IMPROVED EXTRACELLULAR VESICLES FROM NON-MAMMALIAN CELLS, METHODS OF ENHANCED PRODUCTION AND USES THEREOF

Summary

In one aspect, provided herein is a method for enhancing the production of extracellular vesicles (EVs) from non-mammalian cells, the method comprising: (a) subjecting the cells to an acoustic and/or electromagnetic stimulation; and (b) optionally, collecting the EVs produced from the cells after the stimulation.

In another aspect, provided herein is a method of fermenting natural raw material comprising EVs, the method comprising the steps of: (A) obtaining non-mammalian cells by the method as disclosed herein ; and (B) subjecting the cells to a fermentation; wherein preferably the fermentation comprises: (i) admixing the non-mammalian cells with: - an aqueous salt solution; and/or - at least one microorganism selected from the group consisting of microbiome-associated bacteria, yeasts and molds, or any combination thereof; and/or - a carbohydrate substrate selected from the group consisting of fructose, glucose, galactose, maltose and lactose, or any combination thereof; and/or (ii) incubating, under substantially anaerobic atmospheric conditions, for a sufficient amount of time to allow the fermentation to proceed; (C) optionally, collecting the EVs obtained from step (B).

In another aspect, provided herein are EVs obtainable or that have been obtained by the method as disclosed herein.

In another aspect, provided herein are EVs from ginger cells, preferably from ginger rhizome cells, wherein: - said EVs comprise one or more miRNAs corresponding to member(s) of each of the ginger miRNA families *MIR319*, *MIR159*, *MIR396*, *MIR168*, *MIR156* and *MIR164*, and wherein the one or more miRNAs corresponding to each of the families *MIR319* and *MIR159* are comprised at a higher abundance as compared to the one or more miRNAs corresponding to each of the families *MIR396*, *MIR168*, *MIR156* and *MIR164*; and/or - said EVs comprise at least one miRNA from each of the following groups (i) to (vi): (i) SEQ ID NOs: 1-13; (ii) SEQ ID NOs: 14-17; (iii) SEQ ID NOs: 18-24; (iv) SEQ ID NOs: 25-30; (v) SEQ ID NOs: 31-44; and (vi) SEQ ID NOs: 45-30, wherein the total amount of the miRNA(s) corresponding to each of the groups (ii) and (v) is higher relative to the total amount of the miRNA(s) corresponding to each of the groups (i), (ii), (iv), and (vi).

In another aspect, provided herein is a use of the EVs as disclosed herein in the manufacture of a beverage, a non-beverage food, a dietary supplement, and/or a cosmetic or personal care product.

In another aspect, provided herein are EVs as disclosed herein for use as a medicament.

In another aspect, provided herein is a use of the EVs as disclosed herein in the manufacture of a medicament for preventing and/or treating a pathological condition selected from the group consisting of a cancerous disease, a cardiovascular disease, a neurodegenerative disease, a metabolic disease, an inflammatory disease, an immune disease and an infectious disease.

In another aspect, provided herein is a use of the EVs as disclosed herein in the manufacture of a medicament for - stimulating and/or strengthening the immune defense; - promoting hematopoiesis; - treating inflammation; - promoting health and/or longevity; - enhancing mitochondrial function, preferably for enhancing mitochondrial ATP synthesis; - anti-aging and/or skin regeneration; - reducing oxidative stress, preferably oxidative stress and inflammation in skin pathologies; - reducing oxidative-stress-mediated cell damage, preferably skin damage; - treating and/or preventing neuro-degeneration; - treating and/or preventing a metabolic disorder, preferably selected from diabetes, hypertension, obesity and arteriosclerosis; - treating and/or preventing an acute or chronic respiratory disease (e.g., a lung disorder), preferably selected from asthma and chronic obstructive pulmonary disease (COPD); - treating and/or preventing an eye disorder, preferably selected from astigmatism, dry eyes, and conjunctivitis; - treating and/or preventing a skin disease, preferably selected from atopic eczema and open wounds; - treating and/or preventing an (auto-)immune disorder and/or an allergy; - treating and/or preventing a joint disease; - improving cell function and/or organ function; - promoting (gastro-)intestinal (GI) health and/or amelioration of (gastro-)intestinal (GI) illness; - treating and/or preventing neoplasm; - as an anti-microbial agent; - for promoting hair growth and/or preventing hair loss; - treating and/or preventing an erectile dysfunction and/or for increasing sexual potency; - enhancing physical or mental performance; and/or - diagnosing and/or prognosing a disease.

