

DESCRIPTION

ADIPOCYTE DIFFERENTIATION INHIBITOR, FOOD COMPOSITION FOR
INHIBITING ADIPOCYTE DIFFERENTIATION, AND METHOD FOR PRODUCING
ADIPOCYTE DIFFERENTIATION INHIBITOR

TECHNICAL FIELD

[0001]

The present invention relates to an adipocyte differentiation inhibitor, a PPAR γ expression inhibitor, a C/EBP α expression inhibitor, a food composition for inhibiting adipocyte differentiation, a cosmetic composition for inhibiting adipocyte differentiation, and a method for producing the adipocyte differentiation inhibitor.

BACKGROUND ART

[0002]

So-called lifestyle-related diseases include, for example, an obesity disease, hypertension, diabetes, hyperlipidemia, myocardial infarction, cerebral stroke, and the like. Recently, it becomes clear that these diseases are developed by various causes affecting each other rather than developed by individual causes, and such complex lifestyle-related diseases are also called metabolic syndrome.

[0003]

The term "metabolic syndrome" represents a state in which a series of disease conditions caused by insulin resistance such as type 2 diabetes, hyperlipidemia, hypertension, visceral obesity, and a fatty liver occur together.

In the metabolic syndrome, an adipocyte, in particular, an adipocyte in an internal organ (a visceral fat), is accumulated to cause an insufficient action of insulin, resulting in a state in which a risk factor for arteriosclerosis such as high blood sugar, dyslipidemia, and hypertension is

accumulated.

[0004]

It has been found that accumulation of the adipocyte leads to the obesity disease, hypertension, diabetes, hyperlipidemia, myocardial infarction, cerebral stroke, and the like, thus preventing the accumulation of the adipocytes is regarded to be very important for preventing and treating these diseases.

The term "obesity disease" described herein is a condition in which a health problem caused by or related to obesity is developed or a condition in which the health problem is predicted to occur due to excessive accumulation of the visceral fats, thus requiring a medical treatment for weight loss.

[0005]

Further, with a recent growing interest in health and beauty, increasing number of people desire to maintain the healthy and skinny body regardless of age or gender. Even non-obese people have a great concern about a local fat accumulation.

[0006]

Conventionally, most of anti-obesity agents for treating or preventing the obesity disease exhibit an adipose accumulation inhibition effect by decomposing the accumulated fats.

The obesity disease is caused by a process in which a preadipocyte is differentiated into an adipocyte (a mature adipocyte), followed by an increase in the number and size of the adipocyte. Further, as a master regulator of the adipocyte differentiation in the differentiation process of the adipocyte, a transcription factor such as PPAR γ and C/EBP α are known. Inhibiting the differentiation of the preadipocyte into the adipocyte is effective for preventing and treating the obesity

disease.

As an adipocyte differentiation inhibitor, a water extract of hop tissue (Patent literature 1), acidic mucopolysaccharides (Patent literature 2), a plant extract (Patent literature 3), and the like are known. Of these, Patent literature 1 discloses that the water extract of hop tissue inhibits the expression of PPAR γ and, through this action, inhibits the differentiation of the adipocyte.

[0007]

On the other hand, as a biological resource, *Euglena* (generic name: *Euglena*, Japanese name: Midorimushi), which is expected to be used as promising food, feed, fuel, and the like, is gaining attention.

Euglena contains 59 kinds of nutrients such as a vitamin, a mineral, an amino acid, and an unsaturated fatty acid, which correspond to a majority of nutrients necessary for humans to maintain life. Thus, it has been proposed that *Euglena* can be used as a supplement for facilitating well-balanced intake of a variety of nutrients and as a food supply source in an impoverished region where people cannot take necessary nutrients.

[0008]

It has been considered difficult to perform a mass culture of *Euglena* for reasons such that *Euglena*, which is positioned as a primary producer in the food chain, is predated by a predator and that setting culture conditions such as light, a temperature condition, and a stirring speed is difficult as compared with other microorganisms. Recently, extensive studies by the present inventors have established a technique for the mass culture, thereby opening a door to a large supply of *Euglena* and a *Euglena*-derived substance such as paramylon extracted from *Euglena*.

Euglena is a unique organism in that it has an animal-like feature with flagellum movement while it also contains chloroplast to perform photosynthesis as a plant. Thus, it has been expected that Euglena itself and a substance derived from Euglena have many functionalities.

Therefore, it has been desirable to develop a method for utilizing Euglena and the substance derived from Euglena which now can be supplied in large quantities.

CITATION LIST

PATENT LITERATURE

[0009]

PATENT LITERATURE 1: JP 2010-173942 A

PATENT LITERATURE 2: JP 2014-9161 A

PATENT LITERATURE 3: JP 2011-6347 A

SUMMARY OF INVENTION

TECHNICAL PROBLEM

[0010]

The present invention has been made in view of the above-mentioned problems and an object of the present invention is to provide a novel adipocyte differentiation inhibitor, PPAR γ expression inhibitor, C/EBP α expression inhibitor, food composition for inhibiting adipocyte differentiation, and cosmetic composition for inhibiting adipocyte differentiation.

Another object of the present invention is to provide the adipocyte differentiation inhibitor, PPAR γ expression inhibitor, C/EBP α expression inhibitor, food composition for inhibiting adipocyte differentiation, and cosmetic composition for inhibiting adipocyte differentiation, serving as a novel method for utilizing an aqueous solvent extract of Euglena.

SOLUTION TO PROBLEM

[0011]

The present inventors have found, as a result of their intensive studies, that an aqueous solvent extract of *Euglena* has an effect of inhibiting differentiation of a preadipocyte to an adipocyte.

More specifically, the present inventors have found that, in a process of the differentiation of the preadipocyte to the mature adipocyte, the aqueous solvent extract of *Euglena* inhibits the expression of PPAR γ and C/EBP α belonging to a key transcription factor group and thus inhibits the differentiation of the preadipocyte to the adipocyte, thereby completing the present invention.

[0012]

Thus, according to the present invention, the above-mentioned problems can be solved by the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the food composition for inhibiting adipocyte differentiation, and the cosmetic composition for inhibiting adipocyte differentiation, characterized by including the aqueous solvent extract of *Euglena* as an active ingredient.

In the above, the aqueous solvent extract of *Euglena* is preferably a water extract of *Euglena* or a hot water extract of *Euglena*.

[0013]

The present invention uses the aqueous solvent extract of *Euglena* on which no side effects have been reported and which has a level of safety in compliance with the Food Sanitation Act of Japan as an active ingredient, allowing continuous administration and continuous intake of the extract for a long period of time.

[0014]

According to the present invention, the above-mentioned problems can be solved by the method for producing the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, and the C/EBP α expression inhibitor, characterized by including a dispersion preparation step in which an aqueous solvent is added to Euglena and shaken to obtain a dispersion and an extraction step in which the dispersion is subjected to centrifuge separation to obtain a supernatant as the aqueous solvent extract of Euglena.

[0015]

The method may include a heating step for heating the dispersion after the dispersion preparation step but before the extraction step.

ADVANTAGEOUS EFFECTS OF INVENTION

[0016]

According to the present invention, it becomes possible to provide the novel adipocyte differentiation inhibitor, PPAR γ expression inhibitor, C/EBP α expression inhibitor, food composition for inhibiting adipocyte differentiation, and cosmetic composition for inhibiting adipocyte differentiation.

The present invention uses the aqueous solvent extract of Euglena on which no side effects have been reported and which has a level of safety in compliance with the Food Sanitation Act of Japan as the active ingredient, allowing continuous administration and continuous intake of the extract for a long period of time.

BRIEF DESCRIPTION OF DRAWINGS

[0017]

FIG. 1 is a schematic explanatory diagram illustrating a life cycle of an adipocyte and protein expression levels of transcription factors.

FIG. 2 is an explanatory diagram illustrating a procedure of an adipose differentiation inhibition test used to examine an adipose differentiation inhibition effect of a water extract or hot water extracts of Euglena in Example 1 and Examples 2 to 6 according to an embodiment of the present invention.

FIG. 3 is a graph showing a result of the adipose differentiation inhibition test of the hot water extract of Euglena in Example 1 of the present invention examined in Test example 1.

FIG. 4 is an image showing a result of staining of lipid droplets after the adipose differentiation inhibition test of the hot water extract of Euglena in Example 1 of the present invention examined in Test example 1.

FIG. 5 is a graph showing a result of the adipose differentiation inhibition test of the water extract or hot water extracts of Euglena in Examples 2 to 6 of the present invention examined in Test example 2.

