described below relate to only some embodiments of the present application. Those skilled in the art will arrive at other figures according to these figures without any creative efforts.

FIG. 1 shows representative images of liver fat staining intensity in zebrafish treated with Wuling Capsule according to Example 1 of the present application, wherein the area within the yellow dashed line represents the liver.

FIG. 2 shows representative images of the liver tissue structure in zebrafish treated with Wuling Capsule according to Example 1 of the present application, wherein the area within the yellow dashed line indicates the analyzed zebrafish liver region, the red arrow points to hepatocellular swelling, and the black arrow points to fatty vacuolar degeneration.

Detailed Description of the Invention

The following examples are given to further describe the embodiments of the present application in detail. The detailed description of the following examples is intended to illustrate the principles of the present invention, but should not be construed as limiting the scope of the present application, i.e., the application is not limited to the examples described.

As analyzed in the section of Background of the present disclosure, although there have been some compositions of traditional Chinese herbal medicine for treating non-alcoholic fatty liver disease in the prior art, their components are complex. In addition, the applicant has conducted research on the medicines currently used in clinical for treating chronic hepatitis, hepatitis B, cirrhosis, hepatitis B-related liver fibrosis, compensatory cirrhosis and the like, and found that the medicines effective for the aforementioned diseases do not provide guidance for treating non-alcoholic fatty liver disease. For example, Fufang Biejia Ruangan Tablets have shown remarkable therapeutic effects for the conditions mentioned above, but exhibit no efficacy against non-alcoholic fatty liver disease.

In one embodiment of the present application, a use of a Wuling formulation in the preparation of a medicament for preventing or treating non-alcoholic fatty liver disease is provided.

The effective components of the Wuling formulation comprise Bupleuri radix, Ganoderma, Salviae miltiorrhizae radix et rhizome and Schisandrae fructus. Despite its simple composition, the four medicinal components act synergistically to exert a therapeutic action in the prevention or treatment of non-alcoholic fatty liver disease.

Bupleuri radix, with its pungent dispersing and bitter draining properties, effectively

soothes liver qi and relieves liver stagnation, serving as the sovereign (or “monarch”) drug in the composition. *Ganoderma*, is sweet in flavor and neutral in nature, has the effects of tonifying qi and strengthening the spleen, and reinforces healthy qi and supports the body’s upright energy; *Salviae miltiorrhizae radix et rhizome* invigorates blood circulation, resolves stasis, unblocks meridians, and alleviates pain; the two ones act together as the minister (or “assistant”) drug. *Schisandrae fructus* is sour-sweet in flavor and warm in nature, tonifies qi and nourishes yin. It can assist *Ganoderma* in supplementing qi and fortifying the middle jiao (spleen and stomach), at the same time can calm the mind, eliminate depression, and further prevent *Bupleuri radix*’s dispersing and ascending action from excessively consuming qi and damaging yin. It also protects the liver and lowers transaminase. Therefore, *Schisandrae fructus* fulfills the role of an adjuvant and modulator (or “mediating /regulating” drug). When the four herbs are used in combination, they simultaneously regulate the liver and spleen, harmonize qi and blood, and balance consolidating and dispersing actions. Together, they work synergistically to soothe the liver and relieve stagnation, tonify qi and strengthen the spleen, and activate blood circulation to resolve stasis. Their combination can significantly improve symptoms in patients with non-alcoholic fatty liver disease, such as pain in the liver region, abdominal fullness and discomfort, fatigue and weakness, and poor appetite, while also lowering abnormal blood biochemical markers.

To further enhance the synergistic effect of the four herbal medicines, in some embodiments of the present application, the Wuling formulation preferably comprises, in parts by weight: 200 to 400 parts of *Bupleuri radix*, 100 to 200 parts of *Ganoderma*, 200 to 350 parts of *Salviae miltiorrhizae radix et rhizome* and 250 to 350 parts of *Schisandrae fructus*. More preferably, the Wuling formulation comprises, in parts by weight: 300 to 350 parts of *Bupleuri radix*, 150 to 180 parts of *Ganoderma*, 300 to 350 parts of *Salviae miltiorrhizae radix et rhizome* and 300 to 350 parts of *Schisandrae fructus*. Further preferably, the Wuling formulation comprises, in parts by weight: 342 parts of *Bupleuri radix*, 173 parts of *Ganoderma*, 342 parts of *Salviae miltiorrhizae radix et rhizome* and 342 parts of *Schisandrae fructus*.