Detailed Description

The present invention generally relates to a method for enhancing the production of extracellular vesicles (EVs) from non-mammalian cells, the method comprising: (a) subjecting the cells to an acoustic and/or electromagnetic stimulation; and (b) optionally, collecting the EVs produced from the cells after the stimulation. The present invention relates further to a method of fermenting non-mammalian cells. Moreover, the present invention relates to EVs obtainable by the methods of the invention, to the use of the EVs of the invention in the manufacture of a beverage, a non-beverage food, a dietary supplement, a drug and/or a cosmetic or personal care product, as well as to the EVs of invention for use as a medicament and in the prevention and/or treatment of a pathological condition selected from the group consisting of a cancerous disease, a cardiovascular disease, a neurodegenerative disease, a metabolic disease, an inflammatory disease, an immune disease and an infectious disease.

In this specification, a number of documents including patent applications and manufacturer's manuals are cited. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was

specifically and individually indicated to be incorporated by reference.

Extracellular vesicles (EVs) are a heterogeneous group of nanoscale lipid bilayer-delimited particles that are naturally secreted by cells into the extracellular space and, unlike a cell, cannot replicate. They can be broadly classified into the three main subtypes “exosomes”, “microvesicles (MVs)” and “apoptotic bodies”, which are differentiated based upon their biogenesis, release pathway, size, content, and function. They carry a cargo of (poly)peptides, nucleic acids (DNA, RNA, including mRNA and many types of small non-coding RNAs, such as microRNAs (miRNAs), Piwi-interacting RNA (piRNA), small nucleolar RNA (snoRNA)), lipids, metabolites, and/or even organelles originating from the parent cells from which they are released. Evidence has been brought forward that EVs are released from nearly all living cells, including those from plants, animals and microbes. Whereas it was initially thought that EVs were simply a mechanism of eliminating waste (including protein aggregates and damaged organelles) from the cell to maintain homeostasis, a growing body of evidence has meanwhile been accumulated confirming that EVs – by shuttling molecular information in the form of their cargoes from donor cells to recipient cells – function as mediators of intercellular communication, cell maintenance, and tumor progression and thereby act as important regulators of normal physiology as well as pathological processes.

Growing interest has therefore been devoted towards an exploitation of EVs, pre-eminently exosomes, in the diagnosis, prognosis and treatment of disease, especially of cancer. For example, as exosomes are found in nearly all body fluids, increasing studies suggest a great promise for exosomes to serve as

CLAIMS

1. A method for enhancing the production of extracellular vesicles (EVs) from non-mammalian cells, the method comprising:
   (a) subjecting the cells to an acoustic and/or electromagnetic stimulation; and
   (b) optionally, collecting the EVs produced from the cells after the stimulation.

2. The method of claim 1, wherein the non-mammalian cells comprise, consist essentially of, or consist of algae cells, plant cells and/or at least one microorganism.

3. The method of claim 2, wherein the non-mammalian cells comprise, consist essentially of, or consist of plant cells and at least one microorganism selected from the genus *Lactobacillus*.

4. The method of claim 2 or 3, wherein the non-mammalian cells are comprised in a maintenance or culture medium, preferably a maintenance or culture medium comprising, consisting essentially of, or consisting of an aqueous solution or water.

5. The method of any one of claims 1 to 4, further comprising, after the stimulation in step (a), and before the optional collecting in step (b), an incubation;
   wherein preferably the incubation is conducted:
   (i) at a temperature in the range of between, with increasing preference, 1°C and 42°C, 1°C and 37°C, 1°C and 35°C, 1°C and 30°C, 1° and 25°, 1°C and 20°C, 1°C and 15°C, 1°C and 10°C, 2°C and 9°C, 3°C and 8°C, 4°C and 7°C, most preferably at about 6°C; and/or
   (ii) for a time period in the range of between, with increasing preference, 1 hour to 72 hours, 2 hours to 60 hours, 6 hours to 48 hours, 12 hours to 36 hours, 18 hours to 30 hours, 20 hours to 28 hours, most preferably about 24 hours.

6. The method of any one of claims 1 to 5, wherein the collecting in step (b) is conducted by:
   (i) centrifugation, thereby obtaining a centrifugation supernatant comprising the EVs;
   (ii) precipitation of the EVs from the centrifugation supernatant by contacting the supernatant with a water excluding polymer;
   (iii) pelleting the precipitated EVs by centrifugation and disposal of the centrifugation supernatant; and
   (iv) resuspending the pelleted EVs in an aqueous buffer.