FIG. 6 is an image showing a result of staining of lipid droplets after the adipose differentiation inhibition test of the water extract or hot water extracts of Euglena in Examples 2 to 6 of the present invention examined in Test example 2.

FIG. 7 is a graph showing a result of the adipose differentiation inhibition test of the hot water extract of Euglena in Example 1 of the present invention and EPA in Comparative example 1 examined in Test example 3.

FIG. 8 is an image showing a result of staining of lipid droplets after the adipose differentiation inhibition test of EPA in Comparative example 1 examined in Test example 3.

FIG. 9 is an explanatory diagram illustrating a procedure of the adipose differentiation inhibition test in which a time period of adding the hot water extract of Euglena in Example 1 of the present invention is changed and a graph showing a result

of the adipose differentiation inhibition test, examined in Test example 4.

FIG. 10 is an image showing a result of staining of lipid droplets after the adipose differentiation inhibition test in which the time period of adding the hot water extract of Euglena in Example 1 of the present invention is changed, examined in Test example 4.

FIG. 11 is a graph showing a result of comparing PPAR γ gene expression levels when the hot water extract of Euglena in Example 1 of the present invention is added, examined in Test example 5.

FIG. 12 is a graph showing a result of comparing C/EBP α gene expression levels when the hot water extract of Euglena in Example 1 of the present invention is added, examined in Test example 5.

DESCRIPTION OF EMBODIMENTS

[0018]

An embodiment of the present invention will be described below with reference to FIG. 1 to FIG. 12.

The present embodiment relates to the invention of an adipocyte differentiation inhibitor, a PPAR γ expression inhibitor, a C/EBP α expression inhibitor, an anti-obesity agent, and an anti-metabolic syndrome agent, each including an aqueous solvent extract of Euglena as an active ingredient.

[0019]

In the present specification, the term "obesity" refers to a condition in which body weight is greater than the normal state or a condition in which excess body fats have been accumulated. As a judging criterion of obesity, a body mass index (BMI) is used. According to the judging criterion of obesity defined by the World Health Organization (WHO), having BMI of 30 or higher is defined as obesity. On the other hand,

in Japan, having BMI of 25 or higher is defined as obesity. The judging criterion in Japan, which is defined by the Japan Society for the Study of Obesity, is based on the fact that, for Japanese, the likelihood of the onset of complication such as impaired glucose tolerance, dyslipidemia, and hypertension increases at around a point where BMI passes 25.

In the present specification, the term "obesity disease" refers to a condition in which a health problem caused by or related to obesity is developed or a condition in which the health problem is predicted to occur due to excessive accumulation of the visceral fats, thus requiring a medical treatment for weight loss. The obesity disease, which is caused by an increase in the number of the adipocyte and enlargement of the adipocyte, is related to an adipocyte life cycle, such as propagation, differentiation, and enlargement of the preadipocyte or cell death and removal from the living body of the adipocyte.

In the present specification, the term "anti-obesity" is used to describe prevention or treatment of the obesity disease described above.

[0020]

In the present specification, the term "metabolic syndrome" represents a condition in which a series of disease conditions caused by insulin resistance such as type 2 diabetes, hyperlipidemia, hypertension, visceral fat obesity, and a fatty liver occur together, and is also known under other names such as syndrome X, insulin resistance syndrome, visceral fat syndrome, and multiple risk factor syndrome.

In the present specification, the term "anti-metabolic syndrome" is used to describe prevention or treatment of at least one or more disease conditions selected from the above-mentioned disease conditions.

[0021]

<Summary of adipocyte>

The adipocyte is a cell having a lipid droplet in the cytoplasm and classified into two types, a unilocular adipocyte (a white adipocyte) and a multilocular adipocyte (a brown adipocyte).

The white adipocyte converts unconsumed excess energy excessively taken into a neutral fat and store it as an energy source. The white adipocytes, which are widely distributed in the human body, are accumulated particularly in the lower abdomen, the buttocks, the thigh, the back, the upper arm, peripheries of internal organs, and the like, and stored as subcutaneous fats and visceral fats.

[0022]

It was considered that the number of the white adipocytes increased only in a limited time, for example, in the infancy and puberty stages, and the lifetime number of the white adipocytes was determined in such stages. However, recent studies have showed that, when the existing white adipocytes are filled with the neutral fats due to excess energy intake or a lack of exercise, the number of the adipocytes increases even after the puberty stage and the fat is further taken into the adipocytes. When the size of the adipocyte reaches the upper limit at which further intake of the fat cannot be expected, the surrounding preadipocytes are stimulated by PPAR γ and the like to become mature adipocytes. The mature adipocytes gradually become larger and further undergo cell division, resulting in an increase in the number of the adipocytes.

[0023]

The number of the white adipocytes in an adult around twenty years old is about 40 billion, while the number of the white adipocytes in a person with obesity reaches as large as

40 to 60 billion.

When the energy is required, the white adipocyte decomposes the accumulated fat into a free fatty acid and glycerol to supply them to the whole body. In this process, the white adipocyte is returned to the preadipocyte by releasing the fat and, when the energy becomes excessive again, such a preadipocyte is converted to the mature adipocyte to take in the fat.

[0024]

On the other hand, the brown adipocyte, which has a function of generating heat by decomposing the excess fat, is present in specific parts of the human body, such as the back side of the neck, peripheries of the scapula, the armpit, and peripheries of the heart and kidney. The number of the brown adipocytes is the largest immediately after the birth, but decreases according to age in the adult, particularly, significantly decreases at the age of 40 or above. It is reported that the decrease in the number of the brown adipocytes is the major cause of the middle-age spread.

[0025]

<Life cycle and transcription factor cascade of adipocyte and adipocyte differentiation inhibition effect>

As shown in FIG. 1, a life cycle of the adipocyte can be considered as being divided into: (1) a process in which the mesenchymal stem cell is determined to differentiate into the preadipocyte, (2) a process in which the preadipocytes are propagated and differentiated into the mature adipocyte, and (3) a process in which the enlarged adipocyte is subjected to apoptosis and removed from the living body.

Transcription factors closely involved in the adipocyte differentiation process have been elucidated by a system using a cultured cell and a genetically modified individual and the

like.

[0026]

Peroxisome proliferator-activated receptor (PPAR), CCAAT/enhancer-binding protein (C/EBP), and sterol regulatory element binding protein 1 or adipocyte determination and differentiation-dependent factor 1 (SREBP-1/ADD1) are known as the most important transcription factors in the differentiation of the adipocyte.

[0027]

PPAR is known to constitute a family including PPAR α , PPAR γ , and PPAR δ . Of these, PPAR γ is particularly important for the adipocyte differentiation. When PPAR γ is forcefully expressed in lipoblastoma and the preadipocyte, these cells are differentiated into the adipocytes.

[0028]

C/EBP is known to constitute a family including C/EBP α , C/EBP β , C/EBP δ , C/EBP γ , C/EBP ϵ , and C/EBP ξ , and, like PPAR γ , C/EBP α functions as a master regulator of the adipocyte differentiation. Further, C/EBP β and C/EBP δ are expressed in the early stage of the differentiation to control the expression of C/EBP α and PPAR γ .

[0029]

SREBP-1/ADD1 is known to promote the differentiation of the adipocyte. Further, SREBP-1/ADD1 is also involved in the production of a ligand of PPAR γ in the differentiation process of the adipocyte.

[0030]

These three transcription factor groups (PPAR, C/EBP, and SREBP-1/ADD1) undergo crosstalk to promote the differentiation process of the adipocyte.

[0031]

<Euglena>

In the present embodiment, the term "Euglena" includes a microorganism taxonomically classified in genus *Euglena*, a variety thereof, a variant thereof, and a related species in family Euglenaceae.

The genus *Euglena* herein refers to a group of organisms belonging to family Euglenaceae, order Euglenales, class Euglenoidea, division Euglenozoa, Excavata in Eukaryotes.

[0032]

Specific examples of the species belonging to genus *Euglena* include *Euglena chadefaudii*, *Euglena deses*, *Euglena gracilis*, *Euglena granulate*, *Euglena mutabilis*, *Euglena proxima*, *Euglena spirogyra*, *Euglena viridis*, and the like.