The Wuling formulation of the present application may be used in any conventional dosage

form. In some embodiments, the Wuling formulation is prepared in a dosage form selected from the group consisting of a decoction, a pill, an oral liquid, a tablet, a capsule, a granule, a medicated electuary (i.e., Herbal Paste for oral use), and a powder. The preparation processes for the aforementioned dosage forms are well established, facilitating the application of the Wuling formulation in preparing a medicament for preventing or treating the non-alcoholic fatty liver disease.

In some embodiments, the Wuling formulation is selected from any one of Wuling Capsule, Wuling Pill and Wuling Powder. The aforementioned formulations are all official preparations, which has advantage in the implementation of the application described herein.

Through repeated experimental verification, the inventor of the present application has derived the following formula. Using the effective safe concentration for zebrafish described in this application, the equivalent theoretical human dose can be calculated by the following formula:

$$\text{human dose (g/time)} = [\text{concentration for zebrafish } (\mu\text{g/mL}) \times 6/100] \times 2$$

Based on the above conversion, in some embodiments, the Wuling formulation is administered to an adult in a unit dose of 3 g to 6 g, three times per day. The aforementioned dosage regimen demonstrates significant therapeutic efficacy against non-alcoholic fatty liver disease and exhibits minimal side effects in humans.

The beneficial effects of the present application will be further illustrated below in conjunction with examples and comparative examples. The scope of the present invention, however, is not limited to these examples.

Example 1

The Wuling capsules used were provided by Tsing Hua De Ren Xingfu Pharmaceutical Co., Ltd..

1. Principle and methods of experiment

Experimental Principle: Zebrafish exhibits high similarity to human in terms of liver structure, function and genetics. Except for immune-related Kupffer cells, zebrafish possesses all other cell types found in the human liver and perform the same functions which include bile secretion, glycogen and lipid storage, insulin response, xenobiotic and ammonia metabolism, as well as the secretion of serum proteins such as complement and coagulation factors, transferrin and albumin-like proteins. Within five days post-fertilization, the zebrafish liver becomes fully functional, allowing for comprehensive liver function assessments, which makes zebrafish an important model for studying liver diseases. Zebrafish is fed with a high-sugar and high-fat diet providing energy exceeding the fish needs. The surplus energy is stored as fat in the liver, leading to a continuous increase in hepatic lipid content, so that the fat content in the liver is continuously increased, which induces hepatocyte steatosis and ultimately results in fatty liver disease.

Sample preparation information:

Wuling Capsule, solvent: standard dilution water.

Positive Control: Atorvastatin Calcium Tablets (hereinafter referred to as Atorvastatin Calcium), white tablets, batch No. FR7909, Pfizer Pharmaceuticals Ltd., solvent: DMSO.

Experimental Animals:

Zebrafish were all reared in fish culture water at 28°C (water quality: 200 mg instant sea salt per 1 L of reverse osmosis water, conductivity 450-550 $\mu\text{S}/\text{cm}$, pH 6.5-8.5, hardness 50-100 mg/L CaCO_3). The zebrafish were bred and provided by the company's aquaculture center. The experimental animal use license number is SYXK (Zhe) 2022-0004. Housing and management are complied with the requirements of international AAALAC accreditation (certification number 001458). The IACUC ethics review number is IACUC-2023-7689-01.

Instruments, Consumables and Reagents:

Dissecting microscopy (SZX 7, OLYMPUS, Japan);

CCD camera (Model VertA 1, Shanghai Tussen Vision Technology Ltd., China);

Precision electronic balances (Model CP 214, OHAUS, America);
6-well plate (Zhejiang Beilanbo Biotechnology Co., Ltd., China,);
Double-sided clean bench for two persons (Model SW-CJ-215 KS, Shanghai Sujing Industrial Co., Ltd., China);
Microtome (Model KD 2258, Jinhua Kedi Medical Equipment Co., Ltd., China);
Biological microscope (Model CX 31, OLYMPUS, Japan).
Dimethyl sulfoxide (DMSO, Batch No. BCCD8942, Sigma, Switzerland);
Methylcellulose (Batch No. C2004046, Shanghai Aladdin Biochemical Technology Ltd., China);
Absolute ethanol (Batch No. 20230329, Sinopharm Chemical Reagent Co., Ltd., China);
Oil Red O (Batch No. SHBN4926; Sigma, USA);
1, 2-propanediol (Batch No. 20211117, Sinopharm Chemical Reagent Co., Ltd., China);
4% Tissue Cell Fixative Solution (Batch No. 20221014, Beijing Solarbio Science & Technology Co., Ltd., China);
Xylene (Batch No. C14165111; Shanghai Macklin Biochemical Technology Co., Ltd., China);
PBS Buffer (Batch 70115000; Biosharp, China);
Mayer's Hematoxylin Staining Solution (Batch No. 20220120; Shanghai Yihe Biotechnology Co., Ltd., China);
Eosin Staining Solution (Batch No. 20220120; Shanghai Yihe Biotechnology Co., Ltd., China);
Neutral balsam (Batch No. 330A021; Solarbio, China);
High-performance embedding paraffin (Melting point 54-56°C, Batch No. 20201020; Shanghai Huayong Paraffin Co., Ltd., China)
High-performance embedding paraffin (Melting point 62-64°C, Batch No. 20210828; Shanghai Huayong Paraffin Co., Ltd., China).