7. The method of any one of claims 1 to 6, wherein the acoustic stimulation in step (a) is conducted by applying a continuous or pulsed, preferably pulsed, ultrasound:
   (i) at a frequency in the range of between, with increasing preference, 20 kHz and 10 MHz, 25 kHz and 9 MHz, 50 kHz and 8 MHz, 100 kHz and 7 MHz, 250 kHz and 6 MHz, 500 kHz and 4 MHz, 1000 kHz and 2 MHz, 0.2 MHz and 1.8 MHz, 0.4 MHz and 1.6 MHz, 0.6 MHz and 1.4 MHz, 0.8 MHz and 1.2 MHz, most preferably at a frequency of about 1 MHz;

(ii) with an intensity in the range of between, with increasing preference, 0.5 mW/cm^2 and 3 W/cm^2, 10 mW/cm^2 and 750 mW/cm^2, 10 mW/cm^2 and 750 mW/cm^2, 50 mW/cm^2 and 600 mW/cm^2, 100 mW/cm^2 and 500 mW/cm^2, 200 mW/cm^2 and 360 mW/cm^2, 230 mW/cm^2 and 330 mW/cm^2, 260 mW/cm^2 and 300 mW/cm^2, most preferably of about 280 mW/cm^2;

(iii) with a duty cycle in the range of between, with increasing preference, 1% and 100%, 2% and 90%, 3% and 80%, 4% and 70%, 5% and 60%, 6% and 50%, 7% and 40%, 8% and 35%, 10% and 30%, 15% and 25%, most preferably about 20%;

(iv) with a pulse duration in the range of between, with increasing preference, 1 s and 20 min, 2 s and 10 min, 3 s and 5 min, 5 s and 60 s, 10 s and 50 s, 20 s and 40 s, most preferably about 30 s;

(v) for a total time period in the range of between, with increasing preference, 5 seconds to 12 hours, 10 seconds to 6 hours, 30 seconds to 3 hours, 1 minute to 1.5 hours, 2 minutes to 1 hour, 3 minutes to 40 minutes, 4 minutes to 30 minutes, 5 minutes to 25 minutes, most preferably about 15 minutes; and/or

(vi) at a temperature in the range of between, with increasing preference, 1°C and 42°C, 1°C and 37°C, 1°C and 35°C, 1°C and 30°C, 1° and 25°, 1°C and 20°C, 1°C and 15°C, 1°C and 10°C, 2°C and 6°C, 3°C and 5°C, and most preferably at about 4°C.

8. The method of any one of claims 1 to 7, wherein the electromagnetic stimulation in step (a) is conducted by applying a continuous or pulsed, preferably pulsed, electromagnetic radiation:

(i) at a frequency in the range of between, with increasing preference, 20 kHz and 500 kHz, 30 kHz and 450 kHz, 80 kHz and 400 kHz, 130 kHz and 350 kHz, 180 kHz and 300 kHz, 200 kHz and 280 kHz, 220 kHz and 260 kHz, most preferably about 240 kHz;

(ii) with an energy output in the range of between, with increasing preference, 54 joule per pulse and 96 joule per pulse, 55 joule per pulse and 90 joule per pulse, 55 joule per pulse and 80 joule per pulse, 55 joule per pulse and 70 joule per pulse, 55 joule per pulse and 65 joule per pulse, most preferably about 60 joule per pulse;

(iii) with a pulse duration in the range of between 10 µs and 120 µs, 20 µs and 100 µs, 30 µs and 80 µs, 40 µs and 60 µs, most preferably about 50 µs;

(iv) with a pulse repetition rate in the range of between, with increasing preference, 0.1 Hz and 20 Hz, 0.5 Hz and 15 Hz, 0.6 Hz and 10 Hz, 0.8 Hz and 5 Hz, 1 Hz and 3 Hz, most preferably about 2 Hz;

(v) for a total time period in the range of between, with increasing preference, 5 seconds to 12 hours, 10 seconds to 6 hours, 30 seconds to 3 hours, 1 minute to 1.5 hours, 2 minutes to 1 hour, 3 minutes to 40 minutes, 4 minutes to 30 minutes, 5 minutes to 25 minutes, most preferably about 15 minutes; and/or

(vi) at a temperature in the range of between, with increasing preference, 1°C and 42°C, 1°C and 37°C, 1°C and 35°C, 1°C and 30°C, 1° and 25°, 1°C and 20°C, 1°C and 15°C, 1°C and 10°C, 2°C and 6°C, 3°C and 5°C, and most preferably at about 4°C.