As *Euglena*, *Euglena gracilis* (*E. gracilis*), in particular, a *Euglena gracilis* (*E. gracilis*) Z strain can be used. In addition, it is also possible to use an SM-ZK strain (a chloroplast deficient strain) as a mutant strain of the *Euglena gracilis* (*E. gracilis*) Z strain, *E. gracilis* var. *bacillaris* as a variety of *Euglena gracilis*, a gene mutation strain such as chloroplast mutant strains of these species, other *Euglenas* such as *Astaia longa*, and a substance derived therefrom such as β -1,3-glucanase.

[0033]

Euglenas are widely distributed in fresh water such as a pond and a swamp, brackish water, and sea water, and *Euglenas* separated therefrom may be used. Alternatively, any of *Euglenas* which have already been isolated may be used.

Euglenas include all mutant strains thereof. Further, these mutant strains include those obtained through a genetic method such as, for example, recombination, transduction, and transformation.

[0034]

For culturing *Euglena* cells, a culture liquid to which

nutrient salts, such as, for example, a nitrogen source, a phosphorus source, and a mineral, are added can be used. As an example of such a culture liquid, a modified Cramer-Myers medium ($(\text{NH}_4)_2\text{HPO}_4$ 1.0g/L, KH_2PO_4 1.0g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2g/L, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.02g/L, $\text{Fe}_2(\text{SO}_4)_3 \cdot 7\text{H}_2\text{O}$ 3mg/L, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 1.8mg/L, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ 1.5mg/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4mg/L, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.2mg/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.02g/L, thiamin hydrochloride (vitamin B_1) 0.1mg/L, and cyanocobalamin (vitamin B_{12}), (pH3.5)) can be used. It is noted that $(\text{NH}_4)_2\text{HPO}_4$ can be replaced with $(\text{NH}_4)_2\text{SO}_4$ or NH_3aq . Further, in addition, a known Hunter medium and Koren-Hunter medium prepared based on the description of "Euglena-Physiology and Biochemistry" (edited by Shozaburo KITAOKA, Gakkai Shuppan Center) may be used.

[0035]

The pH of the culture liquid is preferably 2 or more, further, the upper limit of the pH is preferably 6 or less, more preferably 4.5 or less. Adjusting the pH on an acidic side allows a photosynthetic microorganism to grow more predominantly than other microorganisms, thus making it possible to reduce contamination.

Euglena cells may be cultured by an open pond system directly utilizing sunlight, a light-condensing system in which sunlight condensed by a light-condensing device is transmitted through an optical fiber or the like and irradiated to a culture tank to be utilized for photosynthesis, and the like.

Further, Euglena cells can be cultured by, for example, a supply batch method. However, any liquid culture method such as flask culture, culture using a fermenter, a batch culture method, a semi-batch culture method (a fed-batch culture method), and a continuous culture method (a perfusion culture method) may be used for culturing.

[0036]

Culturing can be carried out by using a known culturing apparatus such as an open pond type apparatus, a raceway type apparatus, and a tube type apparatus and a laboratory culturing container such as a Sakaguchi flask, an Erlenmeyer flask, and a reagent bottle. When *Euglena* assimilating CO₂ is cultured using the Cramer-Myers medium, an autotrophic culture medium, air containing 1 to 5% CO₂ is preferably passed through the medium. Further, ammonium phosphate is preferably added to the medium in an amount of about 1 to 5g per liter of medium to allow chloroplasts to sufficiently develop. The culture temperature is normally 20 to 34°C, particularly preferably 28 to 30°C. Further, although it may vary depending on culture conditions, *Euglena* normally reaches a logarithmic growth phase 2 to 3 days after the start of the culture and a stationary phase 4 to 5 days after the start of the culture.

Euglena may be cultured under light irradiation (light culture) or without light irradiation (dark culture).

[0037]

Separation of *Euglena* cells is carried out by a method such as, for example, centrifugal separation, simple sedimentation, or membrane filtration of the culture liquid.

A dried alga product of *Euglena* is prepared by washing the separated *Euglena* cells and then freeze-drying the washed *Euglena* cells under vacuum by a known method. However, the dried alga product may be prepared by spray drying, heating vacuum drying, and the like.

As *Euglena*, the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent of the present embodiment may use the alga body of *Euglena* separated from the culture liquid by centrifugal separation, sedimentation, membrane filtration, or the like, or the dried

alga product. Further, a dried powder of Euglena prepared by crushing the dried alga product by a known crusher may be used.

[0038]

<Aqueous solvent extract of Euglena>

In the present embodiment, the term "aqueous solvent extract of Euglena" refers to an extract extracted from Euglena using an aqueous solvent. In particular, it is preferable that water is used as the aqueous solvent and a water extract or hot water extract of Euglena which is extracted at 5°C to 600°C for several seconds to several tens of hours is used.

Water used for extraction is not necessarily distilled water, pure water, or ultrapure water, and it may be, for example, tap water or water containing impurities. However, water preferably does not include an ingredient that hinders the extraction of the active ingredient.

[0039]

In the present embodiment, the term "water extract" refers to an extract extracted with water of 0 to 50°C (excluding 0°C).

The term "water" described herein refers to water of 0 to 50°C (excluding 0°C).

The temperature of the water is not particularly limited as long as it is within a range capable of sufficiently extracting the active ingredient without affecting the active ingredient. However, it is preferably 1 to 40°C, more preferably 5 to 35°C, particularly preferably 10 to 30°C.

[0040]

In the present embodiment, the term "hot water extract" refers to an extract extracted with water having a temperature higher than 50°C, and it can be also called "warm water extract".

The term "hot water" described herein refers to high temperature water of higher than 50°C and its concept includes

"boiling water", thus, water in a boiling state is also included. Further, the hot water is not limited to hot water in a liquid state, and it also includes hot water in a gaseous state and a supercritical state.

The temperature of the hot water is not particularly limited as long as it is within a range capable of sufficiently extracting the active ingredient without affecting the active ingredient. However, it is preferably higher than 50°C and not higher than 120°C, more preferably higher than 50°C and not higher than 100°C.

[0041]

The pH of the water used for extraction is not particularly limited as long as it is within a range capable of sufficiently extracting the active ingredient without affecting the active ingredient. However, it is preferably pH 4 to 10, more preferably pH 5 to 9, particularly preferably pH 6 to 8.

[0042]

Note that, in the present embodiment, the water alone is used as the aqueous solvent, however, one or more types of solvents normally usable for extraction may be selected and used as long as they are capable of sufficiently extracting the active ingredient without affecting the active ingredient. Examples of such solvents include, but are not limited to, water, alcohols, and glycols. Examples of the alcohols include ethanol, methanol, n-propanol, isopropanol, and the like. Examples of the glycols include butylene glycol, propylene glycol, and the like. Examples of other aqueous solvents include acetone and the like. These solvents may be used alone, as an aqueous solution thereof, or as a mixed solvent of any two or more thereof.

[0043]

The temperature of the aqueous solvent used for

extraction is, for example, 0°C or higher, and it is not particularly limited unless affecting the active ingredient. Although the aqueous solvent in a boiling state or in a supercritical state can be used, the aqueous solvent of 5°C to 600°C is preferably used and the aqueous solvent of 10°C to 200°C is more preferably used.

Thus, the aqueous solvent for extraction includes the aqueous solvent in a boiling state and in a supercritical state. The amount of the aqueous solvent used for extraction is preferably an amount capable of sufficiently dissolving a water-soluble active ingredient contained in Euglena.

[0044]

There is no particular limitation on an extraction method. For example, extraction can be performed by the following methods. However, the extraction method is not limited thereto and the normal extraction methods can be freely selected and used. Examples of the extraction methods include: a method in which a dried alga powder of Euglena is immersed into the aqueous solvent for a predetermined time and then the resulting mixture is subjected to centrifuge separation or filtration; a method in which a dried alga powder of Euglena is added to the aqueous solvent and uniformly dispersed by shaking, and then the resulting dispersion is subjected to centrifuge separation or filtration; and the like.

Further, the aqueous solvent in which Euglena is added can be heated to facilitate extraction.

[0045]

The water extraction of Euglena can be carried out by the normal methods described below without being limited thereto. For example, a Euglena tissue and water are put in a container and the resulting mixture is allowed to stand for a predetermined time while being appropriately stirred or shaken.

An extraction liquid thus obtained can be directly used as the water extract. Further, for example, a supernatant obtained from the extraction liquid by centrifugation can be used as the water extract. Further, the extraction liquid or the supernatant is concentrated and dried to remove the water content, and the resulting product can be used as the water extract. The water extraction may be carried out by adding a small amount, for example, 10mass% or less, of an alcohol, preferably, ethanol, to water to improve the extraction efficiency and shorten the extraction time.