The experimental procedure was as follows:

(1) Determination of the MTC for Wuling Capsule

Albino (AB) strain zebrafish with melanin allele mutation at 5 days post-fertilization (5 dpf) was randomly selected and placed in beakers, with 30 zebrafish in each beaker. Five test concentration groups of Wuling Capsule were set up, and the sample was administered

to the zebrafish in microplates/beakers in form of aqueous solution. A normal control group and a model control group were also included, making a total of seven experimental groups. Each beaker contained 25 mL of solution. The test concentration groups received Wuling Capsule dissolved in water (concentrations are listed in Table 1). Except for the normal control group, all other groups were fed a high-sugar and high-fat diet in form of aqueous solution to establish a diet-induced fatty liver model in zebrafish (specifically, zebrafish was exposed to 1.5 mg/mL egg yolk powder solution during the day and 30 mg/mL glucose solution at night to establish the zebrafish diet-induced fatty liver model). After 2 days of treatment at 28 °C, the number of dead zebrafish and toxicity manifestations in each group were recorded to determine the Maximum Tolerated Concentration (MTC) of the sample. The results are recorded in Table 1.

TABLE 1 Experimental Results of Concentration Screening for the Hepatoprotective Effect of Wuling Capsule against Diet-Induced Fatty Liver (n = 30)

Group	Concentration ($\mu\text{g/mL}$)	Mortality (n)	Mortality Rate (%)	Phenotype
Normal Control	-	0	0	No observable abnormalities
Model Control	-	0	0	No observable abnormalities
Test concentration	25	0	0	Comparable to the model control group
	50	0	0	Comparable to the model control group
	100	6	20	-
	200	30	100	-
	400	30	100	-

Therefore, the MTC for Wuling Capsule on the zebrafish with diet-induced fatty liver is

50.0 µg/mL.

(2) Assessment of hepatic steatosis

Albino (AB) strain zebrafish with melanin allele mutation at 5 days post-fertilization (5 dpf) was randomly selected and placed in beakers, with 30 zebrafish in each beaker. Wuling capsule was administered in form of aqueous solution (the concentration is shown in Table 2). Six experimental groups were established: normal control group, model control group, positive control group (Atorvastatin Calcium, 11.6 µg/mL), and three test concentration groups (MTC 50 µg/mL, 1/2 MTC 25.0 µg/mL, and 1/4 MTC 12.5 µg/mL). Each beaker contained 25 mL of solution. Except for the normal control group, all other groups were fed a high-sugar and high-fat diet in form of aqueous solution to establish a diet-induced fatty liver model in zebrafish (specifically, zebrafish was exposed to 1.5 mg/mL egg yolk powder solution during the day and 30 mg/mL glucose solution at night to establish the zebrafish diet-induced fatty liver model). After 2 days of treatment at 28 °C, the fish was subjected to whole-mount lipid staining using Oil Red O. Then, from each group, 10 zebrafish were randomly selected and photographed under a dissecting microscope. Images were acquired and analyzed using NIS-Elements D3.20 advanced image processing software to quantify hepatic staining intensity. The statistical results of this indicator were used to evaluate the auxiliary protective effect of Wuling Capsule against diet-induced fatty liver disease. Data are expressed as mean ± SE. Statistical analysis was performed using SPSS26.0 software, with $p < 0.05$ considered statistically significant. Representative images of hepatic lipid staining intensity are shown in FIG. 1, and the corresponding quantitative data are summarized in Table 2.

TABLE 2

Group	Concentration (µg/mL)	Hepatic Staining Intensity (pixel, mean ± SE)
Normal control	-	6137 ± 165***
Model control	-	24601 ± 695
Positive control	11.6	11925 ± 650***
Test concentration	12.5	19332 ± 871***

25.0	12023 ± 910*
50.0	9243 ± 792***

***: $p < 0.001$ compared to model control group.

It is indicated that Wuling capsules have the auxiliary protective effect on the diet-induced fatty liver disease.