9. The method of any one of claims 1 to 8, wherein the non-mammalian cells, prior to step (a), are or have been subjected to a fermentation.

10. A method of fermenting natural raw material comprising EVs, the method comprising the steps of:
    (A) obtaining non-mammalian cells by the method of any one of claims 1 to 9; and
    (B) subjecting the cells to a fermentation;
    wherein preferably the fermentation comprises:
    (i) admixing the non-mammalian cells with:
    - an aqueous salt solution; and/or
    - at least one microorganism selected from the group consisting of microbiome-associated bacteria, yeasts and molds, or any combination thereof; and/or
    - a carbohydrate substrate selected from the group consisting of fructose, glucose, galactose, maltose and lactose, or any combination thereof;
    and/or
    (ii) incubating, under substantially anaerobic atmospheric conditions, for a sufficient amount of time to allow the fermentation to proceed;
    (C) optionally, collecting the EVs obtained from step (B).

11. EVs obtainable or that have been obtained by the method of any one of claims 1 to 10.

12. EVs from ginger cells, preferably from ginger rhizome cells, wherein:
    - said EVs comprise one or more miRNAs corresponding to member(s) of each of the ginger miRNA families *MIR319*, *MIR159*, *MIR396*, *MIR168*, *MIR156* and *MIR164*, and wherein the one or more miRNAs corresponding to each of the families *MIR319* and *MIR159* are comprised at a higher abundance as compared to the one or more miRNAs corresponding to each of the families *MIR396*, *MIR168*, *MIR156* and *MIR164*; and/or
    - said EVs comprise at least one miRNA from each of the following groups (i) to (vi): (i) SEQ ID NOs: 1-13; (ii) SEQ ID NOs: 14-17; (iii) SEQ ID NOs: 18-24; (iv) SEQ ID NOs: 25-30; (v) SEQ ID NOs: 31-44; and (vi) SEQ ID NOs: 45-30, wherein the total amount of the miRNA(s) corresponding to each of the groups (ii) and (v) is higher relative to the total amount of the miRNA(s) corresponding to each of the groups (i), (ii), (iv), and (vi).

13. Use of the EVs of claim 11 or 12 in the manufacture of a beverage, a non-beverage food, a dietary supplement, and/or a cosmetic or personal care product.

14. The EVs of claim 11 or 12 for use as a medicament.

15. Use of the EVs of claim 11 or 12 in the manufacture of a medicament for preventing and/or treating a pathological condition selected from the group consisting of a cancerous disease, a

cardiovascular disease, a neurodegenerative disease, a metabolic disease, an inflammatory disease, an immune disease and an infectious disease.

16. Use of the EVs of claim 11 or 12 in the manufacture of a medicament for
    - stimulating and/or strengthening the immune defense;
    - promoting hematopoiesis;
    - treating inflammation;
    - promoting health and/or longevity;
    - enhancing mitochondrial function, preferably for enhancing mitochondrial ATP synthesis;
    - anti-aging and/or skin regeneration;
    - reducing oxidative stress, preferably oxidative stress and inflammation in skin pathologies;
    - reducing oxidative-stress-mediated cell damage, preferably skin damage;
    - treating and/or preventing neuro-degeneration;
    - treating and/or preventing a metabolic disorder, preferably selected from diabetes, hypertension, obesity and arteriosclerosis;
    - treating and/or preventing an acute or chronic respiratory disease (e.g., a lung disorder), preferably selected from asthma and chronic obstructive pulmonary disease (COPD);
    - treating and/or preventing an eye disorder, preferably selected from astigmatism, dry eyes, and conjunctivitis;
    - treating and/or preventing a skin disease, preferably selected from atopic eczema and open wounds;
    - treating and/or preventing an (auto-)immune disorder and/or an allergy;
    - treating and/or preventing a joint disease;
    - improving cell function and/or organ function;
    - promoting (gastro-)intestinal (GI) health and/or amelioration of (gastro-)intestinal (GI) illness;
    - treating and/or preventing neoplasm;
    - as an anti-microbial agent;
    - for promoting hair growth and/or preventing hair loss;
    - treating and/or preventing an erectile dysfunction and/or for increasing sexual potency;
    - enhancing physical or mental performance; and/or
    - diagnosing and/or prognosing a disease.