The extraction time of the water extraction is not particularly limited as long as the active ingredient can be extracted, and it can be appropriately set within a range of several seconds to several tens of hours depending on the extraction temperature.

[0046]

Extraction using the hot water can be carried out by the normal methods described below without being limited thereto. Euglena and water are put in a commonly used extractor and extraction is performed by heating. In a case where boiling water or water in a supercritical state is used for extraction, it is necessary to use an extractor resistant to a water vapor pressure. The pressure at the time of extraction can be set to 1 to 5,000 atmospheres, preferably 60 to 400 atmospheres.

In a case where extraction is carried out under a high-temperature and high-pressure condition, the active ingredient may be decomposed or undergo a chemical reaction by a prolonged extraction time. Thus, when extraction is carried out under a high-temperature and high-pressure condition, the extraction time is set to a short time, for example, preferably within 3 minutes, more preferably within 1 minute, particularly preferably within 30 seconds.

[0047]

The Euglena extract thus extracted can be directly used as the active ingredient of the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the agent for preventing or treating metabolic syndrome according to the present embodiment. However, the extract can be further fractionated into a fraction having higher activity by an appropriate separation method (e.g., partitioned extraction, a gel filtration method, a silica gel chromatography, a reverse phase or normal phase high-performance liquid chromatography, etc.), and such a fraction can be used.

Further, the Euglena extract or the fraction thereof is concentrated and dried to remove the water solvent, and the resulting product can be used as the aqueous solvent extract.

[0048]

<Adipocyte differentiation inhibitor, PPAR γ expression inhibitor, and C/EBP α expression inhibitor>

The adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, and the C/EBP α expression inhibitor according to the present embodiment include the aqueous solvent extract of Euglena as the active ingredient.

The adipocyte differentiation inhibitor according to the present embodiment inhibits expression of PPAR γ and C/EBP α , the master regulators of the adipocyte differentiation, in the differentiation process of the adipocyte, thereby inhibiting the differentiation of the preadipocyte to the adipocyte.

[0049]

<Anti-obesity agent and anti-metabolic syndrome agent>

The anti-obesity agent and the anti-metabolic syndrome agent according to the present embodiment include the aqueous solvent extract of Euglena as the active ingredient.

The aqueous solvent extract of Euglena inhibits accumulation of the adipocytes by inhibiting the expression of PPAR γ and C/EBP α , the master regulators of the adipocyte differentiation, and thus inhibiting the differentiation of the preadipocyte to the adipocyte. As a result, the aqueous solvent extract of Euglena exhibits the effect of preventing or treating anti-obesity and metabolic syndrome.

[0050]

<Application>

The adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena according to the present embodiment as the active ingredient are administered to a patient who has definite diagnosis of the obesity disease and/or metabolic syndrome.

Further, the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent of the present embodiment are configured as a medical composition, a health food, and the like, and prophylactically administered to a reserve patient of the obesity disease and metabolic syndrome such as an individual who is aware of a subjective symptom associated with the obesity disease and/or metabolic syndrome, an individual whose BMI tends to increase, and an individual who is highly susceptible to the obesity disease and/or metabolic syndrome judging from a genetic factor or a living environment.

[0051]

The aqueous solvent extract of Euglena can be taken as a food without any side effect, thus the aqueous solvent extract of Euglena can be administered even before the definitive

diagnosis of the obesity disease and/or metabolic syndrome. Further, the aqueous solvent extract of Euglena can be continuously administered from the time point before the definitive diagnosis to any time point after the definitive diagnosis, for example, a time point when the BMI value is lowered to less than 25, a time point when the differentiation of the adipocyte seems to be less active, a time point when the aqueous solvent extract of Euglena is switched to another anti-obesity agent, and the like.

[0052]

Further, an individual who has completed the treatment of the obesity disease and/or metabolic syndrome is prone to relapse of the obesity disease and/or metabolic syndrome. Thus, the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent of the present embodiment can be continuously administered to the patient of the obesity disease and/or metabolic syndrome who has the BMI value down to less than 25 after having undergone the treatment and achieved the BMI value down to less than 25 for the purpose of inhibiting the relapse of the symptom as an agent for preventing and inhibiting the relapse of the obesity disease and/or metabolic syndrome.

[0053]

It is generally known that the obesity disease and/or metabolic syndrome are caused by various factors such as stress, a dietary habit, and a genetic factor.

Thus, the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent of the present embodiment can be continuously administered for a long period of time to an individual in an environment easily

causing mental stress and social stress, for example, an individual in a workplace or living environment easily causing psychogenic stress and an individual who is preparing an examination or the like.

[0054]

Further, the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent of the present embodiment can be continuously administered for a long period of time to an individual in an environment likely causing the obesity disease and/or metabolic syndrome due to a dietary habit and a genetic factor, for example, an individual whose family has an irregular dietary life and an individual whose family member such as a parent has been affected by the obesity disease and/or metabolic syndrome.

[0055]

Further, a subject to which the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena according to the present embodiment as the active ingredient are administered is not limited to a person and an animal other than human which have the symptoms or conditions described above.

[0056]

For example, the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena as the active ingredient can be administered to or taken by an obese person having BMI not less than 25, who is in the infancy/early childhood or puberty stage when the white adipocytes

particularly increase.

In the person at the age in the infancy/early childhood or puberty stage, the white adipocytes tend to increase. However, the increase of the white adipocytes can be inhibited by the adipocyte differentiation inhibition effect, the PPAR γ expression inhibition effect, and the C/EBP α expression inhibition effect provided by the aqueous solvent extract of Euglena.

[0057]

The term "infancy stage" described herein refers to the time from birth to less than 1 year old, and the term "early childhood" refers to the time from 1 year old or above to less than 6 years old.

Further, the term "puberty" refers to the time during which secondary sex characteristics start and the person becomes mature and has an increase in height to his/her final height. This happens between about the ages of 12 and 17 for males. For females, the puberty is defined according to the Japan Society of Obstetrics and Gynecology as "the time starting with development of the sexual function (elevation of the breasts, development of pubic hair, etc.) and ending with the completion of the secondary sex characteristics through menarche and the establishment of the almost regular menstrual cycle. An average age of the puberty of the current Japanese females is between the ages of 8 or 9 and 17 or 18". According to the World Health Organization (WHO), the puberty is defined between the ages of 10 and 19, thus the puberty is an ambiguous concept which varies depending on a gender and an individual difference. Collectively, the puberty is the time between the ages of 8 and 19.

[0058]

Further, the adipocyte differentiation inhibitor, the

PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena as the active ingredient can be administered to a person of 40 years old or above.

In the person of 40 years old or above, the activity of generating heat by decomposing excess fats is decreased, thus the fats are easily accumulated, making the person susceptible to middle-age spread. The accumulation of the adipocytes and the middle-age spread can be inhibited by the adipocyte differentiation inhibition effect, the PPAR γ expression inhibition effect, and the C/EBP α expression inhibition effect provided by the aqueous solvent extract of Euglena.

[0059]

Further, the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena according to the present embodiment as the active ingredient can include a pharmacologically acceptable additive, and such a mixture can be used as a composition such as a medical composition, a cosmetic composition, and a food composition, and the like.

[0060]

(Medical composition)

The adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena according to the present embodiment as the active ingredient can be preferably used as the medical composition by utilizing the excellent adipocyte differentiation inhibition effect, PPAR γ expression inhibition effect, C/EBP α expression inhibition

effect, anti-obesity effect, and anti-metabolic syndrome effect.

The medical composition can be applied to pharmaceutical products or quasi-pharmaceutical products in any dosage form. For example, the medical composition can be applied to an oral agent such as a powder, a fine granule, a granule, a tablet, a capsule, a suspension, an emulsion, a syrup, an extract, and a pill; an external agent such as an external liquid, an external gel, a cream, an ointment, a spray, a nasal drop, a liniment, a lotion, a poultice agent, a plaster, a spray, and an aerosol; or an injection.

[0061]

The medical composition according to the present embodiment can include one or more freely selected pharmacologically acceptable additives.

For example, in a case where the medical composition according to the present embodiment is applied to the oral agent, the oral agent can include any additive normally usable in the field of formulation of pharmaceuticals, such as, for example, an excipient, a binder, a disintegrant, a surfactant, a preservative, a colorant, a flavoring agent, a perfume, a stabilizer, an antiseptic, and an antioxidant. Further, the oral agent can be provided as a sustained-release preparation or the like by using the drug delivery system (DDS).