(3) Hepatic histopathological sections

Wild-type Albino (AB) strain zebrafish at 5 days post-fertilization (5 dpf) was randomly selected and placed in beakers, with 30 zebrafish in each beaker. All test samples were administered in form of aqueous solution. Each beaker contained 25 mL of solution. Six experimental groups were established: normal control group, model control group, positive control group (Atorvastatin Calcium, 11.6 $\mu\text{g/mL}$), and three test concentration groups (MTC 50 $\mu\text{g/mL}$, 1/2 MTC 25.0 $\mu\text{g/mL}$, and 1/4 MTC 12.5 $\mu\text{g/mL}$). Except for the normal control group, all other groups were fed a high-sugar and high-fat diet in form of aqueous solution to establish a diet-induced fatty liver model in zebrafish (specifically, zebrafish was exposed to 1.5 mg/mL egg yolk powder solution during the day and 30 mg/mL glucose solution at night to establish the zebrafish diet-induced fatty liver model). After 2 days of treatment at 28 °C, zebrafish from each group underwent fixation, dehydration, embedding, sectioning, H & E staining and the like, and then were subjected to histopathological observation and analysis.

Histopathological images are shown in FIG. 2. Under the described experimental conditions, histopathological observation of zebrafish liver from the normal control group revealed regularly arranged hepatocytes, normal hepatocellular structure and clear cellular margins. Histopathological examination of zebrafish from the model control group showed blurred hepatocyte structure, swollen hepatocyte nuclei (indicated by red arrows), and fatty vacuolar degeneration in liver tissue (indicated by black arrows), which indicates that the model was successfully established. Histopathological examination of zebrafish liver from the positive control group (Atorvastatin Calcium) displayed clearer hepatocellular structure compared to the model control group, reduced hepatocyte swelling and decreased fatty vacuolation, demonstrating that Atorvastatin Calcium has an ameliorative effect on high-

sugar high-fat-induced (diet-induced) fatty liver in zebrafish. Histopathological examination of the Wuling Capsule groups at concentration of 12.5, 25.0 and 50.0 $\mu\text{g/mL}$ revealed clearer hepatocellular structure, diminished hepatocyte swelling and reduced fatty vacuolar degeneration in liver tissue compared to the model control group. This suggests that Wuling Capsule has an auxiliary protective effect against high-sugar high-fat-induced (diet-induced) fatty liver in zebrafish, specifically manifested as restoration of liver tissue morphology.

It should be noted that the present application is not limited to the above-described embodiments. The aforementioned embodiments are merely illustrative, and any embodiments having a configuration substantially identical to the technical concept of the present application and achieving the same effects fall within the technical scope of the present application. Furthermore, within the scope of the present application without departing from its essence, various modifications applied to the embodiments that can be conceived by those skilled in the art, as well as other implementations constructed by combining some constituent elements of the embodiments, are also included within the scope of the present application.

CLAIMS

1. A use of a Wuling formulation in the preparation of a medicament for preventing or treating non-alcoholic fatty liver disease.
2. The use according to claim 1, wherein the Wuling formulation comprises, in parts by weight: 200 to 400 parts of Bupleuri radix, 100 to 200 parts of Ganoderma, 200 to 350 parts of Salviae miltiorrhizae radix et rhizome and 250 to 350 parts of Schisandrae fructus.
3. The use according to claim 2, wherein the Wuling formulation comprises, in parts by weight: 300 to 350 parts of Bupleuri radix, 150 to 180 parts of Ganoderma, 300 to 350 parts of Salviae miltiorrhizae radix et rhizome and 300 to 350 parts of Schisandrae fructus.
4. The use according to claim 3, wherein the Wuling formulation comprises, in parts by weight: 342 parts of Bupleuri radix, 173 parts of Ganoderma, 342 parts of Salviae miltiorrhizae radix et rhizome and 342 parts of Schisandrae fructus.
5. The use according to any one of claims 1 to 4, wherein the Wuling formulation is prepared in a dosage form selected from the group consisting of a decoction, a pill, an oral liquid, a tablet, a capsule, a granule, a medicated electuary, and a powder.
6. The use according to claim 5, wherein the Wuling formulation is selected from any one of Wuling Capsule, Wuling Pill and Wuling Powder.
7. The use according to any one of claims 1 to 6, wherein the Wuling formulation is administered to an adult in a unit dose of 3 g to 6 g, three times per day.