[0062]

Further, in a case where the medical composition according to the present embodiment is applied to the external agent, the external agent can include any additive normally usable in the field of formulation of pharmaceuticals, such as a base, a surfactant, a preservative, an emulsifier, a colorant, a flavoring agent, a perfume, a stabilizer, an antiseptic, an antioxidant, a lubricant, a solubilizer, and a suspending

agent.

[0063]

Further, in a case where the medical composition according to the present embodiment is applied to the injection, the injection can include any additive normally usable in the field of formulation of pharmaceuticals, such as, for example, a solvent, a stabilizer, a solubilizer, a suspending agent, a preservative, an isotonizing agent, an antiseptic, and an antioxidant.

[0064]

The adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent according to the present invention exhibit their effects systemically or locally by oral administration, transdermal administration, intracutaneous administration, subcutaneous administration, intramuscular administration, intravenous administration, and the like, or exhibit their effects locally at the administration site.

[0065]

In the medical composition according to the present embodiment, the contents of the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent are not particularly limited and can be freely set according to their purposes.

[0066]

(Food composition)

The adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena according to

the present embodiment as the active ingredient, which exhibit the excellent adipocyte differentiation inhibition effect, PPAR γ expression inhibition effect, C/EBP α expression inhibition effect, anti-obesity effect, and effect of preventing or treating metabolic syndrome, can be compounded into various foods to provide food compositions having such effects.

[0067]

These agents can be used in any food composition such as, for example, drinks (a refreshing beverage, an alcoholic beverage, a carbonated beverage, a milk beverage, a fruit juice beverage, tea, coffee, an energy drink, etc.), a seasoning such as soy sauce, soups, creams, various dairy products, a processed meat product, a processed agricultural product, a frozen confectionery such as ice cream, various powdered foods (including drinks), a concentrated beverage, a preserved food, a frozen food, breads, cereals, and confectioneries (a candy (a cough drop), a cookie, a biscuit, chewing gum, gummy, a chocolate, etc.).

[0068]

Alternatively, these agents can be also used in the foods with health claims (the foods for specified health uses, the food with nutrient function claims, and the foods with functional claims), a so-called health food (including a drink), a concentrated nutrient, a liquid food, and an infant/baby food.

The term "foods for specified health uses" described herein refers to foods which contain health functional ingredients that affect physiological functions and the like and are allowed to be labelled as being suitable for specific health uses with a permission of the Commissioner of the Consumer Affairs Agency, Japan. In the present embodiment, the food is expected to be on sale for the specified health uses

with a label such as food "for reducing body fats", food for preventing and ameliorating fat accumulation, food for inhibiting adipocyte growth, and food for preventing and ameliorating obesity.

Further, the term "foods with nutrient function claims" refers to foods which are used for supplementing nutritional ingredients (vitamins and minerals) and have labels indicating functions of the nutritional ingredients. In order to sell the foods with nutrient function claims, it is necessary that an amount of the nutritional ingredient included in a recommended consumption per day is within a range between a predetermined upper limit value and lower limit value, and that, not only the nutrient function claims, but also warning notices or the like are indicated.

Further, the term "foods with functional claims" refers to foods having labels indicating functionalities based on scientific grounds on responsibility of business owners. Information regarding grounds of safety and functionalities and the like are notified to the Commissioner of the Consumer Affairs Agency, Japan, before sale.

[0069]

In the above, the present embodiment including the aqueous solvent extract of Euglena as the active ingredient can be used as the food for specified health uses for preventing fat accumulation/the food for specified health uses for inhibiting adipocyte differentiation, the food with nutrient function claims for preventing fat accumulation/the food with nutrient function claims for inhibiting adipocyte differentiation, and the food with functional claims for preventing fat accumulation/the food with functional claims for inhibiting adipocyte differentiation, intended for a patient with the obesity disease.

Further, the present embodiment including the aqueous solvent extract of Euglena as the active ingredient can be used as the food for specified health uses for preventing fat accumulation/the food for specified health uses for preventing adipocyte differentiation, the food with nutrient function claims for preventing fat accumulation/the food with nutrient function claims for preventing adipocyte differentiation, and the food with functional claims for preventing fat accumulation/the food with functional claims for preventing adipocyte differentiation, intended for a living body, for example, a person before suffering from the fat accumulation and a reserve patient of the metabolic syndrome caused by the fat accumulation.

[0070]

The food composition according to the present embodiment can be compounded with one or more freely selected ingredients usable for a normal food composition in addition to the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent according to the present embodiment. For example, any additive normally usable in the food field such as various seasonings, a preservative, an emulsifier, a stabilizer, a perfume, a colorant, an antiseptic, and a pH regulator can be included.

[0071]

The contents of the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent in the food composition are not particularly limited and can be freely set according to their purposes.

[0072]

(Cosmetic composition)

The adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent including the aqueous solvent extract of Euglena according to the present embodiment as the active ingredient can be preferably used as a cosmetic composition by utilizing the excellent adipocyte differentiation inhibition effect, PPAR γ expression inhibition effect, C/EBP α expression inhibition effect, anti-obesity effect, and effect of preventing or ameliorating metabolic syndrome.

The cosmetic composition can be applied to any form of cosmetics. For example, the cosmetic composition can be applied to a skin care cosmetic such as a lotion, an emulsion, a cream, and a beauty essence; a makeup cosmetic such as a foundation, a concealer, a makeup base, a rouge, a cheek rouge, an eye shadow, and an eyeliner; a sunscreen cosmetic; and the like.

[0073]

The cosmetic composition according to the present embodiment can be compounded with one or more freely selected ingredients usable for a normal cosmetic composition in addition to the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent according to the present embodiment.

For example, any additive normally usable in the cosmetic field such as a base, a preservative, an emulsifier, a colorant, an antiseptic, a surfactant, an ultraviolet absorber, an antioxidant, a moisturizer, an ultraviolet absorber, a perfume, an antiseptic and fungicide, an extender pigment, a coloring pigment, an alcohol, and water can be included.

[0074]

In the cosmetic composition according to the present embodiment, the contents of the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent are not particularly limited and can be freely set according to their purposes.

[0075]

<Method for producing adipocyte differentiation inhibitor, PPAR γ expression inhibitor, C/EBP α expression inhibitor, anti-obesity agent, and anti-metabolic syndrome agent>

The adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent of the present embodiment can be produced by the following method.

First, a dispersion preparation step in which an aqueous solvent is added to Euglena and the resulting mixture is stirred and/or shaken to obtain a dispersion is carried out. In this dispersion preparation step, a Euglena powder is preferably used as Euglena.

Next, an extraction step in which the dispersion is subjected to centrifuge separation to obtain a supernatant as the aqueous solvent extract of Euglena is carried out.

[0076]

Further, a heating step for heating the dispersion using a heating means such as a high-pressure steam sterilizer may be carried out after the dispersion preparation step but before the extraction step.

Specifically, first, the dispersion preparation step in which the aqueous solvent is added to Euglena and the resulting mixture is stirred and/or shaken to obtain the dispersion is carried out.

Next, the heating step in which heat extraction is

performed by heating the dispersion using the heating means is carried out.

Further, the extraction step in which the dispersion is subjected to centrifuge separation to obtain the supernatant as the aqueous solvent extract of Euglena is carried out.

[0077]

In the heating step, a setting temperature of the heating means can be set to a room temperature or higher, for example, 20°C or higher and 50°C or lower, 50°C or higher and 80°C or lower, 80°C or higher and 100°C or lower, 100°C or higher and 120°C or lower, 120°C or higher and 150°C or lower, and 150°C or higher and 200°C or lower.

[0078]

In the heating step, in a case where the aqueous solvent dispersion of Euglena is in a sealed container, the setting temperature of the heating means is equivalent to the temperature of the aqueous solvent dispersion of Euglena.

Further, in heating step, in a case where the aqueous solvent dispersion of Euglena is in an open container, the temperature of the aqueous solvent dispersion of Euglena is determined according to the pressure of the atmosphere around the open container. Under the atmospheric pressure, the maximum temperature is about 100°C.

[0079]

In the heating step, in the case where the aqueous solvent dispersion of Euglena is in the open container, the heating step is simultaneously carried out with a concentration step of the aqueous solvent extract of Euglena.

[0080]

Note that the aqueous solvent extract of Euglena may be obtained by a general separation method instead of subjecting the dispersion to the centrifugal separation. For example, the

dispersion may be filtrated to obtain the aqueous solvent extract of Euglena as a filtrate.

Further, the dispersion obtained by adding and shaking the aqueous solvent can be directly used as the aqueous solvent extract of Euglena without performing centrifugal separation, filtration, or the like.

[0081]

The aqueous solvent extract of Euglena thus obtained may be further subjected to a fractionation step in which a fraction having higher activity is obtained by fractionation by an appropriate separation method (e.g., partitioned extraction, a gel filtration method, a silica gel chromatography, a reverse phase or normal phase high-performance liquid chromatography, etc.).

Further, a concentration step for concentrating the aqueous solvent extract of Euglena or the fraction thereof thus obtained and/or a drying step for performing drying by evaporating the aqueous solvent may be performed.

[0082]

The aqueous solvent extract of Euglena obtained as described above is used as the adipocyte differentiation inhibitor, the PPAR γ expression inhibitor, the C/EBP α expression inhibitor, the anti-obesity agent, and the anti-metabolic syndrome agent of the present embodiment.

Examples

[0083]

The present invention will be described in detail below on the basis of specific examples. However, the present invention is not limited to these examples.

In the following test examples, effects of a hot water extract of Euglena (Example 1) and a water extract or hot water extracts of Euglena (Examples 2 to 6) extracted at a room

temperature and different temperatures (25°C, 50°C, 75°C, 95°C, and 120°C) on differentiation of a human subcutaneous adipose-derived stem cell to an adipocyte were examined to study a Euglena extraction method in which a differentiation inhibition effect on the adipocyte was improved.

Further, effects of the hot water extracts of Euglena on expression levels of the PPAR γ gene and C/EBP α gene functioning as master regulators of adipocyte differentiation were examined to study an adipocyte differentiation inhibition mechanism.
[0084]

<Example 1>

To 0.5g of Euglena powders (Euglena gracilis, EU-1593, manufactured by euglena Co., Ltd.), 20ml of ultrapure water was added, and the resulting mixture was shaken to uniformly disperse the Euglena powders. The mixture was then subjected to heat extraction (95°C, 2 hours) using a dry heat sterilizer. A supernatant obtained by centrifuge separation (4,000rpm, 3 minutes, 25°C) was collected and filtered with a 0.45 μ m sterilizing filter to prepare a hot water extract of Euglena (a hot water extract liquid of Euglena, undiluted). The extract liquid was used as an adipocyte differentiation inhibitor.
[0085]

<Example 2>

To 0.5g of Euglena powders (Euglena gracilis, EU-1593, manufactured by euglena Co., Ltd.), 20ml of ultrapure water was added, and the resulting mixture was shaken to uniformly disperse the Euglena powders. The mixture was then subjected to extraction at the room temperature (25°C) for 2 hours. A supernatant obtained by centrifuge separation (4,000rpm, 3 minutes, 25°C) was collected and filtered with a 0.45 μ m sterilizing filter to prepare a water extract of Euglena (a water extract liquid of Euglena, undiluted). The extract

liquid was used as the adipocyte differentiation inhibitor.
[0086]

<Examples 3 to 5>

To 0.5g of Euglena powders (*Euglena gracilis*, EU-1593, manufactured by euglena Co., Ltd.), 20ml of ultrapure water was added, and the resulting mixture was shaken to uniformly disperse the Euglena powders. The mixture was then subjected to hot water extraction at various temperatures (Example 3: 50°C, Example 4: 75°C, Example 5: 95°C) for 2 hours using the dry heat sterilizer. Supernatants obtained by centrifuge separation (4,000rpm, 3 minutes, 25°C) were collected and filtered with a 0.45µm sterilizing filter to prepare the hot water extracts of Euglena (the hot water extract liquids of Euglena, undiluted). The extract liquids were used as the adipocyte differentiation inhibitor.

[0087]

<Example 6>

To 0.5g of Euglena powders (*Euglena gracilis*, EU-1593, manufactured by euglena Co., Ltd.), 20ml of ultrapure water was added, and the resulting mixture was shaken to uniformly disperse the Euglena powders. The mixture was then subjected to heat extraction (120°C, 20 minutes) using a high-pressure steam sterilizer. A supernatant obtained by centrifuge separation (4,000rpm, 3 minutes, 25°C) was collected and filtered with a 0.45µm sterilizing filter to prepare the hot water extract of Euglena (the hot water extract liquid of Euglena, undiluted). The extract liquid was used as an adipocyte differentiation inhibitor.

[0088]

<Test example 1 Adipose differentiation inhibition test of hot water extract of Euglena using adipose-derived stem cell>

(Medium)

As an adipose-derived stem cell growth medium (a growth medium), a medium containing DMEM, α MEM, 1% FBS, ITS-X, 10ng/mL b-FGF, and hydrocortisone was used.

As an adipocyte differentiation induction medium, a medium containing DMEM, 10% FBS, 1 μ M dexamethasone, 0.5mM 3-isobutyl-1-methylxanthine, 0.2mM indomethacin, 10 μ g/mL insulin, and 33 μ M biotin was used.

As an adipocyte differentiation maintenance medium, a medium containing DMEM, 10% FBS, 10 μ g/mL insulin, and 33 μ M biotin was used.

[0089]

(Preparation of cells)

- 1) To a 15ml tube, 9ml of the growth medium was placed.
- 2) A frozen vial containing human subcutaneous adipose-derived stem cells (manufactured by Lonza, PT-5006; hereinafter referred to as ASC) was taken out of the liquid nitrogen.
- 3) The vial was put in a warm bath of 37°C for 1 to 2 minutes and thawed until the ice piece became the size of about a human fingertip.
- 4) The vial was wiped with alcohol and placed in a clean bench.
- 5) The remaining ice piece was thawed in the medium in 1) and all cells were recovered in the 15ml tube in 1).
- 6) The cells were spun down at 1,200rpm for 3 minutes.
- 7) After removing supernatant by aspirator, 500 μ l to 1ml of the growth medium was added to suspend the cells.
- 8) Trypan blue and the cell suspension were mixed in an amount of 10 μ l each and the mixture was put into OneCell Counter.
- 9) The number of cells was counted under the microscope.
- 10) The cells were inoculated in a culture dish in an amount of 3 to 5x10⁵ cells/dish.
- 11) The cells were incubated in the CO₂ incubator (37°C, 5vol%CO₂) for 3 days.

[0090]

An adipose differentiation inhibition test of the hot water extract of Euglena was carried out by the procedure shown in FIG. 2 using the human subcutaneous adipose-derived stem cells prepared by the above procedure. The test procedure will be described in detail below.

[0091]

(Day -3: cell inoculation)

- 1) The cells were examined under the microscope.
- 2) After removing cell supernatant by aspirator, 5ml of PBS(-) was added to the cells.
- 3) After removing PBS(-) by aspirator, 1ml of a trypsin solution (0.25%(w/v)Trypsin-1mM EDTA, (manufactured by Wako Pure Chemical Industries, Ltd.) was dropped on the cells.
- 4) The cells were allowed to stand in the CO₂ incubator (37°C, 5vol%CO₂) for 2 minutes.
- 5) The cells were examined under the microscope to confirm that the cells became round and started to detach from each other.
- 6) After adding 2ml of 10% FBS and DMEM, the cells were recovered in a 15ml tube.
- 7) Centrifuge separation was performed at 1,200rpm for 3 minutes.
- 8) After removing supernatant, the cells were resuspended in the growth medium and the number of cells was counted with a hemocytometer.
- 9) The cells were adjusted to 25,000 cells/well with the growth medium and inoculated in a 24-well plate in an amount of 500μl each.
- 10) The cells were allowed to stand in the CO₂ incubator.
- 11) The cells were incubated in the CO₂ incubator (37°C, 5vol%CO₂) for 3 days.

[0092]

(Day 0: adipose differentiation induction/addition of Euglena extract)

1) The Euglena extract in each example was added to the adipocyte differentiation induction medium to produce a Euglena extract-added medium solution containing the Euglena extract at a predetermined concentration.

2) After removing culture supernatant by aspirator, the Euglena extract-added medium solution prepared in 1) was added to the cells in an amount of 500 μ l each.

3) As a control, the adipocyte differentiation induction medium was added to the cells.

4) The cells were cultured in the CO₂ incubator (37°C, 5vol%CO₂) for 7 days.

5) The medium was exchanged once two or three days.

[0093]

(Day 7: adipose differentiation induction/addition of Euglena extract)

1) The Euglena extract in each example was added to the adipocyte differentiation maintenance medium to produce a Euglena extract-added medium solution containing the Euglena extract at a predetermined concentration.

2) After removing culture supernatant by aspirator, the Euglena extract-added medium solution prepared in 1) was added to the cells in an amount of 500 μ l each.

3) As a control, the adipocyte differentiation maintenance medium was added to the cells.

4) The cells were cultured in the CO₂ incubator (37°C, 5vol%CO₂) for 7 days.

5) The medium was replaced once two or three days.

[0094]

(Day 14: cell measurement and cell fixation)

1) The cells were examined under the microscope.

- 2) A CCK-8 solution was prepared in a necessary amount with a ratio of DMEM, P/S medium:Cell Counting Kit-8=24:1. Cell Counting Kit-8 described herein is a living cell number count kit (manufactured by Dojindo Laboratories) using a water soluble tetrazolium salt WST-8 as a color developing agent.
- 3) After removing culture supernatant by aspirator, the CCK-8 solution prepared in 2) was added to the cells in an amount of 500 μ l each.
- 4) The cells were allowed to stand in the CO₂ incubator (37°C, 5vol%CO₂) for 1 hour.
- 5) Each supernatant was transferred to a 96-well plate in an amount of 100 μ l for 2 wells.
- 6) The absorbances at wavelengths of 450nm and 650nm were measured using a microplate reader.
- 7) The remaining supernatant in 5) was removed by aspirator, and PBS(-) was added to the cells in an amount of 500 μ l each.
- 8) The operation in 7) was repeated twice.
- 9) After removing PBS(-), 4% paraformaldehyde (PFA) was added to the cells in an amount of 500 μ l/well each to fix the cells.
- 10) The cells were light-shielded using an aluminum foil and stored at 4°C for 24 hours or longer.
- 11) A fat accumulation rate was quantified by performing Oil Red O staining using the following method.

[0095]

(Staining and quantification of adipose differentiation)

An Oil Red O stock solution was prepared with a ratio of Oil Red O:isopropanol=120mg:40ml in a 50ml tube and stored at the room temperature.

An Oil Red O staining solution was prepared by mixing with a ratio of Oil Red O stock solution:ultrapure water=3:2, followed by filtration, and immediately used for the following cell straining.

- 1) The fixing solution in the plate was removed.
- 2) The cells were washed twice with PBS(-) in an amount of 500 μ l/well.
- 3) The cells were added with a 60% isopropanol aqueous solution in an amount of 500 μ l/well and blended well therewith at the room temperature for about 2 minutes.
- 4) After removing the 60% isopropanol aqueous solution, the Oil Red O staining solution was added to the cells in an amount of 250 μ l/well.
- 5) The cells were allowed to stand at the room temperature for 15 minutes.
- 6) After removing the Oil Red O staining solution, the 60% isopropanol aqueous solution was added to the cells in an amount of 500 μ l/well to wash the cells with gentle stirring.
- 7) After removing the 60% isopropanol aqueous solution, PBS(-) was added to the cells in an amount of 500 μ l/well, and images of the cells were taken.
- 8) After removing PBS(-), 100% isopropanol was added to the cells in an amount of 500 μ l/well to extract Oil Red O.
- 9) After completely extracting Oil Red O, the extracted solution was transferred to a 96-well plate in an amount of 200 μ l/well each.
- 10) The absorbance at 520nm was measured using the microplate reader.
- 11) An absorbance value was used as a fat accumulation amount. The fat accumulation amount per unit cell was represented by a value converted by the fat accumulation amount (Oil Red O)/the number of cells (CCK-8).
- 12) The fat accumulation rate was calculated by setting the fat accumulation rate in the control in which the culture was performed using the adipocyte differentiation induction medium to 100%.

[0096]

(Result of Test example 1)

The above test was performed using the hot water extract of Euglena in Example 1 and the test result was analyzed. FIG. 3 is a graph showing a comparison of the fat accumulation rates in different concentrations (5vol%, 10vol%, and 20vol%) of the hot water extract of Euglena.

When the fat accumulation rate in the control was set to 100%, the fat accumulation rates in the different concentrations (5vol%, 10vol%, and 20vol%) were 56.4%, 32.5%, and 7.05%, respectively.

The fat accumulation rate decreased as the concentration of the hot water extract of Euglena increased.

Note that these tests were performed multiple times with high reproducibility.

[0097]

As shown in FIG. 4, it was found that fat droplets (parts represented by Oil Red O staining) decreased more with higher concentration of the hot water extract of Euglena.

[0098]

<Test example 2 Adipose differentiation inhibition test of water extracts of Euglena extracted at different temperatures>

The same test as in Test example 1 was carried out except that the water extract or hot water extracts in Examples 2 to 6 (Example 2: 25°C, Example 3: 50°C, Example 4: 75°C, Example 5: 95°C, and Example 6: 120°C) were used instead of the hot water extract of Euglena in Example 1.

[0099]

(Result of Test example 2)

The result of the test using the water extract or hot water extracts of Euglena in Examples 2 to 6 was analyzed. FIG. 5

is a graph showing a comparison of the fat accumulation rates in different concentrations (5vol%, 10vol%, and 20vol%) of the water extract or hot water extracts of Euglena extracted at different temperatures.

The fat accumulation rates of the water extract or hot water extracts of Euglena at different temperatures decreased more as the concentrations of the water extract or hot water extracts of Euglena increased.

Note that these tests were performed multiple times with high reproducibility.

[0100]

On the other hand, the fat accumulation rates did not significantly decrease even though the extraction temperature was increased. From these results, it was found that the concentration of the extract had a bigger impact on the reduction of the fat accumulation rate than the extraction temperature. This shows that the hot water extract extracted at a relatively low temperature of about 50°C has the effect on the reduction of the fat accumulation rate.

[0101]

As shown in FIG. 6, it was found that fat droplets decreased more with higher concentrations of the water extract or hot water extracts of Euglena in the water extract or hot water extracts of Euglena at different temperatures.

[0102]

<Test example 3 Adipose differentiation inhibition test of hot water extract of Euglena and EPA>

The same test as in Test example 1 was carried out using the hot water extract of Euglena in Example 1 and eicosapentaenoic acid (EPA) known to have the adipose differentiation inhibition effect in Comparative example 1.

[0103]

(Result of Test example 3)

The test result using the hot water extract of Euglena in Example 1 and the test result using EPA in Comparative example 1 were analyzed. FIG. 7 is a graph showing a comparison of the fat accumulation rates of the hot water extract of Euglena (concentrations of 10vol% and 20vol%) and EPA (10 μ M, 50 μ M, and 100 μ M).

Note that the addition amount of EPA in the present test was set to be six or more times more than the amount of EPA contained in the hot water extract of Euglena.

The fat accumulation rate in Comparative example 1 was 80% or higher, and this value was higher than the fat accumulation rate using the hot water extract of Euglena in Example 1.

Further, as shown in FIG. 8, it was found that the fat droplets (the parts represented by Oil Red O staining) slightly decreased as compared with the control.

From this result, it was found that, among the ingredients contained in the hot water extract of Euglena, an active ingredient other than EPA, notably, an unidentified active ingredient, exhibited the effect of inhibiting the differentiation of the adipocyte.

[0104]

<Test example 4 Adipose differentiation inhibition test with different addition periods of hot water extract of Euglena>

The same test as in Test example 1 was carried out using the hot water extract of Euglena in Example 1 except that a period of adding the hot water extract of Euglena to the medium was changed.

Specifically, a period from Day 0 to Day 7 when the culture was performed using the adipocyte differentiation induction medium was defined as a first period and a period from Day 7

to Day 14 when the culture was performed using the adipocyte differentiation maintenance medium was defined as a second period. Then, the fat accumulation rates were compared between a case (i) where the hot water extract of Euglena was added both in the first and second periods, a case (ii) where the hot water extract of Euglena was added only in the first period, and a case (iii) where the hot water extract of Euglena was added only in the second period.

[0105]

(Result of Test example 4)

The result of Test example 4 was analyzed. FIG. 9 is a graph showing a comparison of the fat accumulation rates in the case where the period of adding the hot water extract of Euglena was changed.

It was found that the same level of the fat accumulation rate was observed in the case (i) where the hot water extract of Euglena was added in the first and second periods and in the case (ii) where the extract was added only in the first period. On the other hand, it was found that the fat accumulation rate increased in the case (iii) where the extract was added only in the second period. This result shows that the adipose differentiation inhibition effect of the hot water extract of Euglena was exerted in the first period of the adipose differentiation induction.

[0106]

As shown in FIG. 10, it was found that the added fat droplets decreased in the case (i) where the hot water extract of Euglena was added in the first and second periods and in the case (ii) where the extract was added only in the first period as compared with the case (iii) where the extract was added only in the second period.

[0107]

<Test example 5 Gene expression analysis by real-time PCR>

The same test as in Test example 1 was carried out using the hot water extract of Euglena in Example 1 in order to examine expression of the PPAR γ gene and C/EBP α gene functioning as the master regulators of the adipocyte differentiation. Specifically, the hot water extract of Euglena was added on Day 0 and the culture was performed using the differentiation induction medium. Total RNAs were extracted from the cells on culture Day 1, Day 2, Day 3, Day 6 and Day 13, and the real-time PCR was performed using cDNAs obtained by the reverse transcription reaction to measure expression levels of the PPAR γ gene and the C/EBP α gene.

[0108]

(Recovery and extraction of RNA)

- 1) After removing the medium, the cell were washed twice with PBS(-).
- 2) After removing PBS(-), 0.5ml of the total RNA extraction reagent (RNAiso Plus, manufactured by Takara Bio Inc.) was added and the resulting suspension was transferred to a 1.5ml microtube (storable at -80°C).
- 3) To this, 100 μ l (0.2 times of the total RNA extraction reagent) of chloroform was added, and the resulting mixture was stirred by a vortex mixer for 15 seconds and then allowed to stand at the room temperature for 5 minutes.
- 4) Centrifuge separation was performed at 12,000g at 4°C for 15 minutes.
- 5) During the centrifuge separation, 500 μ l of isopropanol was prepared in a new 1.5ml microtube.
- 6) After the centrifuge separation, a transparent layer was transferred to the 1.5ml microtube, and the resulting mixture was stirred by the vortex mixer for 10 seconds and then allowed to stand at the room temperature for 10 minutes.

- 7) Centrifuge separation was performed at 12,000g at 4°C for 15 minutes.
- 8) During the centrifuge separation, a 75% ethanol aqueous solution was prepared and put on the ice.
- 9) After the centrifuge separation, the supernatant was removed and 75% ethanol was added in an amount of 500µl each, and the resulting suspension was gently stirred by the vortex mixer.
- 10) Centrifuge separation was performed at 7,500g at 4°C for 5 minutes.
- 11) The supernatant was removed as much as possible so as not to suck up the pellet.
- 12) The pellet was dried at the room temperature for 10 minutes to 30 minutes.
- 13) After adding 20µl of sterile water, the pellet was dissolved by pipetting several times. The dissolved product was stored at -20°C.

[0109]

(Synthesis of cDNA)

- 1) Concentration was measured with a spectrophotometer (NanoDrop ND-2000C, manufactured by Thermo Fisher Scientific).
- 2) 5×PrimeScript RT Master Mix and 2µl of total RNAs were added in a microtube, and sterile water was added to the mixture to bring the mixture volume to 10µl, followed by mixing.
- 3) The sample was set in a thermal cycler (TP600, manufactured by Takara Bio Inc.) and the reverse transcription reaction was performed by incubation at 37°C for 15 minutes, 85°C for 5 minutes, followed by lowering the temperature to 25°C.
- 4) The sample was diluted 10 times with 90 µl of sterile water and stored at -20°C.

[0110]

(Gene expression analysis by real-time PCR)

- 1) Reagents were mixed at the following ratio to produce a

primer-SYBR mix with a volume corresponding to the number of wells to be used plus one.

PowerUp SYBR Green Master Mix (manufactured by Applied Biosystems): 5 μ l x number of wells

Primer: 0.6 μ l x number of wells

Sterile water: 1.4 μ l x number of wells

2) Each sample was added in a 96-well plate in an amount of 3 μ l. Sterile water was added for the negative control (NC).

3) The primer-SYBR mix was added in an amount of 7 μ l each and the plate was spun down by a plate centrifuge (PS-020, manufactured by Tomy Co., Ltd.).

4) Real-time PCR was performed using a real-time PCR machine (Step One Plus, manufactured by Applied Biosystems).

As the primer, the following primers were used.

· Primers used for PPAR γ gene expression analysis

PPARG-F: 5'-GAACGACCAAGTAACTCTCCTCAAAT-3' (SEQ ID NO: 1)

PPARG-R: 5'-TCTTTATTTCATCAAGGAGGCCAGCATT-3' (SEQ ID NO:

2)

· Primers used for C/EBP α gene expression analysis

CEBPA-F: 5'-GGGTCTGAGACTCCCTTTCCTT-3' (SEQ ID NO: 3)

CEBPA-R: 5'-CTCATTGGTCCCCCAGGAT-3' (SEQ ID NO: 4)

As an internal standard, mRNAs of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene, was amplified in the same manner.

· Primers used for house-keeping gene (GAPDH) gene expression analysis

GAPDH-F: 5'-TGCACCACCAACTGCTTAGC-3' (SEQ ID NO: 5)

GAPDH-R: 5'-TCTTCTGGGTGGCAGTGATG-3' (SEQ ID NO: 6)

[0111]

(Result of Test example 5)

FIG. 11 shows a result of a comparison of the PPAR γ gene expression levels on Day 1, Day 2, Day 3, Day 6, and Day 13 when

the PPAR γ gene expression level on culture Day 0 was set to 1.

It was found that, when the hot water extract of Euglena was added in an amount of 10vol% and 20vol%, the expression levels of the PPAR γ gene on Day 1, Day 2, Day 3, Day 6, and Day 13 were significantly reduced as compared with those on the corresponding Days in the control.

Further, it was found that the expression of the PPAR γ gene was inhibited more as the concentration of the hot water extract of Euglena increased.

[0112]

FIG. 12 shows a result of a comparison of the C/EBP α gene expression levels on Day 1, Day 2, Day 3, Day 6, and Day 13 when the C/EBP α gene expression level on culture Day 0 was set to 1.

It was found that, when the hot water extract was added in an amount of 10vol% and 20vol%, the expression levels of the C/EBP α gene were significantly reduced as compared with those in the control in which the culture was performed in the medium for inducing differentiation of the adipocyte.

Further, it was found that the expression of the C/EBP α gene was inhibited more as the concentration of the hot water extract of Euglena increased.

[0113]

From the result in Test example 5, it was shown that inhibiting the expression of the PPAR γ gene and the C/EBP α gene caused inhibition of the differentiation to the adipocyte, resulting in the reduction in the fat accumulation amount.

SEQUENCE LISTING

<110> Euglena Co., Ltd <120> Adipocyte differentiation inhibitor, food composition for inhibiting adipocyte differentiation and method for producing adipocyte differentiation inhibitor <130> PCT-403 <150> JP2017-036838 <151> 2017-02-28 <160> 6 <170> PatentIn version 3.5 <210> 1 <211> 26 <212> DNA <213> Artificial Sequence <220> <223> PPARG-F <400> 1 gaacgaccaa gtaactctcc tcaaat	26
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CLAIMS

1. An adipocyte differentiation inhibitor comprising an aqueous solvent extract of *Euglena* as an active ingredient.
2. The adipocyte differentiation inhibitor according to claim 1, wherein the aqueous solvent extract of *Euglena* is a water extract of *Euglena*.
3. The adipocyte differentiation inhibitor according to claim 2, wherein the water extract of *Euglena* is a hot water extract of *Euglena*.
4. A PPAR γ expression inhibitor comprising the aqueous solvent extract of *Euglena* as the active ingredient.
5. A C/EBP α expression inhibitor comprising the aqueous solvent extract of *Euglena* as the active ingredient.
6. A food composition for inhibiting adipocyte differentiation, comprising the aqueous solvent extract of *Euglena* as the active ingredient, wherein the food composition is used for inhibiting differentiation of an adipocyte.
7. A cosmetic composition for inhibiting adipocyte differentiation, comprising the aqueous solvent extract of *Euglena* as the active ingredient, wherein the cosmetic composition is used for inhibiting differentiation of the adipocyte.
8. A method for producing the adipocyte differentiation inhibitor, comprising:

a dispersion preparation step in which an aqueous solvent is added to Euglena and shaken to obtain a dispersion; and

an extraction step in which the dispersion is subjected to centrifuge separation to obtain a supernatant as the aqueous solvent extract of Euglena.

9. The method for producing the adipocyte differentiation inhibitor according to claim 8, wherein a heating step for heating the dispersion is performed after the dispersion preparation step, but before the extraction step.